

Pioglitazone restores phagocyte mitochondrial oxidants and bactericidal capacity in chronic granulomatous disease

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Background: Deficient production of reactive oxygen species (ROS) by the phagocyte nicotinamide adenine dinucleotide (NADPH) oxidase in patients with chronic granulomatous disease (CGD) results in susceptibility to certain pathogens secondary to impaired oxidative killing and mobilization of other phagocyte defenses. Peroxisome proliferator-activated receptor (PPAR) γ agonists, including pioglitazone, approved for type 2 diabetes therapy alter cellular metabolism and can heighten ROS production. It was hypothesized that pioglitazone treatment of gp91^{phox-/-} mice, a murine model of human CGD, would enhance phagocyte oxidant production and killing of *Staphylococcus aureus*, a significant pathogen in patients with this disorder.

Objectives: We sought to determine whether pioglitazone treatment of gp91^{phox-/-} mice enhanced phagocyte oxidant production and host defense.

Methods: Wild-type and gp91^{phox-/-} mice were treated with the PPAR γ agonist pioglitazone, and phagocyte ROS and killing of *S aureus* were investigated.

Results: As demonstrated by 3 different ROS-sensing probes, short-term treatment of gp91^{phox-/-} mice with pioglitazone enhanced stimulated ROS production in neutrophils and monocytes from blood and neutrophils and inflammatory macrophages recruited to tissues. Mitochondria were identified as the source of ROS. Findings were replicated in human monocytes from patients with CGD after *ex vivo* pioglitazone treatment. Importantly, although mitochondrial (mt)ROS were deficient in gp91^{phox-/-} phagocytes, their

restoration with treatment significantly enabled killing of *S aureus* both *ex vivo* and *in vivo*.

Conclusions: Together, the data support the hypothesis that signaling from the NADPH oxidase under normal circumstances governs phagocyte mtROS production and that such signaling is lacking in the absence of a functioning phagocyte oxidase. PPAR γ agonism appears to bypass the need for the NADPH oxidase for enhanced mtROS production and partially restores host defense in CGD. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Chronic granulomatous disease, phagocytes, mitochondria, oxidants, thioglitazones

Chronic granulomatous disease (CGD) is a rare disease attributed to mutations in the genes encoding components of the phagocyte nicotinamide adenine dinucleotide (NADPH) oxidase. The inability of phagocytes¹⁻³ to mount an oxidative burst predisposes to infections with certain bacterial and fungal pathogens, such as *Staphylococcus aureus* and *Aspergillus* species, which are most evident in the lung, skin, lymph nodes, and liver,⁴ and even low levels of residual reactive oxygen species (ROS) production by phagocytes are associated with improved survival.⁵ In addition to direct oxidative killing of pathogens, phagocyte ROS orchestrate other antimicrobial defenses, such as activation of antimicrobial proteins within the phagolysosome⁶ and recruitment of antimicrobial autophagocytic machinery to phagolysosomes. These are deficient in phagocytes from patients with CGD.⁷⁻⁹

Pioglitazone and other thioglitazones are so-called nutrient-restricting drugs approved for type 2 diabetes. As peroxisome proliferator-activated receptor (PPAR) γ agonists, they slowly mimic the actions of insulin with increased peripheral glucose disposal. Additionally, their nutrient restriction signaling is associated with altered macrophage metabolism, such as activation of adenosine monophosphate-activated protein kinase (AMPK),¹⁰ and various anti-inflammatory activities.^{11,12} Intriguingly, PPAR γ agonism has also been associated with enhanced host defense against *Candida albicans*,¹³⁻¹⁵ *Staphylococcus aureus*,¹⁶ *Klebsiella pneumoniae*,¹⁷ and *Streptococcus pneumoniae*.¹⁸ Two mechanisms have been proposed: enhanced pathogen containment by means of phagocytosis and encapsulation and enhanced ROS production by phagocytes (or both), although the source was not identified. With regard to ROS production, PPAR γ ligands/agonists are reported to decrease expression of NADPH oxidase subunits of 22 and 47 kDa in some cells^{19,20} but have also been shown to increase mitochondrial reactive oxygen species (mtROS) output in others.^{21,22} Recent data implicating nutrient restriction or “starvation signaling” in the enhancement of mtROS production and entrainment of mtROS

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Abbreviations used

BADGE: Bisphenol A diglycidyl ether
 CFU: Colony-forming units
 CGD: Chronic granulomatous disease
 DHR: Dihydrorhodamine
 DPI: Diphenylene iodonium
 mtROS: Mitochondrial reactive oxygen species
 NADPH: Nicotinamide adenine dinucleotide
 PMA: Phorbol 12-myristate 13-acetate
 PPAR: Peroxisome proliferator-activated receptor
 ROS: Reactive oxygen species
 SOD: Superoxide dismutase
 WT: Wild-type

for killing of intracellular and extracellular bacteria²³ suggest a possible mechanism through which PPAR γ agonists might contribute to host defense.

Given these observations, we questioned whether pioglitazone treatment of gp91^{phox-/-} mice would enhance oxidant production by phagocytes from patients with CGD and, if so, bolster host defense. We found that pioglitazone treatment induced mtROS production in stimulated neutrophils, monocytes, and inflammatory macrophages of mice with CGD, as well as normal mice, and importantly, the restored phagocytes demonstrated significantly enhanced killing of *S aureus* both *in vitro* and *in vivo*. Crosstalk between the NADPH oxidase and mtROS leading to optimal antimicrobial responses by normal phagocytes was also shown. Consequently, in the absence of a functional NADPH oxidase, this signaling is lost but can be restored at the level of mtROS production by pioglitazone treatment.

METHODS**Animals**

Male C57BL/6 and gp91^{phox-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, Me) or bred in house. They received care in accordance with the institutional animal care and use committee and were given pioglitazone (10 mg/kg/d); bisphenol A diglycidyl ether (BADGE), a PPAR γ antagonist; or vehicle (carboxymethyl cellulose) by oral gavage for 5 days unless otherwise indicated. All agents were well tolerated. Mice were killed with CO₂ inhalation.

Reagents

Pioglitazone, BADGE, MitoTEMPO, phorbol 12-myristate 13-acetate (PMA), diphenylene iodonium (DPI), catalase, and superoxide dismutase (SOD) were from Sigma (St Louis, Mo). Conjugated antibodies to CD115, F4/80, Ly6G, and CD11b were from eBioscience (San Diego, Calif). Antibodies to Nox1, Nox4, Duox, gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} were from Santa Cruz Biotechnology (Santa Cruz, Calif). Zymosan, dihydrorhodamine (DHR), MitoTracker Green, and MitoSOX Red were from Life Technologies (Grand Island, NY).

Isolation of blood and peritoneal leukocytes

Red blood cells were lysed (Pharmlyse; BD Biosciences, San Jose, Calif) from whole blood from mice (terminal cardiac puncture) before staining with markers and analysis by flow cytometry (CyAn ADP analyzer, BD Biosciences). Peritoneal cells were harvested by lavage from mice injected intraperitoneally with 1 mg/mL zymosan in PBS. Cells were washed, suspended in PBS (3% FBS), blocked with anti-mouse Fc (CD16/32,

eBioscience) for 30 minutes, and stained for 1 hour on ice with conjugated antibodies before flow cytometry.

ROS detection

DHR analysis was performed using phagocytes (10⁶) incubated for 15 minutes with 5 μ mol/L DHR (PBS, 0.05% gelatin, 0.09% glucose, and 1 mmol/L EDTA) and stimulated with PMA (200 ng/mL) for 15 minutes at 37°C. Cells were washed and analyzed by flow cytometry.

Cytochrome c reduction, measuring superoxide release, was performed as previously described.²⁴

mtROS were detected with MitoSOX Red. Cells (10⁶) were incubated (37°C) with 25 nmol/L MitoTracker Green in the dark (Dulbecco modified Eagle medium and 10% FBS) followed by 4 μ mol/L MitoSox Red (15 minutes each), washed with PBS, and analyzed by flow cytometry or Zeiss LSM 700 confocal microscopy.

Bactericidal assays

***In vitro* assay.** Peritoneal phagocytes (2 $\times$ 10⁵, 10 hours after zymosan) were cocultured with 2 $\times$ 10⁶ colony-forming units (CFU) of *S aureus* (strain 502A, ATCC #27217) grown overnight in Lauryl Broth and washed twice with saline in 100 μ L of RPMI (phenol red free 1% mouse serum) at 37°C for 1 hour. Phagocytes were lysed (1 mL of water [pH 11]²⁵), bacteria were pelleted at 10,000g for 10 minutes (2 times), and washed with 1 mL of saline. Bacterial numbers were determined by using a modified Alamar blue assay.²⁶ This assay was similarly adapted for killing of *Burkholderia cepacia* (ATCC #15416).

***In vivo* assay.** *S aureus* peritonitis was induced, as described by Pollock et al,²⁷ with minor modifications. Briefly, mice were injected intraperitoneally with 0.5 mL of 2 $\times$ 10⁷ CFU/mL *S aureus* (saline), peritonea underwent lavage at designated times, and cell counts and viable bacteria in lavage fluid and cells (after lysis as above) were determined.

Human blood monocytes

Heparinized blood was obtained from patients with X-linked CGD and healthy control subjects at the National Institutes of Health (Bethesda, Md) after approval of the institutional review board.

The blood was express shipped overnight to Denver, Colorado. PBMCs were isolated using Percoll gradients,²⁸ plated in X-vivo¹⁰ to adhere monocytes for 2 hours, washed (5 times) to remove nonadherent cells, and cultured at 37°C in a 10% CO₂ atmosphere without and with pioglitazone (10 μ mol/L). Cells were then stimulated with PMA and mtROS detected using flow cytometry, as above.

Statistics

Each experiment was performed 3 to 5 times, unless otherwise indicated. Analysis and *P* value calculations were conducted by means of ANOVA (JMP statistical program 4.0.1; SAS Institute, Cary, NC). The Wilcoxon matched-pairs signed-rank test was used for single and multiple comparisons. A *P* value of .05 or less was considered significant. Data are reported as means $\pm$ SEMs.

RESULTS***In vivo* treatment with pioglitazone enhances blood neutrophil and monocyte ROS production**

Wild-type (WT) and gp91^{phox-/-} mice were treated with either pioglitazone or vehicle by oral gavage for 5 days to test whether phagocyte oxidant production would be enhanced/restored. Previously, this treatment resulted in PPAR γ activation in inflammatory macrophages and accelerated resolution of sterile peritonitis in mice with CGD.²⁹ After treatment, blood leukocytes were

collected, incubated with DHR, stimulated with the protein kinase C activator PMA, and analyzed for conversion to rhodamine by flow cytometry. By using this classic test of stimulated ROS production, leukocytes from WT mice demonstrated enhanced DHR fluorescence, as expected (Fig 1, A), and pioglitazone treatment had a minimal effect. Leukocytes from vehicle-treated $gp91^{phox-/-}$ mice showed no evidence of stimulated ROS production. In contrast, a subpopulation of leukocytes from pioglitazone-treated $gp91^{phox-/-}$ mice had significantly enhanced DHR fluorescence after stimulation, indicating some restoration of oxidant production in cells lacking a functional NADPH oxidase.

Blood leukocytes were stained with antibodies to surface markers before DHR loading and stimulation to investigate cell types producing oxidants (see Fig E1, gating strategy, in this article's Online Repository at www.jacionline.org). As expected, nearly all PMA-stimulated neutrophils ($Ly6G^+$) and 90% of monocytes ($CD115^+$) from WT mice, regardless of treatment, demonstrated stimulated ROS production, and these cells from vehicle-treated $gp91^{phox-/-}$ mice showed little enhancement (Fig 1, B-D). In contrast, 27% of neutrophils and 32% of

monocytes from pioglitazone-treated knockout mice demonstrated increased DHR fluorescence comparable with that seen in the respective populations of stimulated WT phagocytes. Lymphocytes of either genotype did not demonstrate increased DHR fluorescence after stimulation (data not shown).

