

# Stimulus-Dependent Impairment of the Neutrophil Oxidative Burst Response in Lactoferrin-Deficient Mice

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**Lactoferrin (LF) is an iron-binding protein found in milk, mucosal secretions, and the secondary granules of neutrophils in which it is considered to be an important factor in the innate immune response against microbial infections. Moreover, LF deficiency in the secondary granules of neutrophils has long been speculated to contribute directly to the hyper-susceptibility of specific granule deficiency (SGD) patients to severe, life-threatening bacterial infections. However, the exact physiological significance of LF in neutrophil-mediated host defense mechanisms remains controversial and has not yet been clearly established *in vivo* using relevant animal models. In this study, we used *lactoferrin knockout (LFKO)* mice to directly address the selective role of LF in the host defense response of neutrophils and to determine its contribution, if any, to the phenotype of SGD. Neutrophil maturation, migration, phagocytosis, granule release, and antimicrobial response to bacterial challenge were unaffected in *LFKO* mice. Interestingly, a stimulus-dependent defect in the oxidative burst response of *LFKO* neutrophils was observed in that normal activation was seen in response to opsonized bacteria whereas an impaired response was evident after phorbol myristate-13-acetate stimulation. Taken together, these results indicate that although LF deficiency alone is not a primary cause of the defects associated with SGD, this protein does play an immunomodulatory role in the oxidative burst response of neutrophils. (*Am J Pathol* 2008, 172:1019–1029; DOI: 10.2353/ajpath.2008.061145)**

The initial level of surveillance and host defense against microbial challenge is provided by the innate immune

system. Neutrophils are critical effector cells of this system in which a combination of oxidative and nonoxidative antimicrobial activities responds robustly to kill the invading pathogen.<sup>1–3</sup> The oxidative arm of the neutrophil defense system is provided by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex that generates superoxide anion after the catalytic transfer of electrons from NADPH to molecular oxygen. Superoxide is in turn rapidly converted spontaneously and/or catalytically to several downstream reactive oxygen species including hydrogen peroxide and hypochlorous acid.<sup>4,5</sup> The nonoxidative arm of the neutrophil defense system is provided by a multitude of proteases, hydrolases, antimicrobial peptides, and protein components present in the neutrophil granules.<sup>6,7</sup>

The central role of neutrophils in host defense is underscored in a number of human<sup>8,9</sup> and mouse models<sup>3,10–15</sup> in which one or more of the neutrophil proteins is defective resulting in increased susceptibility to microbial infection. Human-specific granule deficiency (SGD) is a rare congenital disease in which the patients have an absence of or severe deficiency in neutrophil secondary and tertiary granules and associated components.<sup>8,9,16</sup> Numerous abnormalities exist in the neutrophils from these patients rendering them susceptible to severe, life-threatening microbial infections typically caused by *Staphylococcus aureus* or *Pseudomonas aeruginosa*.<sup>17–20</sup> It has been shown that an underlying cause of two cases of SGD is a loss of function mutation in the CCAAT/enhancer-binding protein  $\epsilon$  (C/EBP $\epsilon$ ).<sup>21,22</sup> C/EBP $\epsilon$  is a basic leucine zipper transcription factor expressed predominantly in myeloid cells that control the developmental expression of several genes associated with late-

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stage neutrophil differentiation.<sup>23</sup> The phenotype of *C/EBP $\epsilon$*  knockout mice closely resembles many aspects of the neutrophil defects observed in human SGD, including abnormal bilobed neutrophil morphology, impaired migration to the site of inflammatory challenge, reduced phagocytosis and bactericidal activity, and an impaired oxidative burst activity.<sup>14,24,25</sup> These mice succumb to opportunistic infections, usually caused by *P. aeruginosa* between 3 to 5 months.<sup>14</sup> Like SGD patients, the corresponding mouse model of SGD is deficient in the iron-binding protein, LF, a highly abundant secondary granule component.<sup>24,25</sup>

In the hematopoietic system, LF is expressed specifically in developing neutrophils during the myelocyte stage of maturation<sup>26,27</sup> and the protein is also a prominent component of most exocrine secretions.<sup>28</sup> Several lines of evidence suggest that LF may play an integral part of the innate immune response of the neutrophil to microbial infection. Because of its high affinity for iron and low iron saturation, LF acts as an effective bacteriostatic agent, depriving bacteria of this essential growth nutrient.<sup>29–31</sup> Further, a direct bactericidal activity was shown for LF whereby the protein binds to the outer membrane of Gram-negative bacteria causing the release of lipopolysaccharides with an associated increase in membrane permeability.<sup>32,33</sup> An isolated N-terminal cationic peptide of LF (lactoferrin) has been shown to exhibit increased potency of bactericidal activity relative to the intact protein, with efficacy against a wide range of microorganisms including Gram-negative and Gram-positive bacteria.<sup>34</sup> It has also been speculated that LF indirectly contributes to the innate immune response of the neutrophil. Various reports indicate that LF may influence neutrophil recruitment to sites of infection by altering aggregation, adhesion, attachment, and/or motility of this cell type.<sup>35–38</sup> In addition, it has been shown that LF stimulates phagocytosis.<sup>39,40</sup> Finally, an enhancement of superoxide production by neutrophils was demonstrated in the presence of LF<sup>37</sup> whereas contradictory reports in neutrophils and cell-free systems support either an enhancement<sup>41–43</sup> or no effect<sup>44,45</sup> of LF on hydroxyl radical formation.

The host defense properties ascribed to LF suggest that the neutrophil defects and increased susceptibility to microbial infection observed in SGD patients and *C/EBP $\epsilon$*  knockout mice may be attributable, at least in part, to the absence of this normally abundant secondary granule component. However, the contribution of LF deficiency per se to this syndrome has been difficult to decipher because of the array of additional antimicrobial components that are ablated in association with the specific granule neutrophil defect. In the present study, we have used *LF* knockout (*LFKO*) mice<sup>46</sup> to analyze the specific consequences of LF ablation on the host defense response of the neutrophil. We show that although LF is dispensable for many neutrophil functions, an impairment of phorbol myristate-13-acetate (PMA)-stimulated neutrophil oxidative burst is clearly associated with LF deficiency.

## Materials and Methods

### Animals

The generation of *LFKO* mice by targeted disruption of the LF gene in embryonic stem cells has been reported.<sup>46</sup> *LFKO* mice used for these studies were backcrossed at least 10 generations onto the C57BL/6 genetic background. *Wild-type (WT)* C57BL/6 mice were obtained from Harlan-Sprague Dawley (Indianapolis, IN) and bred in the same facility as the *LFKO* mice. Mice were maintained in microisolator cages under specific pathogen-free conditions in a 12-hour light/dark cycle and were fed a basal rodent chow *ad libitum* (LabDiet; PMI, Richmond, IN). Age- and sex-matched adult mice were used for experiments and all animal research complied with National Institutes of Health and Baylor College of Medicine guidelines for research with experimental animals.