Pioglitazone doses ranging from 1 to 10 mg/kg/d were tested to simulate levels achieved during treatment of human subjects to determine the optimal pioglitazone dose and treatment duration for maximal ROS production.^{30,31} A dose-dependent decrease in the percentage of stimulated $gp91^{phox-/-}$ neutrophils and monocytes producing ROS was observed at 3 and 1 mg/kg/d pioglitazone (see Fig E2 in this article's Online Repository at www.jacionline.org). Mice were also treated with pioglitazone (10 mg/kg/d) or vehicle for up to 14 days to ensure steady-state levels before oxidant production was assessed. Maximal stimulated ROS production by $gp91^{phox-/-}$ leukocytes was evident by 5 days (see Fig E3 in this article's Online Repository at www.jacionline.org). Thus further experiments were conducted after 5 days of treatment at 10 mg/kg/d, as in initial experiments.

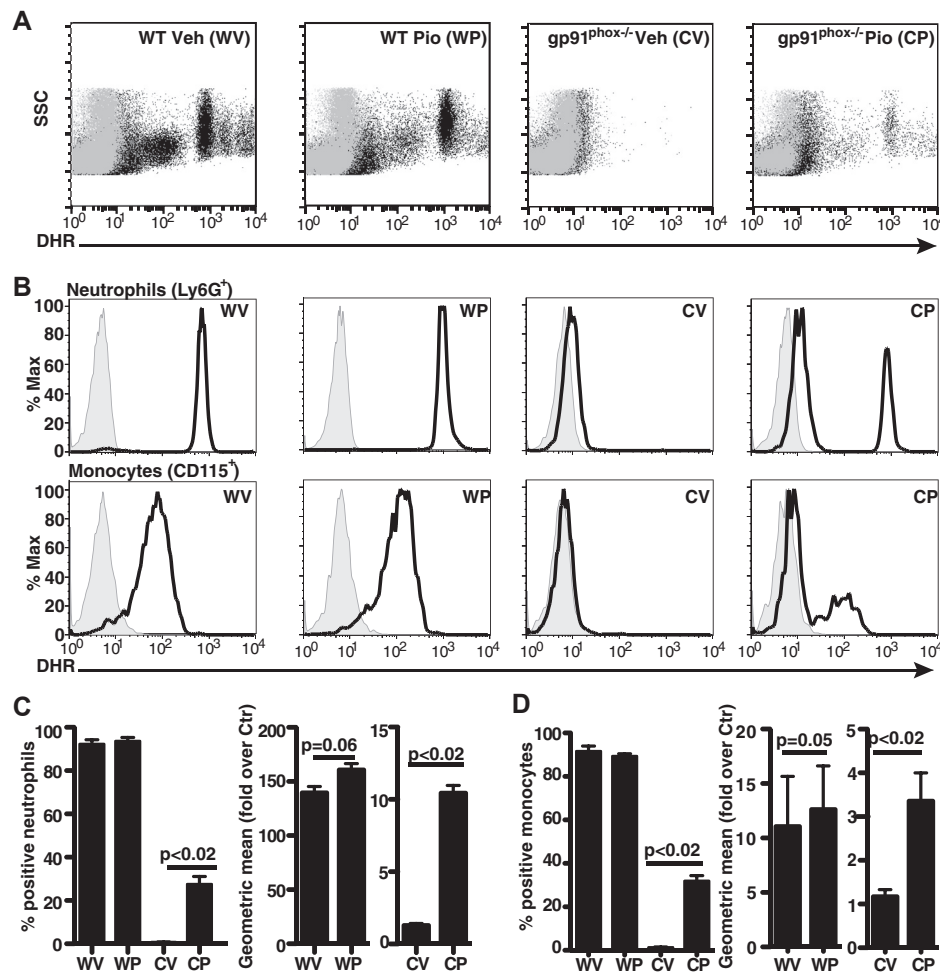


FIG 1. Pioglitazone (Pio) pretreatment of mice enhances PMA-stimulated ROS production by $gp91^{phox-/-}$ neutrophils and monocytes. ROS was measured by means of DHR in blood leukocytes from vehicle (V)- or pioglitazone (P)-treated WT (WV and WP, respectively) and $gp91^{phox-/-}$ (CV and CP, respectively) mice. **A**, Representative dot plots. Gray, No PMA; black, with PMA. **B**, Representative histograms for neutrophils and monocytes. Gray, No PMA; black, with PMA. **C** and **D**, Aggregate data for neutrophils (Fig 1, C) and monocytes (Fig 1, D) are shown as the percentage of cells exhibiting enhanced fluorescence and change in geometric mean fluorescence with PMA. N = 8 mice per group.

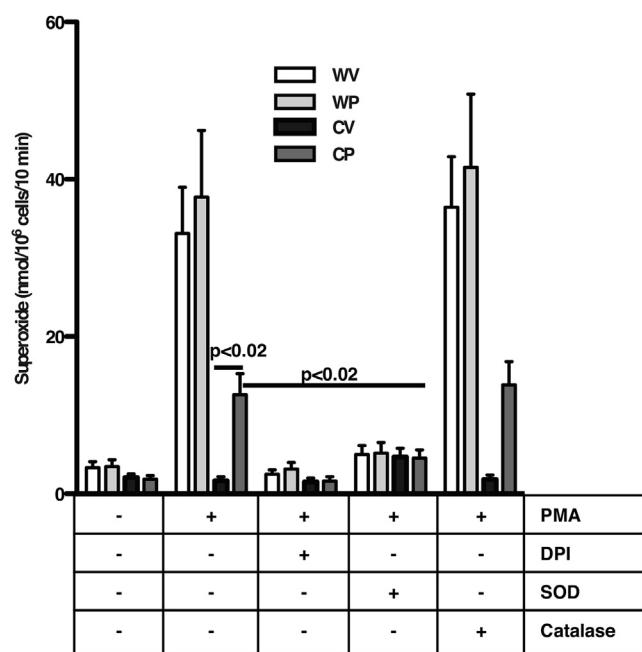


FIG 2. Pioglitazone pretreatment enhances superoxide production by recruited phagocytes from $gp91^{phox-/-}$ mice. Ten hours after intraperitoneal zymosan injection, phagocytes were harvested from vehicle (V)- or pioglitazone (P)-treated WT (WV and WP, respectively) or $gp91^{phox-/-}$ (CV and CP, respectively) mice. Phagocytes were stimulated with PMA in the presence and absence of inhibitors, and oxidants were measured by the reduction of cytochrome c. N = 9 mice per group.

Recruited phagocytes from pioglitazone-treated $gp91^{phox-/-}$ mice show enhanced superoxide production

Greater numbers of phagocytes were needed than were available from blood, and therefore a sterile peritonitis model was used to recruit phagocytes to further investigate oxidant production and potential sources of oxidants. Recruited phagocytes from pioglitazone- or vehicle-treated $gp91^{phox-/-}$ and WT mice were quite comparable in numbers, types, and zymosan ingestion at 6 to 10 hours after zymosan injection (see Fig E4 in this article's Online Repository at www.jacionline.org). Recruited neutrophils ($Ly6G^+$) and inflammatory macrophages ($F4/80^{lo+}$) derived from recruited monocytes¹⁸ were incubated with DHR, stimulated with PMA, and analyzed by flow cytometry, with results similar to those of the blood phagocytes from both genotypes and treatment groups (see Fig E4, D).

The nature of ROS produced in recruited phagocytes was then investigated by using cytochrome c reduction, a relatively specific assay for superoxide. Significant release of stimulated oxidants from vehicle- and pioglitazone-treated WT phagocytes was demonstrated (Fig 2). As in the DHR assay, partial restoration of stimulated ROS production from pioglitazone-treated $gp91^{phox-/-}$ phagocytes was evident. Pretreatment with SOD, but not catalase, inhibited ROS production, supporting superoxide production. The nonspecific flavochrome inhibitor DPI^{32,33} ablated superoxide production by the NADPH oxidase of WT phagocytes and, interestingly, also inhibited oxidant production by pioglitazone-restored $gp91^{phox-/-}$ leukocytes, suggesting a flavochrome as the source.

Flow cytometry and RT-PCR were used to investigate several flavochromes as potential sources of ROS (see Fig E5 in this

article's Online Repository at www.jacionline.org). These included the NADPH oxidases and associated components, the dual oxidases, and other flavochromes. Absence of $gp91^{phox}$ protein was verified in the phagocytes of $gp91^{phox-/-}$ mice with and without pioglitazone treatment. Pioglitazone did not decrease $p22^{phox}$ or $p47^{phox}$ expression, as has been described in other systems.^{19,20} Other NADPH oxidases and dual oxygenases were either undetectable or unchanged after pioglitazone treatment. Pretreatment of the cells with inhibitors for inducible nitric oxide synthase, 12/15 lipoxygenase, cytosolic phospholipase A₂, and COX used at standard doses had no effect on stimulated oxidant production, suggesting a source other than these for oxidant production (data not shown).

Pioglitazone treatment enhances mtROS production in activated phagocytes

PPAR γ agonists have been shown to affect mitochondrial biogenesis and functions in various systems.^{21,34} Mitochondria are rich sources of flavochrome enzymes and have several sites for superoxide production.³⁵⁻³⁷ By using MitoTracker Green, a dye selectively taken up by mitochondria, mitochondrial content was investigated in blood leukocytes of pioglitazone- and vehicle-treated mice. Pioglitazone had little effect on mitochondrial content in blood phagocytes from either WT or $gp91^{phox-/-}$ mice (see Fig E6 in this article's Online Repository at www.jacionline.org).

mtROS production was next assessed by using the probe MitoSOX Red, a triphenylphosphonium-linked dihydroethidium (DHE) compound that concentrates within mitochondria and fluoresces red when oxidized by ROS. Harvested peritoneal phagocytes were loaded with MitoTracker Green and then MitoSOX Red and immediately analyzed by fluorescence microscopy without further stimulation. Recruited phagocytes harvested from vehicle- or pioglitazone-treated WT mice showed heterogeneity in MitoSOX Red staining, with some cells having detectable colocalization of MitoSOX Red with MitoTracker Green and others not. This was irrespective of whether they contained detectable ingested zymosan particles (Fig 3, top 2 rows and insets). Notably, zymosan particles, either free or within the phagolysosome, took up the stains and fluoresced brightly in the green channel and to a lesser degree in the red channel, as previously reported (see Fig 3 and Fig E7, A, in this article's Online Repository at www.jacionline.org).³⁸ In contrast to WT phagocytes, recruited phagocytes of vehicle-treated $gp91^{phox-/-}$ mice showed almost no MitoSOX Red fluorescence (Fig 3, third row). Pioglitazone treatment of $gp91^{phox-/-}$ mice resulted in clearly detectable MitoSOX Red staining of some leukocytes (Fig 3, bottom row), again irrespective of whether they contained obvious zymosan. Flow cytometric analysis of the inflammatory cells quantified and corroborated these findings (Fig 4). First, heterogeneity in MitoSOX Red staining was evident for both recruited phagocyte populations, neutrophils, and inflammatory macrophages but was significantly higher for WT phagocytes than for $gp91^{phox-/-}$ phagocytes. Second, pioglitazone treatment, as compared with vehicle treatment, enhanced MitoSOX Red fluorescence, and this was evident for both WT and $gp91^{phox-/-}$ neutrophils and inflammatory macrophages.

mtROS staining was also assessed after *ex vivo* PMA stimulation (Fig 4 and see Fig E7, B). MitoSOX Red fluorescence was enhanced after stimulation of WT leukocytes regardless of treatment group and for leukocytes of pioglitazone-treated