### Neutrophil Morphology and Blood Cell Analysis

Blood was obtained by cardiac puncture and blood smears were stained with Wright-Giemsa to examine neutrophil morphology. Images were acquired using bright-field microscopy (Zeiss Axioscope; Carl Zeiss, Thornwood, NY). Peripheral blood cells were collected from mice by cardiac puncture and were analyzed using a Bayer Advia 120 hematology analyzer (Bayer Diagnostics, Dallas, TX).

### Isolation of Bone Marrow Neutrophils

Mature bone marrow neutrophils were isolated and purified from mice essentially as described previously with minor modifications.<sup>47,48</sup> Briefly, bone marrow cells were isolated from femurs and tibias and mature neutrophils were purified using a discontinuous Percoll gradient (81%, 62%, 55%, 50%, 45%). Cells at the interphase of the 81% and 62% layer were isolated and washed in NM buffer (Hanks' buffered saline solution minus calcium and magnesium (HBSS<sup>-/-</sup>)/0.1% bovine serum albumin). Contaminating red blood cells were lysed in ACK buffer (BioWhittaker, Walkersville, MD) and the resulting cell population was washed twice in NM buffer. Viable cells were counted with a hemocytometer using trypan blue exclusion and cells were resuspended in HBSS<sup>-/-</sup>/5 mmol/L glucose.

### Granulocyte-Specific Antigen (Gr-1) Analysis

Purified bone marrow neutrophils ( $5 \times 10^5$ ) were incubated with anti Gr-1 (1  $\mu$ g/ml; clone RB6-8C5; eBioscience, Inc., San Diego, CA) for 30 minutes on ice. Cells were washed and resuspended in 1% paraformaldehyde for flow cytometric analysis using a Coulter EPICS XL-MCL flow cytometer and System 2 version 3.0 software (Beckman Coulter, Hialeah, FL). Cells were gated on the neutrophil population based on characteristic forward and side light scatter.

### *PMA Stimulation of Neutrophils and Dihydrorhodamine (DHR123)/Rhodamine 123 (R123) Oxidative Burst Assay*

Quantitative determination of the oxidative burst of purified bone marrow neutrophils was determined using a DHR123/R123 assay essentially as described.<sup>49</sup> DHR123 is the nonfluorescent substrate used in the assay and R123 is the fluorescent oxidation reporter product of this substrate formed during the oxidative burst response of the neutrophil. Briefly,  $5 \times 10^5$  cells in HBSS<sup>-/-</sup>/5 mmol/L glucose were incubated with DHR123 (1  $\mu$ mol/L; Molecular Probes, Eugene, OR) for 5 minutes at 37°C with gentle horizontal agitation. Cells were incubated in PMA (0 to 600 ng/ml; Sigma Chemical Co., St. Louis, MO) at 37°C to stimulate the oxidative burst or incubated with buffer vehicle alone (nonstimulated cells). After 30 minutes, the reactions were stopped by placing the tubes on ice and neutrophil cells were analyzed for conversion of DHR123 to the fluorescent R123 by flow cytometry.

### *Bacterial Stimulation of Oxidative Burst Response in Purified Neutrophils*

*S. aureus* Wood 46 was opsonized with 50% serum (obtained from WT C57BL6) for 15 minutes at room temperature as described.<sup>50</sup> Neutrophils ( $5 \times 10^5$ ) were incubated with DHR123 (1  $\mu$ mol/L) for 5 minutes at 37°C followed by an incubation with a 100-fold excess of opsonized bacteria for 1 hour at 37°C. The reaction was stopped by placing tubes on ice and neutrophils were analyzed for conversion of DHR123 to the fluorescent R123 as described above.

### *Western Immunoblot Analysis*

Purified bone marrow neutrophils (2 to  $4 \times 10^6$  cells/ml) were sonicated in 1 $\times$  sodium dodecyl sulfate loading buffer and proteins corresponding to 1 to  $2 \times 10^5$  cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5 to 15%). Immunoblots were probed with antibodies directed against p22<sup>phox</sup> (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup> (Millipore, Billerica, MA), GP91<sup>phox</sup> (a generous gift from Al Jesaitis, Montana State University, Billings, MT), and GAPDH (loading control; Chemicon International, Temecula, CA) following the manufacturer's recommendations. Signal was detected by ECL Plus detection reagents (GE Health Care, Piscataway, NJ) and relative levels of proteins were determined by using densitometric scanning and ImageQuant software (version 5.2; Amersham Biosciences, Piscataway, NJ).

### *Thioglycollate-Induced Peritonitis*

Analysis of neutrophil migration to the peritoneal cavity after thioglycollate administration was performed as described by Lu and colleagues<sup>51</sup> with minor modifications. Briefly, WT and LFKO mice were injected intraperitoneally with 3% thioglycollate media (1 ml) and were euthanized at 0 or 4

hours after injection. Peritoneal exudates were harvested by lavage using 4 ml of cold EB buffer [1 $\times$  phosphate-buffered saline (PBS) minus calcium and magnesium/40 U/ml heparin] and total cell count was determined using a hemocytometer. The peritoneal extract was washed twice in WB buffer (1 $\times$  PBS, pH 7.2, minus calcium and magnesium/0.1% bovine serum albumin) and differential cell counts were obtained using CamcoQuik-stained (Fisher Scientific, Pittsburgh, PA) cytospin samples. At least 500 cells were counted per slide under oil immersion microscopy ( $\times 100$ ).

### *Phagocytosis*

Phagocytic analysis of blood neutrophils was quantitatively analyzed using a commercially available kit (Phagotest; Orpegen, Heidelberg, Germany). Heparinized whole blood (100  $\mu$ l) obtained from WT and LFKO mice was cooled on ice and mixed with ice-cold opsonized fluorescein isothiocyanate-labeled bacteria (20  $\mu$ l). Samples were incubated on ice (control samples) or 37°C (test samples) for 60 minutes. After bacterial incubation, samples were placed on ice and neutralization solution was added to quench the fluorescence of surface-bound bacteria while leaving internalized bacteria unaltered. Erythrocytes were lysed and the remaining cells were resuspended in DNA staining solution for flow cytometric analysis. Neutrophils were distinguished using forward and light scatter patterns and the percentage of cells undergoing phagocytosis and the number of bacteria ingested per cell was determined.