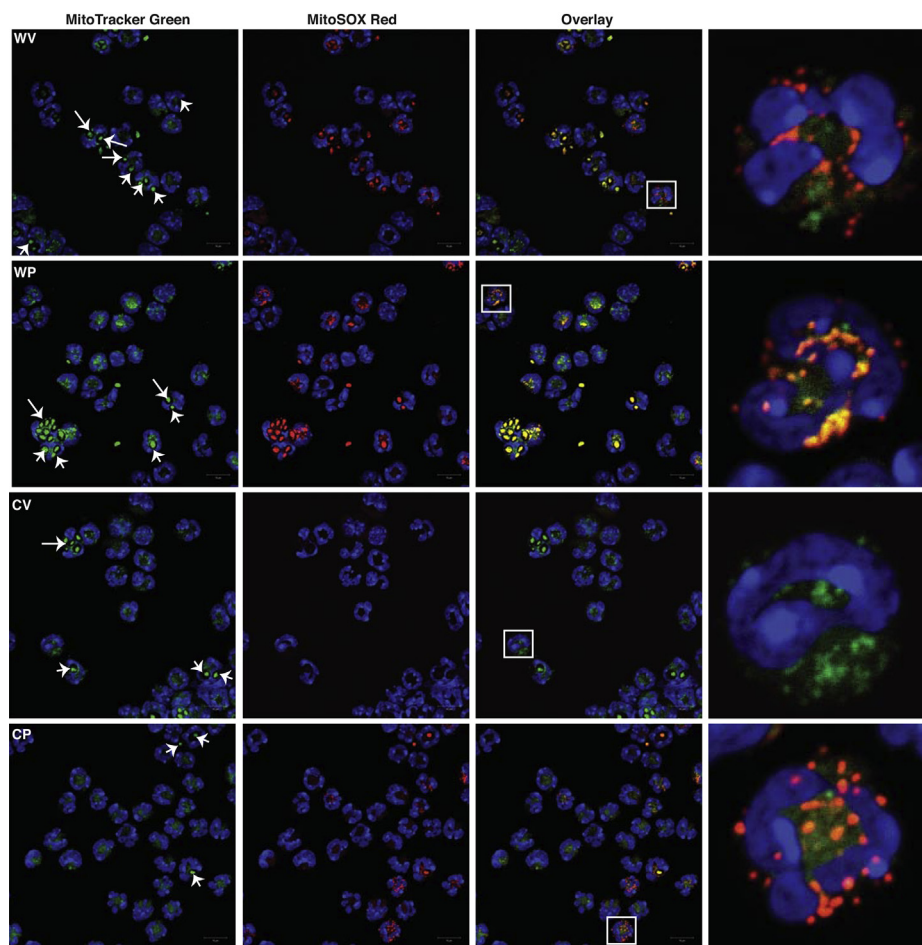


FIG 3. Pioglitazone pretreatment of $gp91^{phox-/-}$ mice enhances production of mtROS in recruited phagocytes, as in Fig 2, from vehicle (V)- or pioglitazone (P)-treated WT (WV and WP, respectively) and $gp91^{phox-/-}$ (CV and CP, respectively) mice were stained with MitoTracker Green, MitoSOX Red, and 4'-6-diamidino-2-phenylindole dihydrochloride and analyzed by confocal microscopy ($\times 63$ magnification). Last panel, High-resolution images of a single cell (white box). Arrows denote ingested zymosan. Representative images are shown. N = 3 mice per group.

$gp91^{phox-/-}$ mice. No enhancement was noted after PMA stimulation of leukocytes from vehicle-treated $gp91^{phox-/-}$ mice (Fig 4 and see Fig E7, B). The enhanced MitoSOX Red fluorescence was ablated when leukocytes were pretreated with DPI for 15 minutes before PMA stimulation. Pretreatment with MitoTEMPO, a mitochondrial antioxidant, also inhibited PMA-stimulated enhancement and suppressed MitoSOX Red fluorescence in recruited cells at baseline (Fig 4 and see Fig E7, C). Finally, rotenone, a mitochondrial complex 1 inhibitor, also suppressed MitoSOX Red staining in recruited neutrophils and inflammatory macrophages, regardless of genotype or treatment group, indicating complex 1 as a key source of mtROS production (Fig 4).

Because zymosan itself fluoresced with the mitochondrial stains (see Fig E7, A), these findings were confirmed in a zymosan-free system by using blood neutrophils and monocytes. Baseline fluorescence of blood leukocytes was generally lower than that of recruited leukocytes (Fig 5 compared with Fig 4). After PMA stimulation, marked enhancement of mtROS production was noted for both WT leukocytes and pioglitazone-restored $gp91^{phox-/-}$ leukocytes compared with that seen with leukocytes from vehicle-treated $gp91^{phox-/-}$ mice. Monocytes stained less

brightly with MitoSOX Red than neutrophils. Notably, minimal heterogeneity was seen for the phagocyte populations under these conditions: essentially all WT and pioglitazone-restored $gp91^{phox-/-}$ phagocytes responded to PMA with mtROS production. As with the recruited peritoneal leukocytes, stimulated mtROS were largely ablated by pretreating the blood cells with either DPI or MitoTEMPO. Taken together, these data demonstrate that pioglitazone treatment appears to largely restore stimulated mtROS production of $gp91^{phox-/-}$ leukocytes, both neutrophils and monocytes/inflammatory macrophages, whether circulating in the blood or recruited to inflamed tissues.

Monocytes from human patients with X-linked CGD and healthy control subjects were similarly tested for stimulated mtROS production with and without *ex vivo* treatment with pioglitazone. Untreated normal human monocytes, either freshly isolated or after several days of culture, showed marked enhancement of mtROS after PMA stimulation (Fig 6 and see Fig E8 in this article's Online Repository at www.jacionline.org). However, monocytes from patients with X-linked CGD did not show enhanced mtROS production in response to PMA (Fig 6), replicating the findings for murine monocytes. After

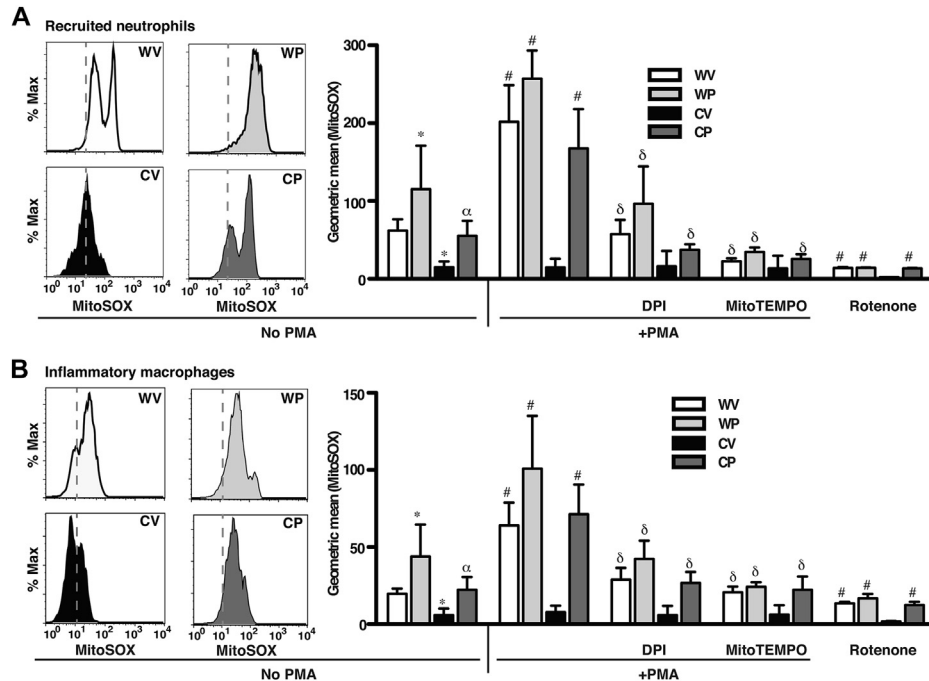


FIG 4. Pioglitazone treatment enhances production of mtROS by neutrophils (A) and macrophages (B) harvested from inflamed peritonea of WT and gp91^{phox}^{-/-} mice. Recruited phagocytes from mice (WV, WP, CV, and CP, as in Figs 2 and 3) were treated with or without DPI or MitoTEMPO (15 minutes), stained with MitoSOX and then stimulated with or without PMA, and analyzed by flow cytometry. Left, Representative histograms without PMA. The line depicts relative fluorescence of CV neutrophils or macrophages. Right, Aggregate data. N = 8 mice per group. $P \leq .02$ for comparisons as follows: *WV without PMA, αCV without PMA, #respective genotype/treatment group without PMA, and δrespective genotype/treatment group with PMA alone.

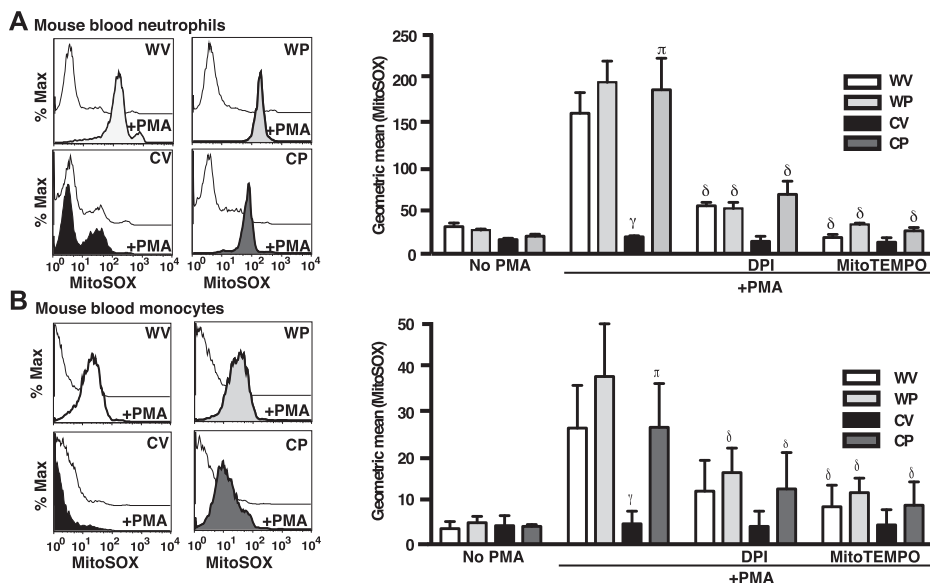


FIG 5. Pioglitazone treatment of gp91^{phox}^{-/-} mice enhances production of mtROS by stimulated blood neutrophils (A) and monocytes (B). Blood leukocytes from WT (WV and WP, respectively) and gp91^{phox}^{-/-} (CV and CP, respectively) mice, as in Fig 1, were treated with and without DPI or MitoTEMPO for 15 minutes, stained with MitoSOX Red, stimulated or not with PMA, and analyzed by means of flow cytometry. Left, Representative histograms. Right, Aggregate data. N = 8 mice per group. $P \leq .02$ for comparisons as follows: γWV with PMA, πCV with PMA, and δrespective genotype/treatment group with PMA alone.

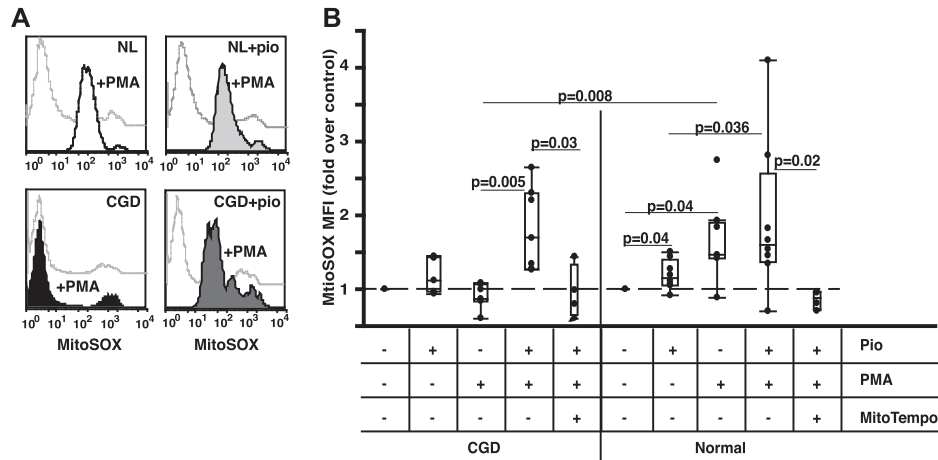


FIG 6. *Ex vivo* treatment of human monocytes from patients with CGD with pioglitazone (*Pio*) enhances stimulated mtROS production. Human monocytes from patients with X-linked CGD and healthy subjects were isolated from heparinized blood, plated, and treated with 10 μ M/L pioglitazone for 2 days. The cells were then stained with MitoSOX Red and stimulated with PMA in the presence or absence of MitoTEMPO and analyzed by flow cytometry. Representative histograms (**A**) and aggregate data normalized to each untreated control subject (**B**) are shown. N = 9 and 7 for healthy subjects (*NL*) and patients with CGD, respectively.

establishing optimal culture conditions for *ex vivo* pioglitazone treatment (10 μ M/L for 2 days, see Fig E8), human monocytes from patients with CGD were then cultured under these conditions and showed marked enhancement of mtROS production after PMA stimulation, with restoration to levels comparable with those of stimulated normal monocytes (Fig 6). As with murine cells, provision of mitoTEMPO before PMA stimulation largely ablated detection of mtROS. Of note, in the absence of phagocyte stimulation, mtROS production was only minimally enhanced by *ex vivo* culture with pioglitazone in either normal monocytes or those from patients with X-linked CGD (Fig 6), which is similar to findings after *in vivo* treatment in the murine model.