### *Primary Granule Exocytosis*

Purified mature bone marrow neutrophils ( $5 \times 10^5$  cells) were suspended in phenol red-free RPMI 1640 media and stimulated with 5  $\mu$ g/ml of cytochalasin B (Sigma) and 5  $\mu$ mol/L fMLP (Sigma) to induce primary granule exocytosis and myeloperoxidase (MPO) secretion.<sup>52</sup> MPO in the supernatants was detected using a tetramethylbenzidine assay as described.<sup>52,53</sup>

### *CD11b Cell Surface Levels in Resting and Activated Neutrophils*

The activation status and secondary/secretory granule release of WT and LFKO neutrophils were assessed by measuring the cell surface expression of CD11b in resting and stimulated neutrophils. Purified bone marrow neutrophils ( $5 \times 10^5$  cells in HBSS<sup>-/-</sup>/5 mmol/L glucose) were incubated at 37°C with 100 ng/ml of PMA (stimulated cells) or buffer vehicle alone (nonstimulated cells). After 10 minutes, the reactions were stopped by placing the tubes on ice. Cells were analyzed by flow cytometry for cell surface expression of CD11b using phycoerythrin-conjugated anti-CD11b (BD PharMingen, San Diego, CA). Cells were gated on the neutrophil population based on characteristic forward and side light scatter and dead cells were excluded using propidium iodide staining. Isotype-specific antibodies were used to assess nonspecific staining.

### Spontaneous Abscess Bacterial Analysis

Swabs of bacterial abscesses were obtained and submitted to Antech Diagnostics (Irvine, CA) or Radil (University of Missouri, Columbia, MO) for bacterial species identification.

### *S. aureus* Bactericidal Assay

The bactericidal response of *WT* and *LFKO* neutrophils was assessed using a bactericidal assay with *S. aureus* (Wood strain) as described by Clemens and colleague<sup>54</sup> with minor modifications. Purified bone marrow neutrophils ( $2 \times 10^6$ ) were incubated with  $2 \times 10^5$  log phase opsonized bacteria in HBSS/10 mmol/L HEPES/10 mmol/L dextrose/1 mmol/L Ca/3. mmol/L Mg, pH 7.44 containing 10% mouse serum. At 0-, 15-, 30-, and 60-minute time points, 20- $\mu$ l aliquots were removed and combined with 180  $\mu$ l of PBS/0.2% Triton X-100 on ice to lyse the neutrophils. Serial dilutions were plated on brain-heart infusion agar and colonies were enumerated after 24 hours. The percent survival is represented as the percentage of colonies remaining at each time point in comparison to the number at time 0 for each condition.

### *S. aureus* in Vivo Models of Infection

To compare the antimicrobial response of *WT* and *LFKO* mice to *S. aureus* challenge, both an airpouch<sup>55,56</sup> and an intraperitoneal<sup>50</sup> model of bacterial infection were performed. For the airpouch study, a subcutaneous pouch was created in anesthetized *WT* and *LFKO* mice on day 0 using 5 ml of sterile air followed by reinflation on day 3 with 2.5 ml of sterile air. Anesthetized mice were inoculated with  $5 \times 10^6$  CFUs or  $5 \times 10^7$  CFUs of early exponential phase *S. aureus* (8325-4) in 100  $\mu$ l of 0.9% saline. At 24 hours after infection, mice were euthanized. The airpouch was lavaged using 5 ml of ice-cold PBS and the lavage fluid was centrifuged and resuspended in PBS/0.05% Triton X-100. Bacterial suspensions were sonicated, serially diluted in tryptic soy broth (TSB) and plated onto TSB agar. Plates were incubated at 37°C and bacterial counts were enumerated 24 hours later. For the intraperitoneal infection study, mice were inoculated with  $5 \times 10^7$  CFUs of logarithmic growth phase *S. aureus* (LS-1) in 200  $\mu$ l of PBS. Four hours after infection, mice were euthanized, the peritoneal cavity was flushed with 6 ml of ice-cold PBS, 5 mmol/L ethylenediaminetetraacetic acid, and 5 U/ml heparin. Peritoneal lavages were sonicated, serially diluted, and plated on TSB agar for bacterial enumeration.<sup>50</sup>

### Experimental Infection with *P. aeruginosa*

*P. aeruginosa* acute infection of adult *WT* and *LFKO* mice was performed using an intranasal challenge.<sup>57,58</sup> Briefly, anesthetized *WT* and *LFKO* mice were infected intranasally with a clinical blood isolate of *P. aeruginosa* ( $10^5$  or  $10^6$  CFUs in 13  $\mu$ l). Mice were sacrificed 6 hours later and lungs were aseptically removed. Lungs were weighed,

homogenized, and strained through a nylon mesh. The homogenate was centrifuged at  $10,000 \times g$  for 5 minutes and incubated in TSB/0.1% Triton X-100. Serial dilutions of the homogenate were plated onto TSB agar. Plates were incubated at 37°C and bacterial colonies were enumerated 24 hours later.

### Statistical Analysis

Statistical analysis was performed using SPSS (version 10) software (SPSS, Chicago, IL). Differences between groups were examined using the Student's *t*-test for equality of means with a *P* value of  $\leq 0.05$  considered statistically significant. When the *F* values for Levene's test for the equality of variance were significant, the Mann-Whitney *U*-test was used to determine the level of significance.

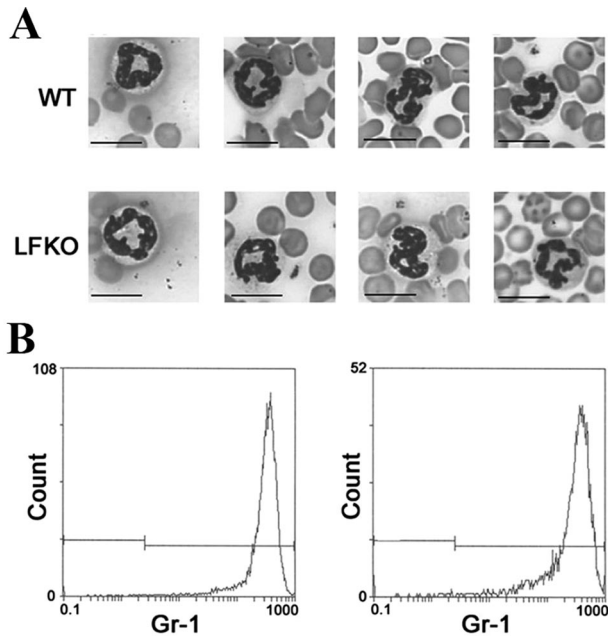
## Results

### *LF* Is Not Required for Neutrophil Differentiation

C/EBP $\epsilon$  is essential for proper differentiation and functional activity of the neutrophil as evidenced by the pleiotropic defects in C/EBP $\epsilon$ -deficient neutrophils.<sup>9,14,24,25</sup> To determine whether ablation of the prominent secondary granule component LF contributes to any of these defects, we examined the differentiation and function of neutrophils in *LFKO* mice. One of the first defects noted on specific granule deficiency was a failure of the neutrophil to terminally differentiate as evidenced by the presence of bilobed nuclei and their deficient granularity.<sup>8,9</sup> To determine whether LF is required for neutrophil differentiation, Wright-Giemsa staining of peripheral blood smears from *LFKO* and *WT* mice was performed. Mature *WT* neutrophils were identified by their pale staining of abundant cytoplasm and segmented ring-shaped nuclei. Neutrophils from *LFKO* mice displayed a similar morphology and were indistinguishable from *WT* neutrophils (Figure 1A). Further, a similar pattern of surface staining for the granulocyte differentiation antigen, Gr-1, a neutrophil maturation marker,<sup>59</sup> was observed in mature *WT* [mean fluorescent intensity (MFI),  $355.15 \pm 21.7$ ] and *LFKO* (MFI,  $329.82 \pm 21.2$ ) neutrophils (Figure 1B). Finally, flow cytometric analysis of mature *LFKO* neutrophils exhibited an indistinguishable forward and side scatter profile to *WT* neutrophils (Figure 2, A and E) indicating that the size and granularity of the neutrophil populations from the two genotype groups were similar. Collectively, these data demonstrate that LF is not required for neutrophil differentiation.

### Oxidative Burst Is Impaired in *LFKO* Neutrophils in Response to PMA Stimulation but Is Normal in Response to Stimulation with Opsonized Bacteria

C/EBP $\epsilon$ -deficient mouse neutrophils display a defective oxidative burst activity in response to stimulation with PMA, a potent stimulator of the NADPH oxidase-depen-



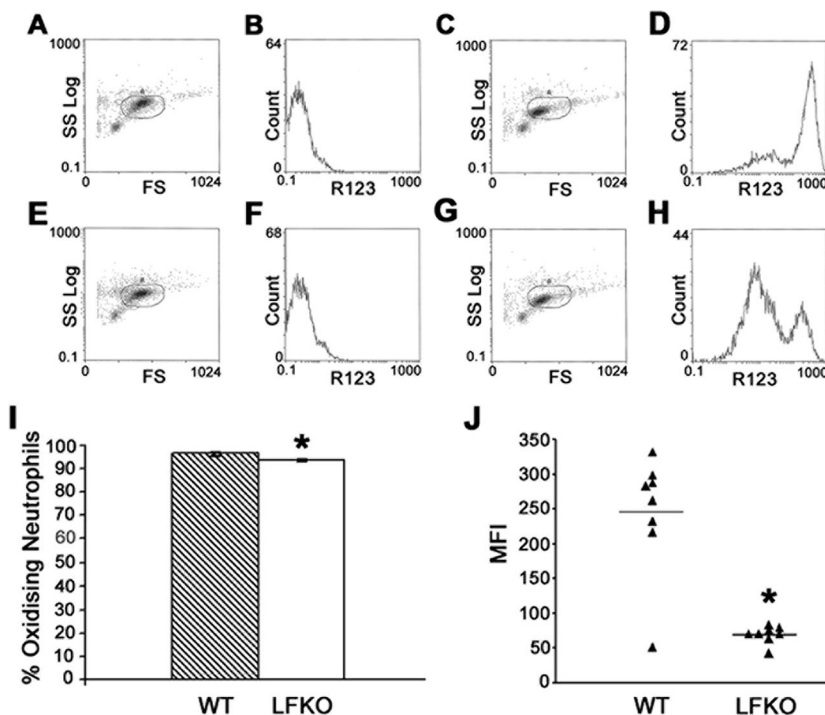
**Figure 1.** Normal maturation of neutrophils in *LFKO* mice. **A:** Peripheral blood neutrophils from *LFKO* mice display normal mature morphology. Representative neutrophils from blood smears stained with Wright-Giemsa from *WT* (**top**) and *LFKO* (**bottom**) mice. Neutrophils from both genotypes have ring-shaped segmented nucleus characteristic of mature morphology in mouse blood. **B:** Expression of Gr-1 in *WT* and *LFKO* neutrophils. Purified bone marrow cells from *WT* and *LFKO* mice ( $n = 8$  per genotype) were stained with anti-Gr-1 as described in the Material and Methods. **Left:** Histogram of representative fluorescence staining for Gr-1 in *WT* neutrophils. **Right:** Histogram of representative fluorescence staining for Gr-1 in *LFKO* neutrophils. Scale bars = 10  $\mu$ m.

dent respiratory burst.<sup>14,24</sup> To establish if the oxidative arm of the neutrophil host defense response was altered in the absence of LF, purified bone marrow neutrophils were stimulated with PMA and the production of reactive

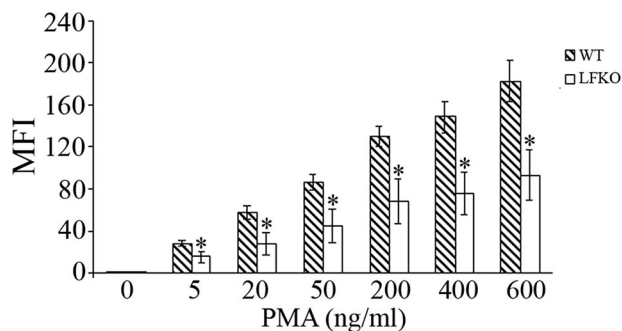
oxygen metabolites was determined by measuring the conversion of the fluorogenic substrate, DHR 123, to fluorescent R123.<sup>49</sup> A dramatic impairment in oxidative burst activity was observed in stimulated *LFKO* neutrophils as compared to *WT* controls (Figure 2). Minimal differences were observed in the total number of neutrophils undergoing burst activity in *LFKO* versus *WT* neutrophils (Figure 2I). However, the MFI, corresponding to the mean oxidative burst activity per cell, was greatly reduced in stimulated *LFKO* neutrophils versus stimulated *WT* neutrophils (*LFKO*,  $70.0 \pm 4.4$  versus *WT*,  $245.6 \pm 30.6$ ;  $P = 0.007$ ) (Figure 2J). To determine whether the significant impairment in the oxidative burst response of *LFKO* neutrophils is observed throughout a range of PMA concentrations, we analyzed the oxidative burst response in *LFKO* neutrophils in response to a PMA dose curve (Figure 3). A similar significant defect in the oxidative burst response of the *LFKO* neutrophils remained throughout a range of PMA concentrations tested (5 to 600 ng/ml). These results suggest that LF is required for optimal oxidative burst activity in neutrophils in response to PMA stimulation. Finally, we characterized the oxidative burst in *WT* and *LFKO* neutrophils in response to opsonized bacteria. Interestingly, using this stimulus, no difference was detected in the oxidative burst response of *WT* and *LFKO* neutrophils (Figure 4).