Pioglitazone enhancement of mtROS production is PPAR γ dependent

Pioglitazone has been shown to have PPAR γ -dependent and independent effects on mitochondria.^{21,22,34} PPAR γ expression is ordinarily low in monocytes and neutrophils³⁹ but upregulated in monocytes on recruitment into inflammatory tissues^{18,39} or after thioglitazone treatment.⁴⁰ As shown in Fig E9, A, in this article's Online Repository at www.jacionline.org and in our earlier observations,²⁹ PPAR γ mRNA expression was lower in recruited gp91^{phox-/-} phagocytes compared with that seen in WT phagocytes, and expression increased in both genotypes after pioglitazone treatment, confirming that pioglitazone activates PPAR γ in these cells.²⁹ Dependence on PPAR γ for pioglitazone-enhanced mtROS production was tested by using transgenic mice with myeloid-specific ablation of PPAR γ (LysMCre x PPAR $\gamma^{fl/fl}$). As shown in Fig E9, B, neutrophils and inflammatory macrophages from LysMCre x PPAR $\gamma^{fl/fl}$ mice, unlike WT mice, did not show enhanced MitoSOX Red fluorescence after pioglitazone treatment, supporting PPAR γ dependence. WT and gp91^{phox-/-} mice were also treated with the PPAR γ antagonist BADGE concurrently with pioglitazone or vehicle. BADGE dose-dependently inhibited the enhancement in mtROS production after pioglitazone treatment in both genotypes (see

Fig E9, C). Finally, rapid (within minutes) PPAR γ -independent mtROS production has been reported for pioglitazone used *ex vivo*.⁴¹ Therefore peritoneal phagocytes of untreated WT and gp91^{phox-/-} mice were incubated for 1 hour *ex vivo* with pioglitazone (10 μ M/L) and assessed for mtROS. As with human monocytes (see Fig E8), no enhancement in mtROS production was detected after this brief exposure to pioglitazone (data not shown). Taken together, these data support the hypothesis that enhancement of mtROS production by pioglitazone treatment is PPAR γ dependent.

Phagocytes of pioglitazone-treated gp91^{phox-/-} mice show enhanced killing of *S aureus*

S aureus is a significant pathogen in patients with CGD, and efficient killing of this bacterium by phagocytes is dependent on ROS production.^{27,42} The ability of pioglitazone treatment to enhance phagocyte killing of *S aureus* was next investigated *in vitro*. Leukocytes were harvested from inflamed peritonea of vehicle- and pioglitazone-treated WT and gp91^{phox-/-} mice 10 hours after zymosan injection and coincubated *ex vivo* with viable *S aureus* for 1 hour, and the percentage of bacteria killed was assessed. As shown, *S aureus* was readily killed by WT leukocytes but not by leukocytes from vehicle-treated gp91^{phox-/-} mice (Fig 7). Pioglitazone treatment of the gp91^{phox-/-} mice enhanced leukocyte killing to approximately 36% of that seen for WT mice. In all cases pretreatment of leukocytes with SOD nearly abolished killing or even slightly enhanced survival of the bacteria. Finally, pretreatment of leukocytes obtained from pioglitazone-treated gp91^{phox-/-} mice with MitoTEMPO ablated most of their ability to kill *S aureus*. Interestingly, MitoTEMPO pretreatment of WT leukocytes reduced *S aureus* killing by almost half, suggesting that mitochondria play a significant role in the provision of oxidants for bacterial killing in normal phagocytes. Killing assays were also performed with *B cepacia*, another pathogen relevant to CGD. As with *S aureus*, leukocytes from vehicle-treated gp91^{phox-/-} mice were unable to kill this

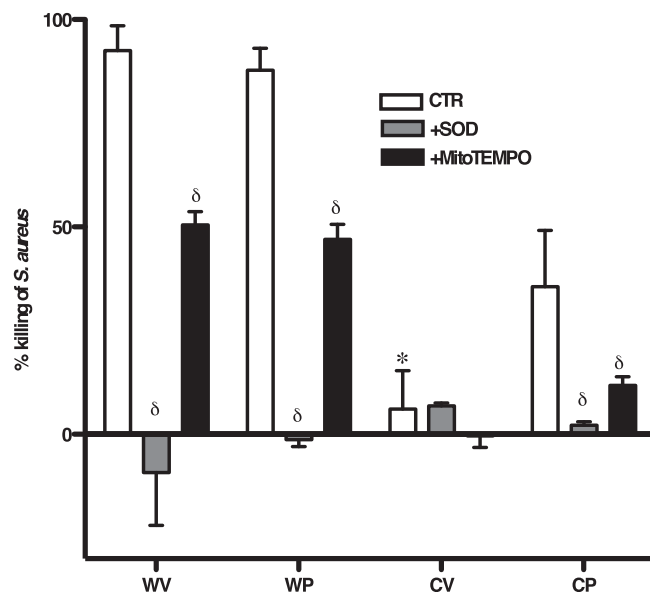


FIG 7. Recruited phagocytes from pioglitazone-treated $gp91^{phox-/-}$ mice show enhanced killing of *S aureus* *ex vivo*. Recruited peritoneal phagocytes from WV, WP, CV, and CP mice, as in Fig 2, were treated with or without SOD or MitoTEMPO for 30 minutes and coincubated with *S aureus* (1×10^6 CFU) at 37°C for 1 hour. The percentage of bacteria killed was determined. $P \leq .02$; *compared with phagocytes from WV mice and δ compared with phagocytes from respective treatment group in the absence of inhibitors. $N = 6$ mice per group.

pathogen, whereas killing by leukocytes of pioglitazone-treated $gp91^{phox-/-}$ mice was restored to approximately 47% of WT killing (see Fig E10 in this article's Online Repository at www.jacionline.org). Again, killing by all phagocytes was ablated in the presence of SOD and MitoTEMPO.

Finally, the ability of pioglitazone treatment to enhance host defense against *S aureus* *in vivo* was tested by using a well-described, nonlethal model of peritonitis.²⁷ WT and $gp91^{phox-/-}$ mice treated with either pioglitazone or vehicle were injected intraperitoneally with viable *S aureus*, and peritonea were lavaged at 24 or 48 hours for CFU enumeration. Similar numbers of inflammatory cells were recruited to the peritoneum for both genotypes and treatment groups at both time points (see Fig E11 in this article's Online Repository at www.jacionline.org). WT mice readily cleared bacteria over 48 hours, regardless of treatment. In contrast, vehicle-treated $gp91^{phox-/-}$ mice showed clearance that was significantly delayed, as described previously (Fig 8). Importantly, pioglitazone treatment of the $gp91^{phox-/-}$ mice resulted in an enhanced clearance of *S aureus* from peritonea at 24 hours, demonstrating that pioglitazone treatment restored early host defenses.

DISCUSSION

We have demonstrated that pioglitazone treatment of $gp91^{phox-/-}$ mice enhances stimulated oxidant production from mitochondria of activated neutrophils, monocytes, and inflammatory macrophages. *Ex vivo* treatment of human monocytes from patients with CGD with pioglitazone similarly enhanced mtROS in response to stimulation (Fig 6). Even phagocytes of pioglitazone-treated WT mice showed some enhancement in mtROS production (Fig 5). Mitochondrial oxidant production in

each case was shown by using MitoSOX Red, a DHE probe that preferentially localizes to mitochondria and reacts with various ROS.⁴³ ROS production was also demonstrated by using 2 other indicators, DHR and cytochrome c, each differing in detection of ROS. DHR, an intracellular probe that is not directed specifically to mitochondria, is capable of reacting with a number of oxidizing species and can be autoamplifying,⁴³ likely explaining the heterogeneity in cellular fluorescence seen with this reagent. Cytochrome c reduction with SOD inhibition is most specific for detecting superoxide but requires extracellular ROS production after a robust stimulus, such as PMA, for detection.²⁴ A key finding of this investigation was that pioglitazone treatment enabled $gp91^{phox-/-}$ phagocytes to kill *S aureus* and *B cepacia*, significant pathogens in patients with CGD, in an mtROS-dependent manner (Figs 7 and 8 and see Fig E10). Clinical trials in human CGD will be required to determine whether pioglitazone has efficacy as an adjunctive treatment to traditional antimicrobial agents for the enhancement of host defense.

Activated neutrophils and monocytes are highly glycolytic cells with limited mitochondrial respiration^{44,45}; nonetheless, electrons are still shuttled to maintain mitochondrial membrane potential and integrity.⁴⁶ Somewhat surprising was our evidence that enhanced mtROS production in stimulated phagocytes appeared to depend on the NADPH oxidase and was lacking in $gp91^{phox-/-}$ phagocytes.

Findings from other studies support these observations. First, reduced oxygen consumption by mitochondria was demonstrated in human alveolar macrophages from patients with CGD during phagocytosis of *S aureus*.¹ Second, crosstalk between the NADPH oxidase for production of mtROS has been demonstrated experimentally: knockdown of $p47^{phox}$ in stimulated glioma cells ablated mtROS production,⁴⁷ and activation of the NADPH oxidase resulted in mitochondrial membrane depolarization, respiratory dysfunction, and mtROS production.⁴⁸

From our investigation, it appears that PPAR γ is a signaling intermediary between the NADPH oxidase and mitochondria during phagocyte activation (see Fig E12 in this article's Online Repository at www.jacionline.org). Oxidized lipids are ligands of this nuclear receptor,^{49,50} and as such, PPAR γ activation can be a downstream target of ROS. In turn, its activation appears to enhance mtROS production,^{21,41} suggesting a feed-forward signaling loop between cellular ROS and PPAR γ . Therefore pioglitazone activation of PPAR γ appears to bypass the requirement for NADPH oxidase-derived ROS in $gp91^{phox-/-}$ phagocytes and results in mtROS production and enhanced bactericidal activity.

Mobilization of mitochondria to phagolysosomes has been suggested by studies showing localization of mitochondrial enzymes in isolated maturing phagosomes of neutrophils.⁵¹ Similarly, West et al⁵² have recently shown that stimulation of macrophages with Toll-like receptor ligands on beads enhanced mtROS production and delivery of mitochondria to phagolysosomes and that inhibition of these events reduced killing of intracellular bacteria *in vitro*. Our data demonstrating zymosan-containing phagolysosomes surrounded by mitochondria (Fig 4) also suggest direct delivery of mtROS. How mitochondria are routed to phagolysosomes is not entirely clear but likely involves assembly of autophagocytic machinery. mtROS, as well as thioglitazones, drive "starvation-induced" autophagy, whereby intracellular organelles are delivered to autophagolysosomes (eg, damaged mitochondria in so-called mitophagy) for degradation and to provide

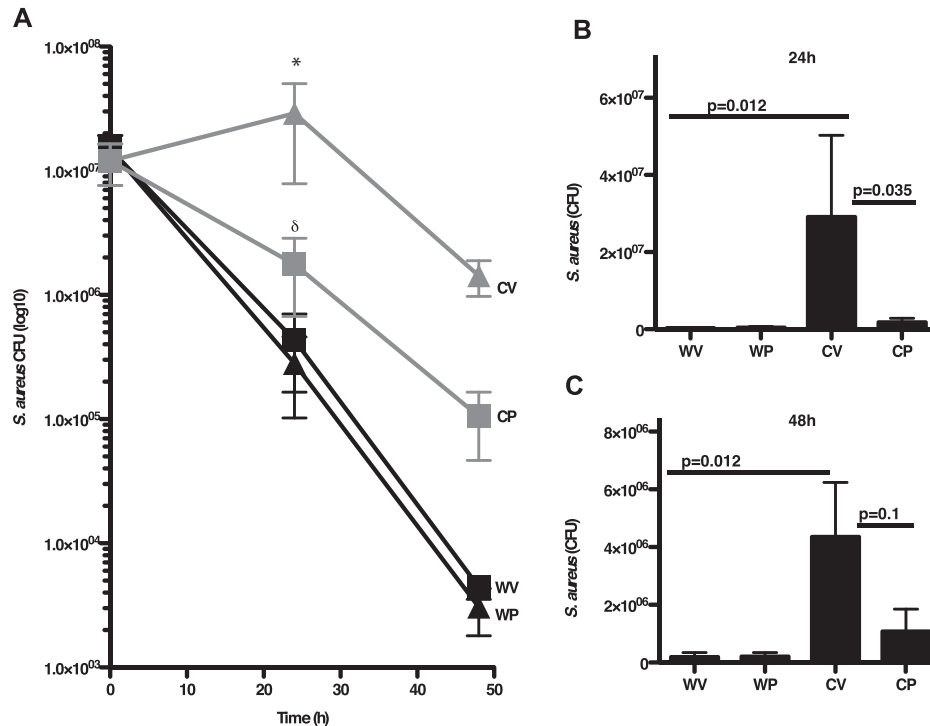


FIG 8. Pioglitazone treatment of gp91^{phox}^{-/-} mice enhances clearance of *S aureus* in vivo. Vehicle (V)- or pioglitazone (P)-treated WT (WV and WP, respectively) and gp91^{phox}^{-/-} (CV and CP, respectively) mice were injected intraperitoneally with *S aureus*, and peritonea were lavaged at 24 and 48 hours. **A**, Bacterial numbers in lavage fluid and cells were determined. $P \leq .02$: comparisons with *WV and ^δCV mice. **B** and **C**, CFU data are depicted on a linear scale for clarity. N = 16 and 11 mice per group at 24 and 48 hours, respectively.

energy to the cell.^{53,54} This autophagocytic process has been demonstrated to require ROS, is inhibitable with DPI, and is deficient in neutrophils from patients with CGD.^{7,55,56} Precise dissection of molecular events leading to pioglitazone restoration of phagocyte mtROS production and enhanced killing of bacteria will require cellular models that allow genetic manipulation and are the focus of future investigation.

Deficient activity of the NADPH oxidase is also associated with exaggerated sterile inflammation and autoimmune consequences that are frequent in patients with CGD (eg, colitis, granuloma, and poor wound healing) and systemic lupus erythematosus, as well as altered tissue and cellular functioning.⁵⁷⁻⁵⁹ For instance, in the absence of a functional NADPH oxidase, macrophages are poorly programmed for inflammation resolving functions, including efferocytosis, the clearance of activated and dying neutrophils, and production of anti-inflammatory mediators.^{29,60-62} Oxidant-dependent PPAR γ activation is critical in programming recruited monocytes and inflammatory macrophages derived from them,¹⁸ and its activation is delayed in macrophages from patients with CGD with exaggerated inflammation as a consequence.²⁹ Oxidants and mitochondrial disruption are also critical to drive neutrophil apoptosis⁴⁴ and for the development of appropriate “eat me” signals on activated and dying neutrophils.⁶³ Lacking these, neutrophils from patients with CGD accumulate in great numbers, ultimately die, deteriorate, enhance inflammation, and might promote autoimmunity. These observations, together with several *in vivo* models, support the hypothesis that oxidants likely play increasingly recognized and important anti-inflammatory signaling roles.⁶⁴⁻⁶⁶

Thioglitzazones are potent PPAR γ agonists currently approved for use in type 2 diabetes and result in production of IL-10, suppression of systemic inflammatory cytokine production (IL-6 and TNF- α), and improved insulin resistance.^{11,67} Although cardiac toxicity in patients with type 2 diabetes raised safety concerns for rosiglitazone, pioglitazone appears safer in this regard.⁶⁸ Short-term or intermittent pioglitazone therapy is less likely to be associated with potential side effects.⁶⁹ Thioglitzazones are currently in clinical trials for treatment of metabolic syndrome, obesity, end-stage renal disease, autoimmunity, rheumatoid arthritis, cystic fibrosis, and severe asthma (<http://www.clinicaltrials.gov>). Recently, we showed that treatment of gp91^{phox}^{-/-} mice with pioglitazone reversed exaggerated sterile inflammation by enhancing clearance of neutrophils and suppression of cytokine production.²⁹ Whether PPAR γ -mediated enhanced mtROS production was responsible for the restored macrophage-resolving activities and/or enhanced development of clearance signals on neutrophils from patients with CGD is an open question currently under investigation. These earlier observations regarding anti-inflammatory effects, along with the demonstration here that pioglitazone treatment restores mtROS production by murine CGD phagocytes and enhances host defense against a relevant pathogen, make a compelling case for the investigation of this and possibly other on-the-shelf “starvation signaling” agents¹⁰ in patients with CGD.

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Key messages

- The NADPH oxidase governs mitochondrial oxidant production in activated phagocytes, and such crosstalk is absent in patients with CGD.
- Pioglitazone, a “nutrient restriction” therapeutic agent, restores mitochondrial ROS production and enhances host defense in murine CGD.

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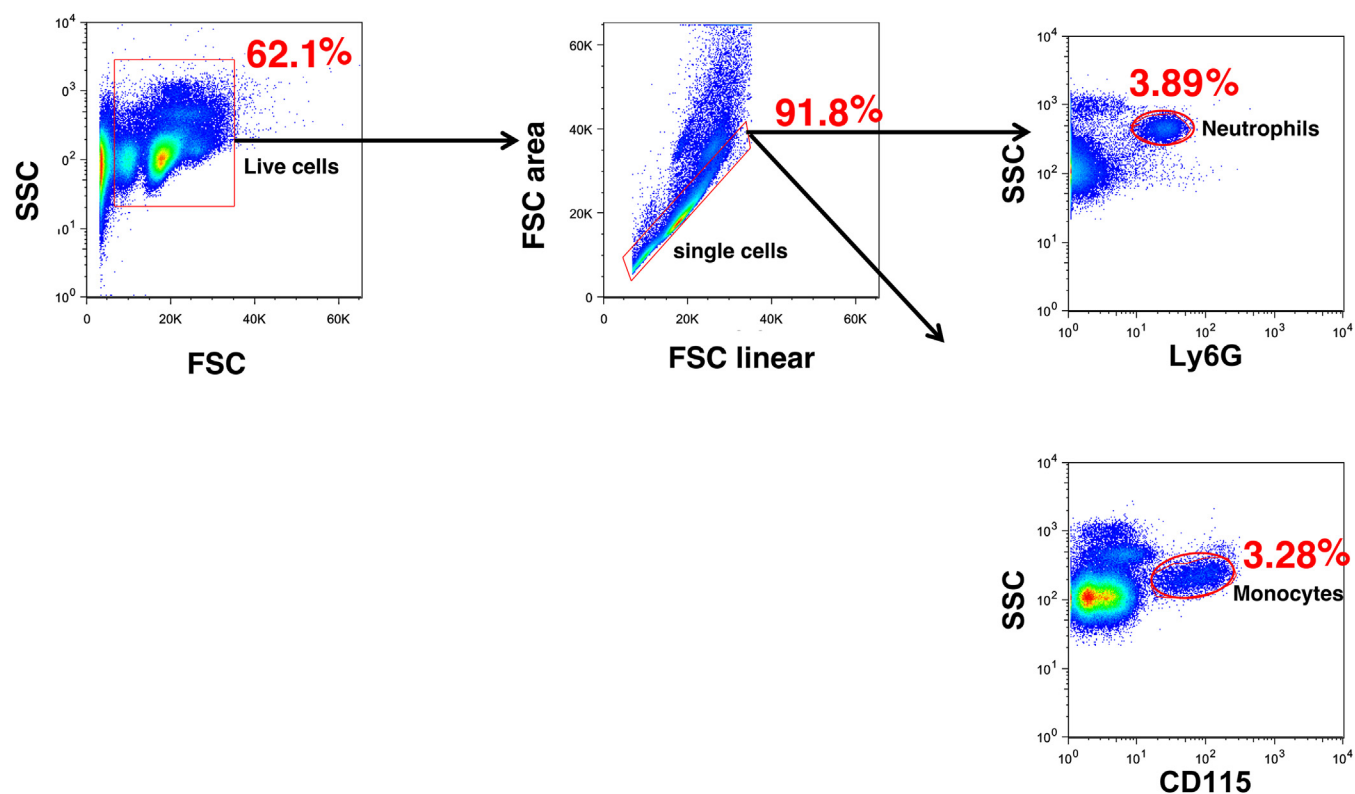


FIG E1. Gating strategy for identification of neutrophils and monocytes from mouse blood. After 5 days of treatment with 10 mg/kg/d pioglitazone or vehicle, blood was obtained by means of cardiac puncture, red cells were lysed, and the remaining leukocytes were stained for surface markers and analyzed by means of flow cytometry. Live cells, with doublets excluded, were plotted as side scatter (SSC) versus Ly6G to identify neutrophils and SSC versus CD115 to identify monocytes. FSC, Forward scatter.

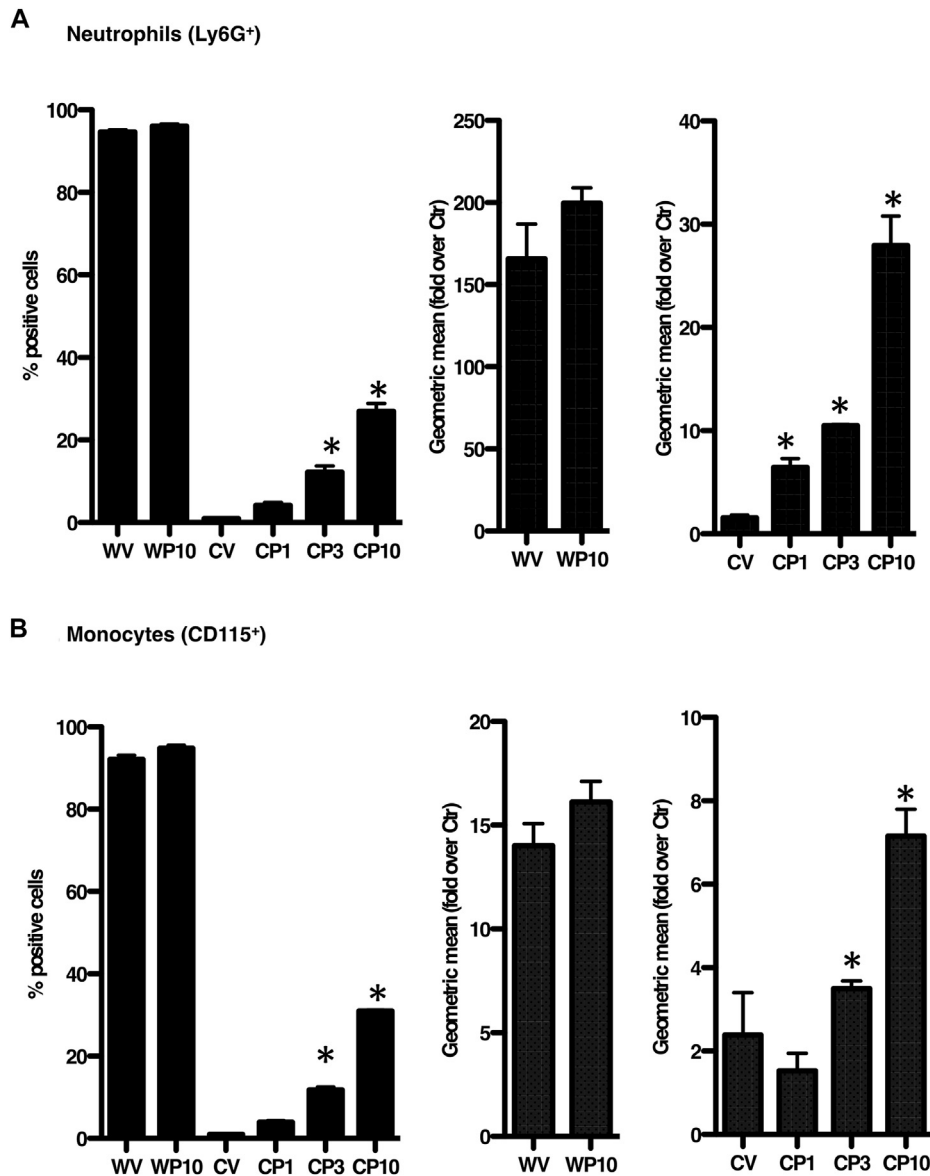


FIG E2. Pioglitazone dose response for production of stimulated ROS by blood phagocytes. After 5 days of treatment with 1, 3, or 10 mg/kg/d pioglitazone or vehicle, blood neutrophils (Ly6G⁺; **A**) and monocytes (CD115⁺; **B**) from WT (WV and WP, respectively) and gp91^{phox-/-} (CV and CP, respectively) mice were harvested and tested for ROS production in response to PMA, as in Fig 1. The percentage of ROS⁺ cells and the geometric mean for the population expressed as fold value over control subjects without PMA stimulation are shown. N = 3 mice for each treatment group. $P \leq .05$ *compared with CV mice.