### Normal Expression of NADPH Oxidase Components in LFKO Neutrophils

The NADPH oxidase is a multisubunit enzyme complex consisting of gp91<sup>phox</sup> and p22<sup>phox</sup> membrane-associated catalytic core components (cytochrome *b*<sub>558</sub>) and



**Figure 2.** Oxidative burst is impaired in *LFKO* neutrophils. The oxidative burst activity of purified bone marrow neutrophils isolated from *WT* and *LFKO* mice was determined using a DHR123/R123 assay and flow cytometric analysis. **A–D:** Representative *WT* cells. **A:** *WT* unstimulated neutrophils showing gated cells based on forward scatter (FS) and side scatter (SS) profiles. **B:** *WT* unstimulated neutrophils showing mean fluorescence intensity (MFI) of rhodamine 123 (R123). **C:** *WT* neutrophils stimulated with PMA showing gated cells. **D:** MFI of R123 in *WT* stimulated cells. **E–H:** Representative *LFKO* cells. **E:** *LFKO* unstimulated neutrophils showing gated cells. **F:** MFI of R123 in *LFKO* unstimulated neutrophils. **G:** *LFKO* neutrophils stimulated with PMA showing gated cells. **H:** MFI of R123 in *LFKO* stimulated cells. **I:** Data represent the mean  $\pm$  SEM percentage of stimulated neutrophils undergoing oxidative burst for *WT* and *LFKO* mice ( $n = 8$  per genotype). **J:** Data represent the MFI of R123 from stimulated neutrophils undergoing oxidative burst for *WT* and *LFKO* mice ( $n = 8$  per genotype). The mean values are indicated by horizontal lines. \*Significantly different ( $P = 0.007$ ) when compared to *WT* mice. Oxidative burst experiments were performed six times with slight variations using three to eight animals per genotype, and in all cases the mean MFI of *LFKO* neutrophils was lower (1.4- to 3.9-fold) than *WT* controls, reaching statistical significance in four of the six experiments.

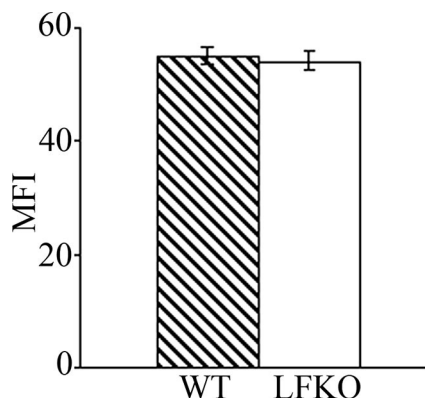


**Figure 3.** Dose response of PMA activation of oxidative burst in *LFKO* neutrophils. The dose response oxidative burst activity of purified bone marrow neutrophils isolated from *WT* and *LFKO* mice was determined using a DHR123/R123 assay and flow cytometric analysis. Data represent the mean MFI ( $\pm$ SEM) of R123 from stimulated neutrophils undergoing oxidative burst for *WT* and *LFKO* mice ( $n = 6$  per genotype). \*Significantly different ( $P \leq 0.05$ ) when compared to *WT* mice.

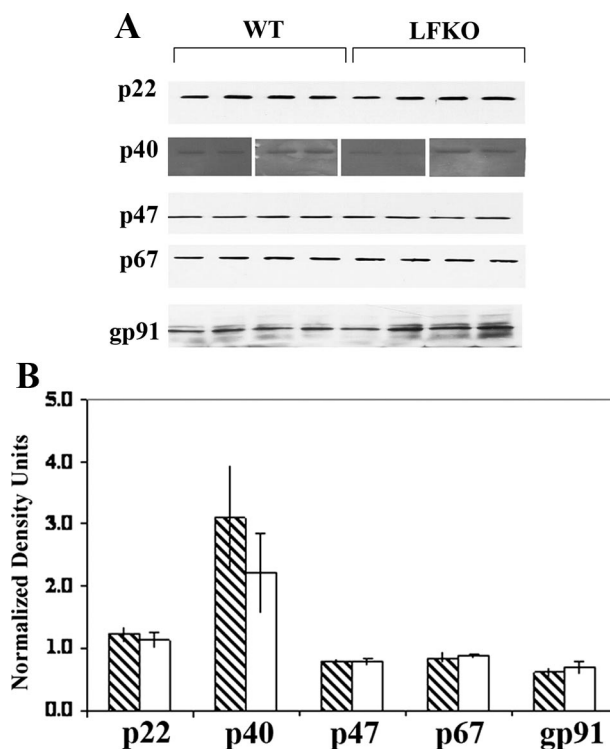
four cytosolic components, p40<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> and a small GTPase (Rac1/2). The cytosolic components translocate to the membrane on neutrophil activation to form a functional oxidase complex with cytochrome b<sub>558</sub>.<sup>60,61</sup> To determine the level of expression of the NADPH oxidase subunits, quantitative Western immunoblot analysis was performed in *WT* and *LFKO* neutrophils. No significant difference in the levels of p22<sup>phox</sup>, p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, or gp91<sup>phox</sup> were detected in the two groups (Figure 5) indicating that the abnormal PMA-stimulated oxidative burst response observed in *LFKO* neutrophils is not because of altered expression of the NADPH oxidase subunits in these mice.

#### Normal Neutrophil Migration and Phagocytosis in *LFKO* Neutrophils

To determine whether the recruitment of neutrophils is altered in the absence of LF, *WT* and *LFKO* mice were challenged with 3% thioglycollate and peritoneal exudate cells were analyzed immediately and at 4 hours after challenge. No neutrophils were detected in the peritoneal cavity in unstimulated *WT* or *LFKO* mice. On thioglycol-



**Figure 4.** Oxidative burst in *LFKO* neutrophils in response to bacterial stimulation. Purified bone marrow neutrophils from *WT* and *LFKO* mice were stimulated with a 100-fold excess of opsonized bacteria. The oxidative burst response was determined using a DHR123/R123 assay and flow cytometric analysis. Data represent the MFI ( $\pm$ SEM) of R123 from stimulated neutrophils undergoing oxidative burst for *WT* and *LFKO* mice ( $n = 6$  per genotype).