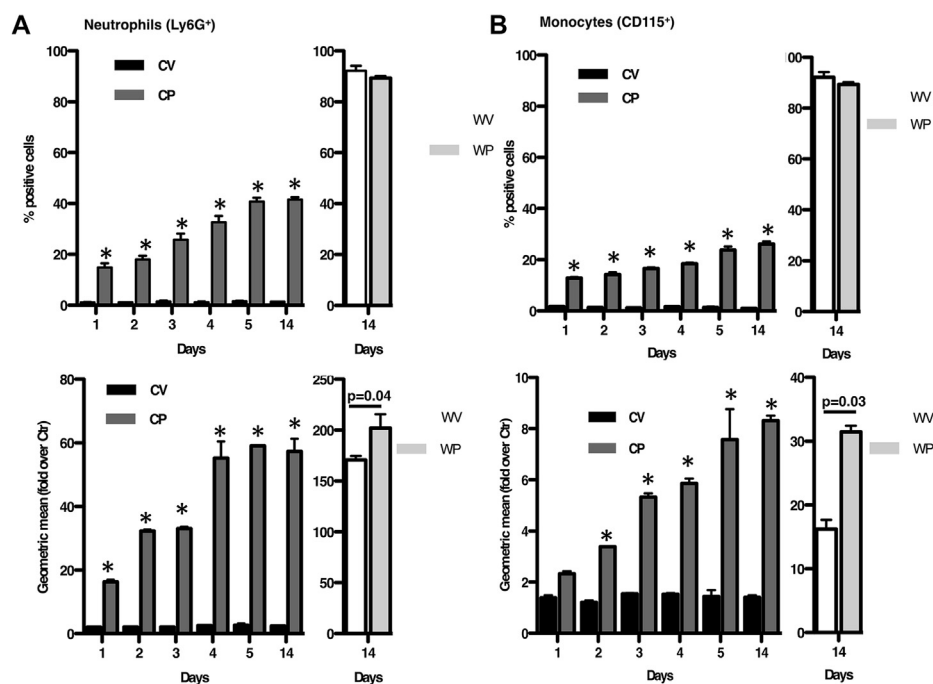


FIG E3. ROS response after prolonged treatment with pioglitazone. Blood neutrophils (Ly6G⁺; **A**) and monocytes (CD115⁺; **B**) from WT (WV and WP, respectively) and gp91^{phox-/-} (CV and CP, respectively) mice were tested for ROS production in response to PMA, as in Fig 1, after 1 to 14 days of treatment with 10 mg/kg/d pioglitazone or vehicle. For simplicity, ROS production for leukocytes from WT mice is shown only for day 14. N = 3 mice each treatment group.

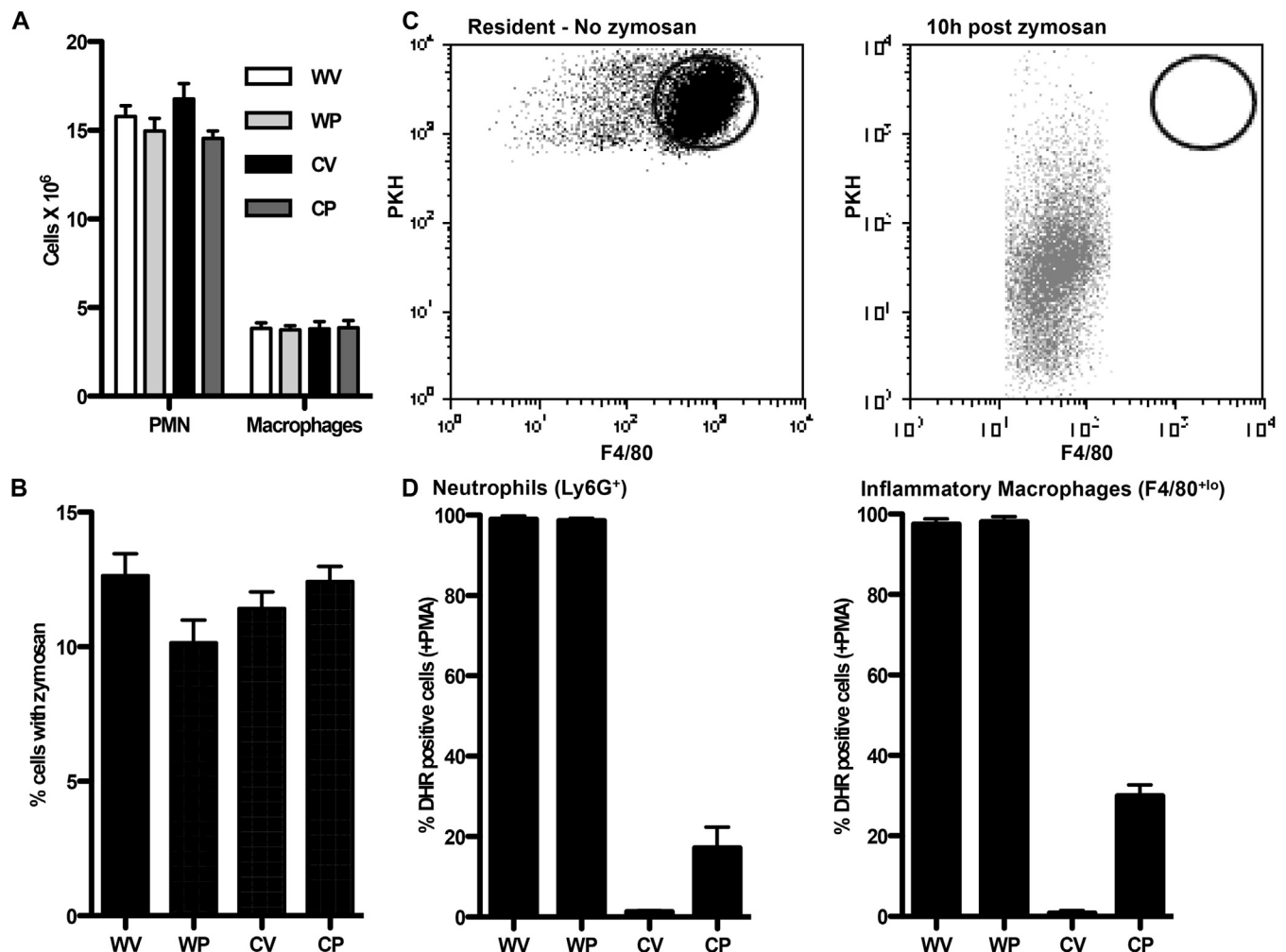


FIG E4. Characterization of recruited phagocytes in patients with zymosan-induced peritonitis. After 5 days of treatment with pioglitazone or vehicle, WT (WV and WP, respectively) and gp91^{phox-/-} (CV and CP, respectively) mice, as in Fig 1, were injected intraperitoneally with zymosan. **A**, Phagocyte numbers and differentials on cytospin preparations were determined at 10 hours after zymosan. Similar numbers and types of inflammatory leukocytes were recruited to the peritonea for both genotypes and treatment groups. **B**, The percentage of phagocytes from each genotype and treatment group positive for zymosan ingestion was not different ($n = 10$).²⁹ **C**, Inflammatory macrophages (F4/80⁺PKH^{lo}) derived from recruited monocytes, but not resident peritoneal macrophages (F4/80^{hi}PKH^{hi}), are recovered in 10-hour harvests from inflamed peritonea of mice injected with intraperitoneal zymosan. Mice were injected with PKH 24 hours before zymosan or PBS and then underwent lavage 10 hours later. Resident peritoneal macrophages F4/80^{hi}PKH^{hi} underwent lavage from mice receiving PBS, whereas only inflammatory macrophages (F4/80⁺PKH^{lo}) underwent lavage from mice receiving zymosan. F480^{hi} resident peritoneal macrophages are not recovered by means of lavage at this early stage of peritonitis. **D**, Vehicle (V)- or pioglitazone (P)-treated WT (WV and WP, respectively) and gp91^{phox-/-} (CV and CP, respectively) mice, as in Fig 1, were injected intraperitoneally with zymosan and phagocytes were harvested at 10 hours, loaded with DHR, stimulated with PMA (200 ng/mL for 15 minutes), and analyzed for DHR fluorescence by using flow cytometry. Aggregate data with PMA stimulation are expressed as the percentage of cells exhibiting a shift in DHR fluorescence.

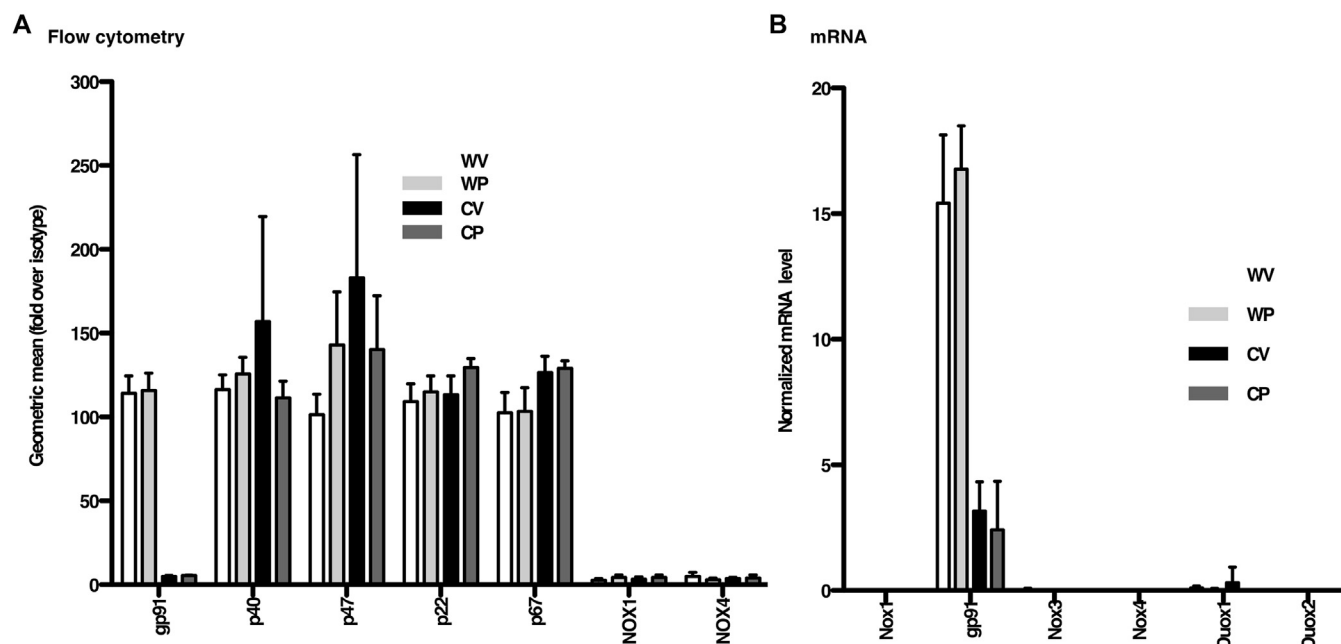


FIG E5. NOX and DUOX proteins in peritoneal phagocytes from gp91^{phox-/-} and WT mice after pioglitazone or vehicle treatment. After 5 days of treatment with pioglitazone (P) or vehicle (V), WT (WV and WP) and gp91^{phox-/-} (CV and CP) mice were injected intraperitoneally with zymosan. **A**, Ten hours after zymosan, cells were harvested, permeabilized, and stained with antibodies as described in the [Methods](#) section and analyzed by using flow cytometry. **B**, Trizol reagent (Life Technologies) was used to prepare total RNA (2 μ g) that was treated with DNase (1 U/mL) and reverse transcribed with a first-strand complementary DNA synthesis kit (Life Technologies). PCR amplification was performed by using fast universal PCR buffer and predesigned primers from Life Technologies on a 7900H platform. Primer pairs targeting at least 2 exons were chosen, and for gp91^{phox}, primer pairs targeting exons 2 and 3 were chosen (exon 3 is deleted in these knockout mice²⁷). Relative expression levels for mRNAs were calculated by using the comparative cycle threshold method normalized to GUSb RNA. N = 7 mice.

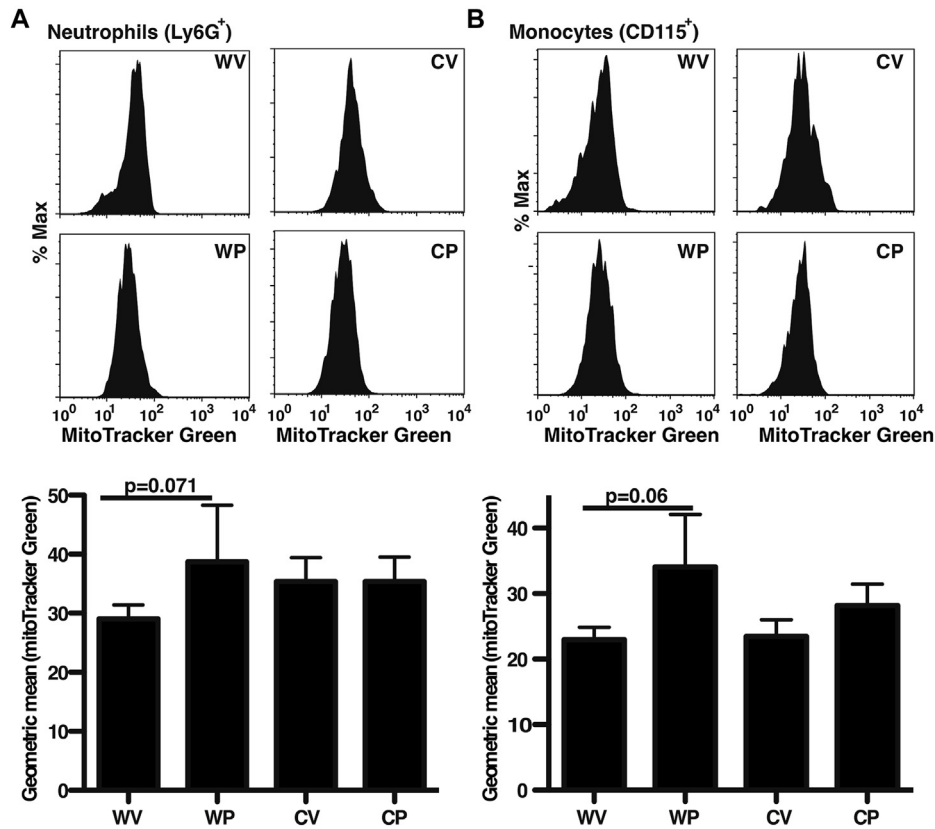


FIG E6. Pioglitazone treatment has little effect on mitochondrial content in blood neutrophils (A) and monocytes (B). Blood leukocytes from vehicle (V)- or pioglitazone (P)-treated WT (WV and WP) and gp91^{phox-/-} (CV and CP) mice were stained with surface markers followed by MitoTracker Green and then analyzed by using flow cytometry. Representative histograms (top) and aggregate data (bottom) are shown. N = 6 mice per group.

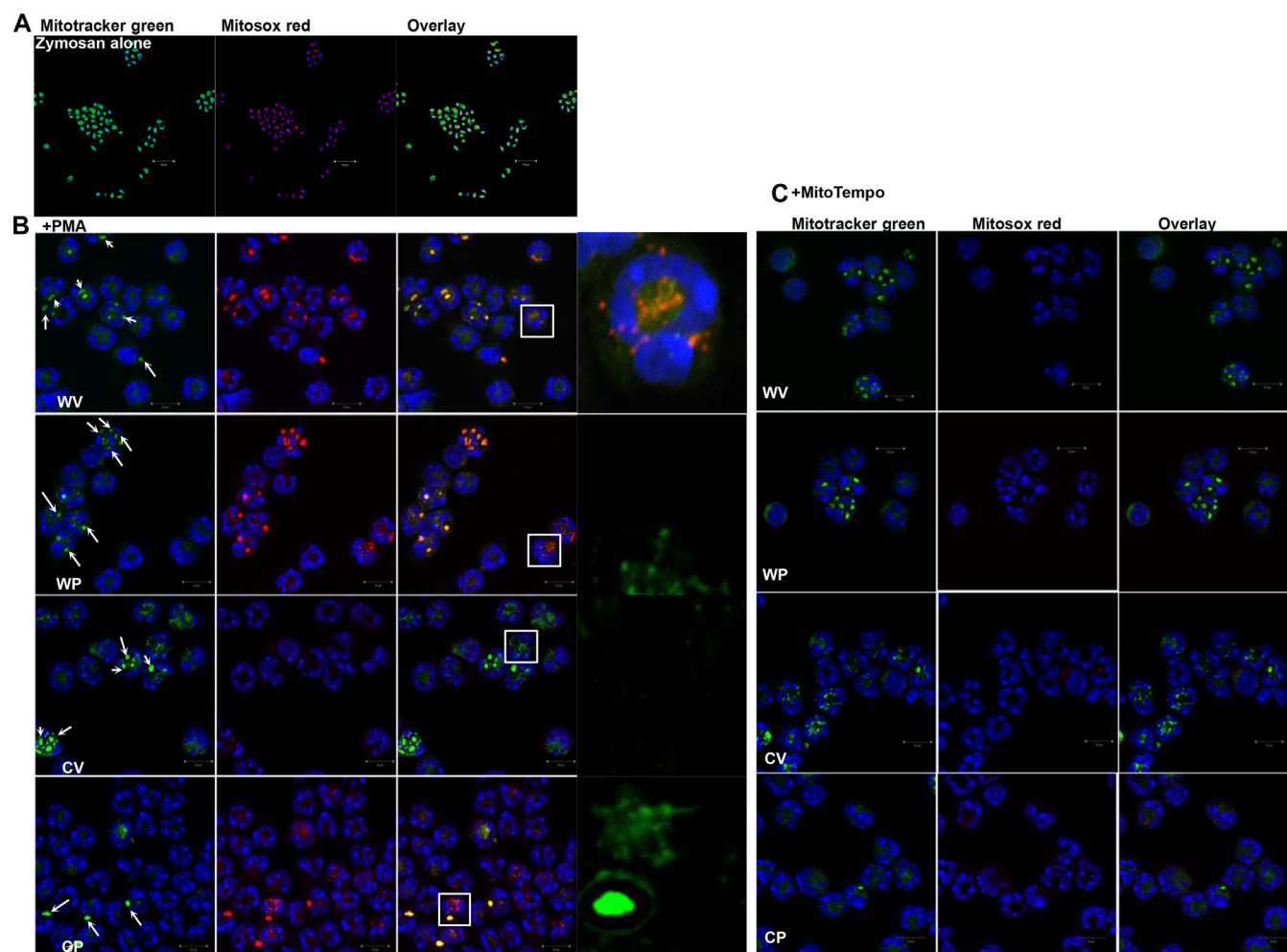


FIG E7. Pioglitazone enhancement of gp91^{phox}^{-/-} murine ROS production in recruited phagocytes is of mitochondrial origin. **A**, Zymosan particles (no cells) were stained with 4'-6-diamidino-2-phenylindole dihydrochloride, MitoTracker Green (25 nmol/L for 15 minutes), and MitoSOX Red (4 mmol/L for 15 minutes); washed; and then analyzed by using confocal microscopy with a $\times 63$ oil immersion lens to determine their absorption of the stains. **B**, Recruited phagocytes were harvested 10 hours after intraperitoneal zymosan injection from vehicle-treated (WV) or pioglitazone-treated (WP) WT mice and vehicle-treated (CV) or pioglitazone-treated (CP) gp91^{phox}^{-/-} mice. Harvested cells were then stained with MitoTracker Green (25 nmol/L for 15 minutes) and MitoSOX Red (4 mmol/L for 15 minutes) with PMA, washed, stained with 4'-6-diamidino-2-phenylindole dihydrochloride, and analyzed by using confocal microscopy with a $\times 63$ oil immersion lens (see the [Methods](#) section). High-resolution images of a single cell (*white box*) are shown in the *last panel*. *Arrows* denote zymosan in the first column. **C**, Harvested cells were pretreated with MitoTEMPO (100 mmol/L) for 30 minutes before staining and PMA stimulation as in [Fig E7, B](#). Representative images are shown for each genotype and treatment group ($n = 3$).

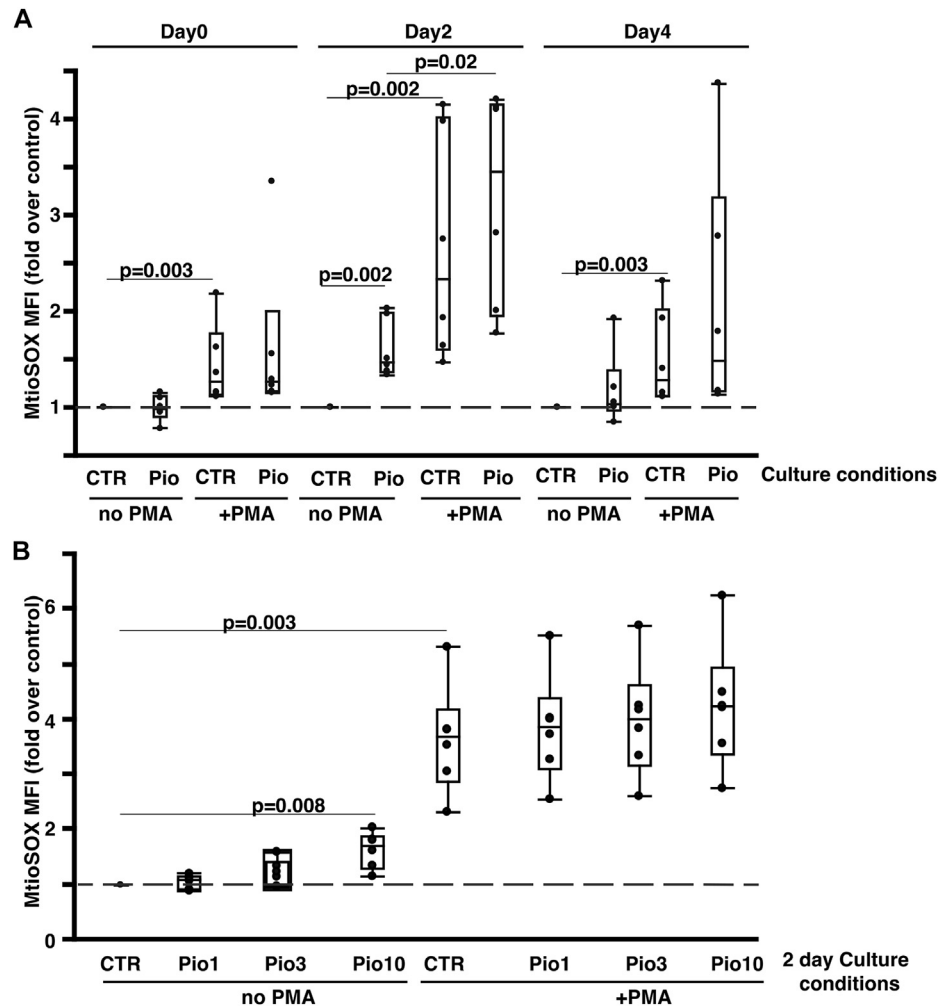


FIG E8. Pioglitazone (*Pio*) enhances production of mtROS by stimulated human monocytes in a time- and dose-dependent manner. Human monocytes were isolated from blood, plated, and treated as indicated. **A**, Pioglitazone (10 mmol/L) was added to monocyte cultures for 1 hour (day 0 cultures), 2 days, or 4 days, and then monocytes were stained with MitoSOX Red, stimulated with or without PMA, and analyzed by using flow cytometry. **B**, Monocytes were cultured for 2 days in different concentrations of pioglitazone (0, 1, 3, and 10 mmol/L) before MitoSOX staining, stimulation with PMA, and analyses as in [Fig E8, A](#). N = 6 monocytes from healthy subjects. Data are normalized to each untreated control subject.