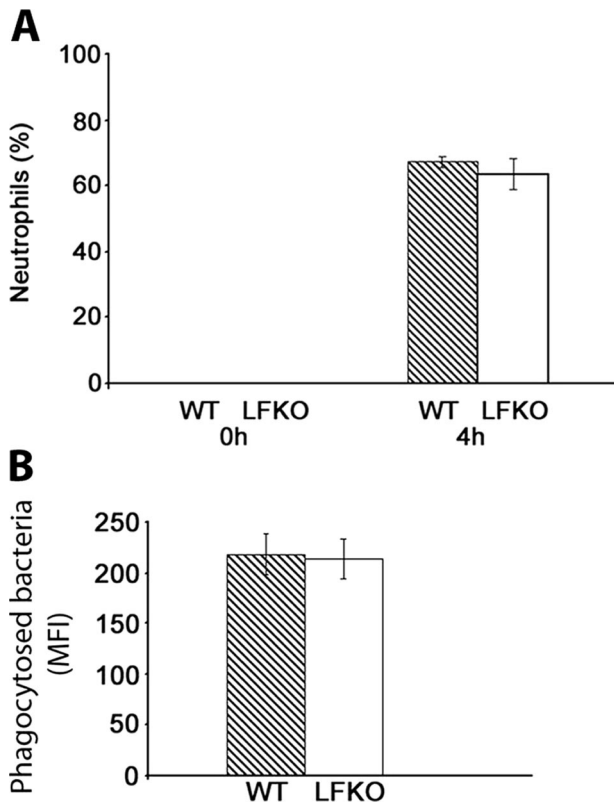
**Figure 5.** Western immunoblot analysis of NADPH oxidase components. Purified mature bone marrow neutrophils were sonicated into loading buffer, and protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5 to 15%). Immunoblots were probed with antibodies directed against NADPH *phox* components. Blots were reprobed with GAPDH as a loading control, and relative levels of proteins was determined by using densitometric scanning. Data show the Western immunoblot results from four *WT* and four *LFKO* mice. The graph shows the quantitation of the relative amounts of the proteins ( $\pm$ SEM) in *WT* and *LFKO* samples after normalization against GAPDH. Note: For the p40<sup>phox</sup> Western, two gels were run with the first gel containing duplicate samples from two *WT* and two *LFKO* mice and the second gel containing duplicate samples from another two *WT* and two *LFKO* mice. The transferred blots were split in half and one half probed with p40<sup>phox</sup> and the other half probed with GAPDH because these two proteins are similar in size.

late challenge, similar neutrophil percentages (Figure 6A) and absolute neutrophil counts (data not shown) was observed in *WT* and *LFKO* animals indicating that LF is not required for neutrophil recruitment.

We next determined if phagocytosis was normal in *LFKO* neutrophils. Heparinized blood obtained from *WT* and *LFKO* mice was incubated with fluorescein isothiocyanate-labeled opsonized bacteria and uptake was assessed by flow cytometric analysis. The MFI, corresponding to the number of ingested bacteria per neutrophil, was similar for both *WT* and *LFKO* mice (Figure 6B). In addition, no difference in the number of neutrophils undergoing bacterial phagocytosis at 37°C were observed between the two genotype groups (data not shown) demonstrating that LF is not required for the uptake of opsonized bacteria by neutrophils.

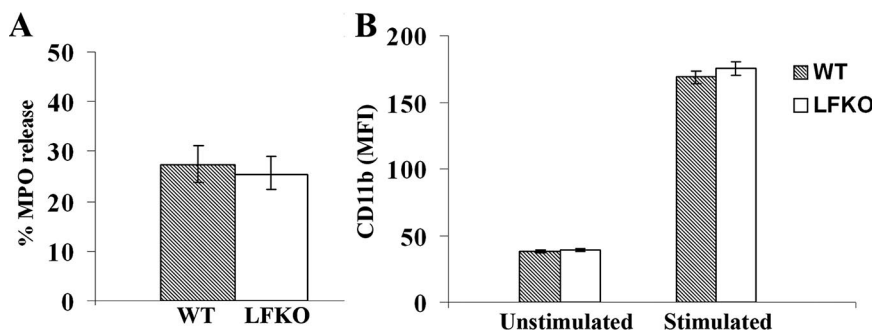
#### *LFKO* Neutrophils Show Normal Granule Mobilization

On stimulation with cytochalasin B and the chemoattractant, fMLP, neutrophils release the contents of their pri-



**Figure 6.** Normal migration and phagocytosis of *LFKO* neutrophils. **A:** Inflammatory influx of neutrophils into the peritoneal cavity after thioglycollate administration. *WT* and *LFKO* mice were injected with thioglycollate and euthanized at the time points indicated. The percentage of neutrophils was obtained from cytospin differentials. Data are represented as mean  $\pm$  SEM. Six *WT* and five *LFKO* mice were used for the 0-hour time point and 10 *WT* and 7 *LFKO* mice were used for the 4-hour time point. **B:** Phagocytosis of peripheral blood neutrophils. Heparinized blood from *WT* and *LFKO* mice was incubated with fluorescein isothiocyanate-labeled bacteria at 37°C for 60 minutes and analyzed by flow cytometric analysis. Neutrophils were distinguished based on light scatter properties. Data show the MFI, corresponding to the number of ingested bacteria per neutrophil, from *WT* and *LFKO* mice ( $n = 12$  per genotype). Data are represented as mean  $\pm$  SEM.

mary granules into the extracellular space.<sup>62</sup> To determine whether LF was required for primary granule exocytosis, we assessed the levels of the major primary granule protein, MPO, in the supernatants of cytochalasin B/fMLP-stimulated neutrophils. No differences were observed in MPO levels in the supernatants of stimulated neutrophils from *WT* and *LFKO* mice (Figure 7A) indicating that the loss of LF does not affect primary granule release.



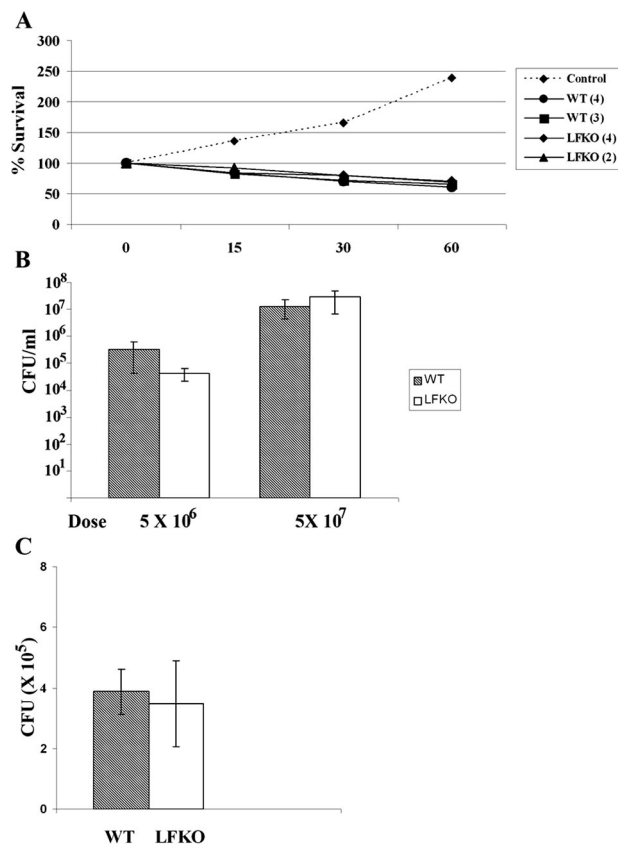
**Figure 7.** Primary granule myeloperoxidase secretion and CD11b up-regulation is normal in *LFKO* neutrophils. **A:** Purified bone marrow neutrophils ( $5 \times 10^6$ /ml) from *WT* and *LFKO* mice were incubated with 5  $\mu$ g/ml cytochalasin B and 5  $\mu$ mol/L fMLP for 15 minutes, and supernatants were collected and analyzed for secretion of (MPO). Data represent the mean percentage of MPO released ( $\pm$ SEM) relative to total cellular MPO. Neutrophils from six *WT* and six *LFKO* mice were used for this analysis. **B:** Expression of CD11b on the surface of resting and PMA-activated neutrophils was detected by staining with phycoerythrin-conjugated anti-CD11b followed by flow cytometric analysis. Data represent the mean ( $\pm$ SEM) of the MFI for CD11b on *WT* and *LFKO* neutrophils ( $n = 6$  per genotype).

CD11b is an integrin that is stored primarily in the secondary and secretory granules of unstimulated neutrophils with lower levels located at the cell surface. On neutrophil activation, the granule CD11b stores are mobilized resulting in an up-regulation of CD11b at the cell surface. To determine whether there was any alteration in the activation status or mobilization of the secondary and secretory granules of *LFKO* neutrophils, we compared the expression of CD11b, on resting and PMA-activated *WT* and *LFKO* neutrophils. Under resting conditions, a similar level of cell surface CD11b was detected in *WT* and *LFKO* neutrophils (Figure 7B). On PMA stimulation, a dramatic increase (4.4-fold) in surface expression of CD11b was detected in *WT* neutrophils reflecting the translocation of neutrophil intracellular granule reserves of this protein to the cell surface.<sup>63</sup> A similar increase (4.48-fold) of cell surface expression of CD11b was also detected in *LFKO* neutrophils indicating that secondary/secretory granule mobilization in PMA-stimulated *LFKO* neutrophils is indistinguishable from *WT* neutrophils.

#### *LFKO* Mice Display No Increased Susceptibility to Acute *S. aureus* and *P. aeruginosa* Challenge

A hallmark of specific granule deficiency is the increased incidence of opportunistic bacterial infections with *S. aureus* and *P. aeruginosa* being two of the most prevalent pathogens identified.<sup>14, 17–20</sup> *LFKO* mice displayed an approximately twofold higher incidence of opportunistic infections than *WT* mice, with 8 of 99 *LFKO* mice infected between 3 to 15 months as compared to 4 of 101 mice infected in this age group for the *WT* group that were housed under similar conditions. The abscesses presented around the eye or mouth and in some cases, more than one site of abscess formations were present in the same animal. In the majority of cases, microbiological analysis demonstrated that the bacterium responsible for the abscess was the Gram-positive opportunistic pathogen, *Staphylococcus*, and in which further conclusive identification was performed, *S. aureus* was the identified species. Although the increased incidence of infection in *LFKO* mice did not reach statistical significance, these findings are noteworthy considering the fact that *LFKO* mice displayed this increased susceptibility to infection even while housed in an SPF facility.

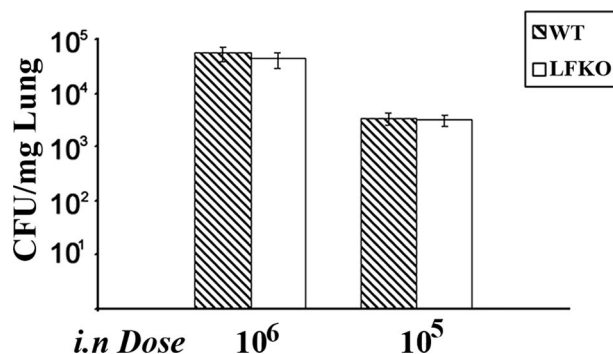
We next assessed the ability of *LFKO* neutrophils to kill *S. aureus* using both *in vitro* and *in vivo* models of *S. aureus*



**Figure 8.** Antimicrobial response to acute *S. aureus* infection is similar in *WT* and *LFKO* mice. **A:** *In vitro* bactericidal assay. Log-phase growth *S. aureus* cells were incubated alone (control) or with purified *WT* or *LFKO* neutrophils. Aliquots were removed at 0, 15, 30, and 60 minutes, serially diluted, and plated on brain-heart infusion agar plates. Bacterial colonies were enumerated 24 hours later. Neutrophils used in this assay came from pooled samples obtained from two to four mice (number of mice indicated in brackets after genotype). The assay was repeated twice with similar results. **B:** *In vivo S. aureus* infection. *WT* and *LFKO* mice were infected in an airpouch with *S. aureus* at the doses indicated. Results are represented as the mean CFUs per ml of lavage fluid  $\pm$  SEM. Eight *WT* and seven *LFKO* mice were used for the  $5 \times 10^6$  CFU dose and eight mice per genotype were used for the  $5 \times 10^7$  CFU dose. **C:** *WT* and *LFKO* mice were infected intraperitoneally with *S. aureus* ( $5 \times 10^7$  CFUs). Peritoneal fluid was extracted 4 hours after infection and bacterial CFUs were recorded. Results represent mean CFUs per mouse  $\pm$  SEM. Six *WT* and six *LFKO* mice were used for the analysis.

infection. The results of this analysis are shown in Figure 8. The bactericidal activity of *LFKO* neutrophils against opsonized *S. aureus* in an *in vitro* assay was similar to that of *WT* neutrophils (Figure 8A). Further, no difference in bacterial CFUs was observed between *WT* and *LFKO* mice using either an airpouch (Figure 8B) or intraperitoneal model (Figure 8C) of acute *S. aureus* infection that are highly contingent on neutrophil-dependent clearance of the bacteria.

Finally, we determined the effect of LF deficiency on susceptibility to an acute bacterial infection with the Gram-negative pathogen, *P. aeruginosa*. *WT* and *LFKO* mice were challenged intranasally with *P. aeruginosa* ( $\sim 10^5$  or  $10^6$  CFUs) and bacterial counts were assessed 6 hours later. No difference in bacterial killing was observed between the two groups with a similar number of CFUs observed in lung homogenates from *WT* and *LFKO* mice (Figure 9). Taken together, these results suggests



**Figure 9.** Acute *P. aeruginosa* lung challenge. *WT* and *LFKO* mice were infected intranasally with *P. aeruginosa* at the doses indicated. Results are represented as the mean CFUs per mg of lung tissue  $\pm$  SEM. Eight mice per genotype were used for the  $10^6$  CFU dose and 14 *WT* and 16 *LFKO* mice were used for the  $10^5$  CFU dose.

that the antimicrobial function of LF may be more responsible for surveillance against opportunistic infections in the exocrine secretions rather than contributing to the host defense response of the neutrophil against infections with high doses of bacteria.