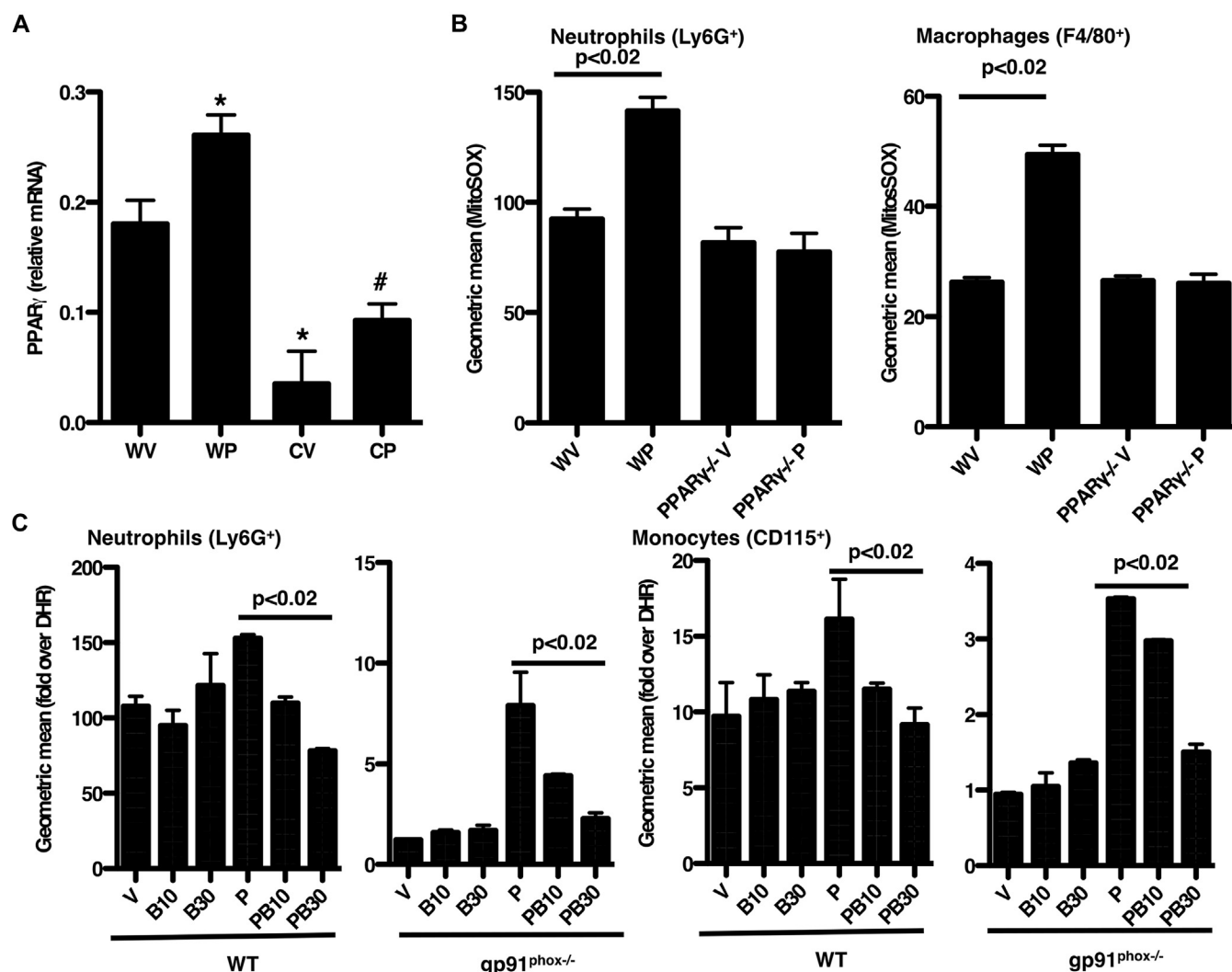


FIG E9. Pioglitazone enhancement of mitochondrial ROS production is PPAR γ dependent. **A**, Vehicle (V)- or pioglitazone (P)-treated WT (WV and WP) or gp91^{phox}^{-/-} (CV and CP) mice were injected intraperitoneally with zymosan and phagocytes harvested at 10 hours. PPAR γ expression (mRNA) was assessed by using quantitative PCR and normalized to GUSb. N = 6. * $P < .02$ compared with WV and # $P < .02$ compared with CV mice. **B**, Recruited neutrophils and inflammatory macrophages were harvested 10 hours after zymosan from WV and WP mice as in Fig E9, A, and vehicle- and pioglitazone-treated mice with genetic deletion of PPAR γ in myeloid cells. Phagocytes were stained for MitoSOX Red and analyzed by means of flow cytometry without further stimulation, as in Fig 5. Geometric mean fluorescence is shown. N = 3. **C**, After vehicle (V), pioglitazone (P), BADGE (10 or 30 mg/kg/d; B10 and B30), or the combination of pioglitazone and BADGE (PB10 and PB30) treatment for 5 days, blood leukocytes were obtained from WT (WV, WP, WB, and WPB) and gp91^{phox}^{-/-} (CV, CP, CB, and CPB) mice, loaded with DHR, stimulated with PMA (200 ng/mL for 15 minutes), and analyzed for DHR fluorescence by using flow cytometry. The change in geometric mean fluorescence (fold over controls without PMA) for the whole population of either neutrophils or monocytes is shown. N = 4 mice in each treatment group.

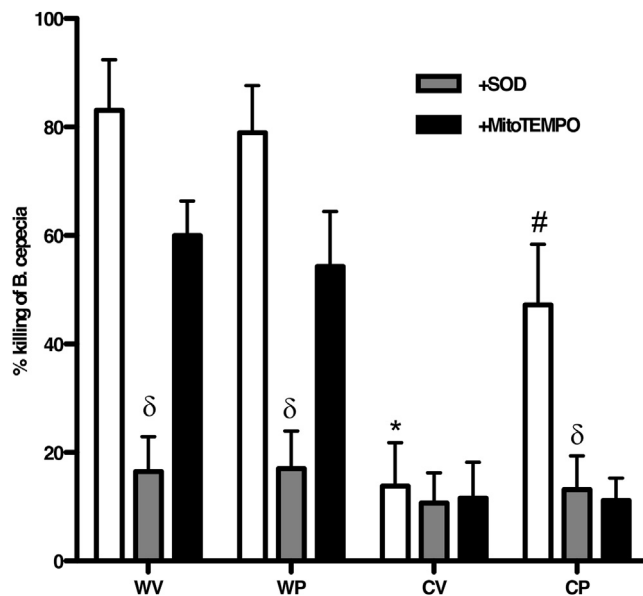


FIG E10. Recruited phagocytes from pioglitazone-treated $gp91^{phox-/-}$ mice show enhanced killing of *B. cepecia* *ex vivo*. Recruited peritoneal phagocytes (1×10^6) from WV, WP, CV, and CP mice, as in Fig 2, were treated with or without SOD or MitoTEMPO *ex vivo* for 30 minutes and coincubated with *B. cepecia* (1×10^7 CFU, as grown overnight in Lauryl Broth and washed twice with saline) at 37°C for 2 hours in 100 mL of RPMI (phenol red free, 1% mouse serum). The phagocytes were then lysed with 0.1% Triton X for 10 minutes, the remaining viable bacteria were enumerated by using the Alamar blue assay, and the percentage of bacteria killed was determined. $P \leq .02$: *compared with phagocytes from WV, #compared with phagocytes from CV mice, and ^δcompared with phagocytes from respective treatment groups in the absence of inhibitors. N = 7 mice per group.

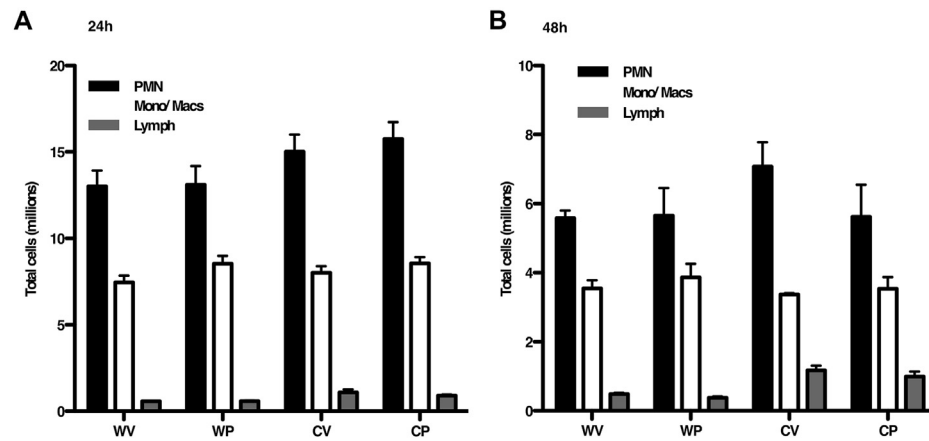


FIG E11. Cell numbers and differentials after intraperitoneal injection of *S aureus*. Vehicle (V)- or pioglitazone (P)-treated WT (WV and WP) and gp91^{phox-/-} (CV and CP) mice were injected intraperitoneally with *S aureus*. Phagocyte numbers and differentials at 24 hours (A) and 48 hours (B) were determined on cytopsin preparations. N = 16 mice at 24 hours and 11 mice at 48 hours.

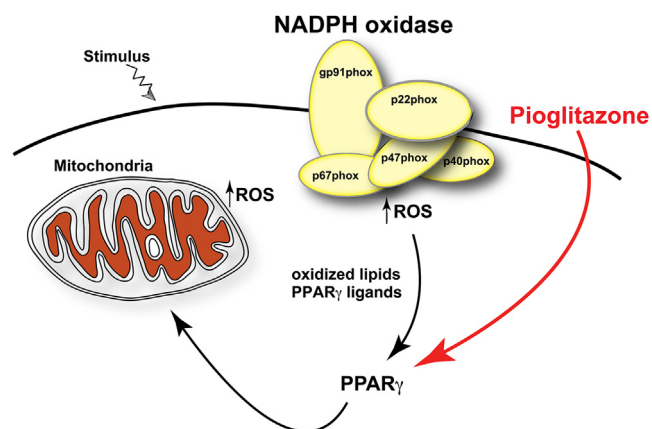


FIG E12. Crosstalk between NADPH oxidase and mitochondria. There is crosstalk between NADPH oxidase and mitochondria under normal circumstances that leads to enhanced mitochondrial ROS and optimal antimicrobial responses by phagocytes. In the absence of a functional NADPH oxidase (CGD), this signaling is lost but, importantly, can be largely restored at the level of mitochondrial ROS production by pioglitazone treatment.