#### Alterations in Peripheral White Blood Cells in *LFKO* Mice

To determine whether LF was required for normal neutrophil cell distributions, we examined the percentages of white blood cells in the peripheral blood of *WT* and *LFKO* mice. The total white blood cell (WBC) counts were slightly decreased in *LFKO* mice versus *WT* controls (*WT*,  $7.7 \pm 0.4$  versus *LFKO*,  $6.5 \pm 0.4$ ;  $P = 0.046$ ). Interestingly, a decrease in lymphocyte percentages and a more marked elevation in neutrophil and eosinophil percentages were also observed in response to LF ablation (Table 1) suggesting that LF may play an immunomodulatory role in these leukocyte cell populations.

#### Discussion

In the present study, we have used *LFKO* mice to decipher the role of LF in the host defense response of the neutrophil, with particular emphasis on determining its contribution to the phenotype of SGD syndrome. Previous studies have shown that *C/EPB $\epsilon$*  is required for terminal differentiation of neutrophils and for elab-

**Table 1.** White Blood Cell Indices in *WT* and *LFKO* Mice

Percent	<i>WT</i>	<i>LFKO</i>
Lymphocytes	$84.8 \pm 1.0^*$	$79.6 \pm 1.4$
Neutrophils	$8.7 \pm 0.8^*$	$12.7 \pm 1.1$
Monocytes	$1.5 \pm 0.1$	$1.7 \pm 0.2$
Eosinophils	$1.8 \pm 0.2^*$	$3.4 \pm 0.4$
Basophils	$0.6 \pm 0.1$	$0.6 \pm 0.1$
Large unstained cells	$2.7 \pm 0.3$	$2.0 \pm 0.2$

Mice (*WT*,  $n = 46$ ; *LFKO*,  $n = 50$ ) were between 7 and 11 weeks of age at the time of analysis. Values are presented as mean of percent cells  $\pm$  SEM.

\*Significant difference ( $P < 0.05$ ) between *WT* and *LFKO*.



oration of a variety of neutrophil functions including migration, phagocytosis, oxidative burst, and antimicrobial activity.<sup>9,14,24,25</sup> Of these activities, we show here that the major secondary granule protein, LF, is a stimulus-dependent regulator of the oxidative burst response of the differentiated neutrophil, but it is not required for neutrophil maturation, migration, phagocytosis, granule mobilization and release, or for antimicrobial activity against acute *S. aureus* or *P. aeruginosa* bacterial challenges.

Intriguingly, we show that the oxidative burst is impaired in *LFKO* neutrophils in response to treatment with PMA, supporting an essential role for this protein in the production and/or metabolism of reactive oxygen species after stimulation with this potent protein kinase C agonist.<sup>64</sup> Interestingly, despite the latter defect, we show that the oxidative burst of neutrophils in response to opsonized *S. aureus* does not require LF. The reason for this selective stimulus-dependent defect is currently unknown but it has been reported previously that activation of the oxidative burst in neutrophils can result in quantitative, qualitative, and subcellular differences in the pattern of reactive oxygen species formed depending on the stimulus used.<sup>50,64–66</sup> Future work is warranted to determine the exact cellular and molecular mechanism by which LF regulates the production and/or metabolic fate of reactive oxygen species and to determine whether its ability to bind iron and/or impinge on cellular signaling pathways<sup>67–71</sup> plays a role in this regulation. The negative effect of LF ablation on the oxidative burst activity is at variance with a study by Hock and colleagues<sup>72</sup> in which expression of neutrophil secondary granule proteins, including LF, was ablated secondary to disruption of the transcription factor, Gfi-1, and normal oxidative burst activity was reported in response to PMA stimulation. This discrepancy may be explained by technical differences in the measurement of the reactive oxygen species and/or may be more reflective of the fact that Gfi-1-deficient atypical myeloid cells have acquired traits of the monocyte/macrophage lineage, which may compensate for the lack of LF.

*LFKO* mice displayed an approximately twofold increased incidence of spontaneous abscess formation compared to *WT* control mice housed in the same SPF facility. The bacterium identified in the majority of the abscesses was *Staphylococcus* and in which species determination was performed, *S. aureus* was the species identified. *Staphylococcus* infections have also been observed in SGD patients<sup>17,18,20</sup> and a decreased intracellular bactericidal activity against this pathogen was reported *in vitro* using neutrophils from *C/EPBε* knockout mice.<sup>25</sup> However, the incidence of opportunistic infection in *LFKO* mice was not statistically significant and was lower (~8% of mice aged 3 to 15 months) than that reported for *C/EPBε* knockout mice (60% of mice by 2 to 5 months) housed under similar SPF conditions<sup>14</sup> or SGD patients of which all patients are infected early in life.<sup>8,9,17</sup> In addition, the neutrophil oxidative burst response to opsonized *S. aureus* was similar in *WT* and *LFKO* mice and neutrophils from both genotype groups exerted a similar bactericidal response against *S. aureus in vitro*.

Further, no increased susceptibility to acute challenges with *S. aureus* in *LFKO* mice was observed using either airpouch or intraperitoneal models of infection that are highly dependent on neutrophils for killing. These results, together with the likely oral and ocular origins of the spontaneous *S. aureus* abscesses observed in *LFKO* mice, suggest that LF may be redundant for the antimicrobial response of the neutrophil against high doses of this pathogen whereas LF may play a more nonredundant role in the sentinel antimicrobial defenses at the mucosal surface. Future work is warranted to examine the protective role of LF by monitoring spontaneous infection rates in larger groups of *LFKO* and *WT* mice housed in nonbarrier facilities.

LF has previously been postulated to play a role in lymphocyte and neutrophil development and function<sup>67–71</sup> and in eosinophil activation.<sup>73</sup> Interestingly, peripheral blood analysis uncovered a significant decrease in the white blood cells of *LFKO* mice. Increases in the percentages of neutrophils and eosinophils (~1.5-fold and 2-fold elevation, respectively, versus *WT* controls) and slighter decreases in the percentage of lymphocytes were also observed on LF ablation. Although the physiological significance of these findings remains to be established, these results suggest that LF may play an immunomodulatory role in these leukocyte subtypes.

In sum, the results presented here demonstrate that LF is a positive modulator of the neutrophil oxidative burst in response to PMA stimulation. However, LF is dispensable for many neutrophil functions and LF ablation alone is not sufficient to recapitulate the disease severity of SGD syndrome. This suggests that the diverse array of antimicrobial defense mechanisms present in the neutrophil provides considerable redundancy in the host response to infection. The observation that *LFKO* mice trend toward increased incidence of spontaneous infections suggest that LF may play an important role in the antimicrobial surveillance at the mucosal barrier, protecting against opportunistic infections, in particular, those caused by *S. aureus*. Finally, the alterations observed in the leukocytes in the peripheral blood of *LFKO* mice suggest that LF might play a broader regulatory role in the immune system.

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