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Impaired p47phox phosphorylation in neutrophils from p67phox-deficient chronic granulomatous disease patients

Short title: p67phox regulates p47phox phosphorylation

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Key Points:

- Phosphorylation of the NADPH oxidase component p47phox is decreased in neutrophils from p67phox-deficient CGD patients
- Recombinant p67phox and p40phox potentiate the phosphorylation of p47phox by PKCs *in vitro*
- p67phox and p40phox are novel regulators of p47phox-phosphorylation

Abstract

Superoxide production by the phagocyte NADPH oxidase is essential for innate immunity as shown in chronic granulomatous disease (CGD), an immunodeficiency disease due to mutations in one of its genes. The NADPH oxidase is composed of two membrane proteins (gp91phox/NOX2 and p22phox) and four cytosolic proteins (p47phox, p67phox, p40phox, and Rac1/2). The phosphorylation of p47phox is required for NADPH oxidase activation in cells. As p47phox and p67phox can form a tight complex in cells, we hypothesized that p67phox could regulate p47phox phosphorylation. To investigate this hypothesis, we used phospho-specific antibodies against five major p47phox-phosphorylated sites (Ser304, Ser315, Ser320, Ser328 and Ser345) and neutrophils from healthy donors and from p67phox^{-/-}-CGD patients. Results showed that fMLF and PMA induced the time- and concentration-dependent phosphorylation of p47phox on Ser304, Ser315, Ser320 and Ser328 in healthy human neutrophils. Interestingly, in neutrophils and Epstein-Barr virus-transformed B-lymphocytes from p67phox^{-/-}-CGD patients, phosphorylation of p47phox on serine residues was dramatically reduced. In COSphox cells, the presence of p67phox led to increased phosphorylation of p47phox. *In vitro* studies showed that recombinant p47phox was phosphorylated on Ser304, Ser315, Ser320 and Ser328 by different PKC isoforms and the addition of recombinant p67phox alone or in combination with p40phox potentiated this process. Thus, p67phox and p40phox are required for optimal p47phox phosphorylation on Ser304, Ser315, Ser320 and Ser328 in intact cells. Therefore, p67phox and p40phox are novel regulators of p47phox-phosphorylation.

Introduction

Professional phagocytes, such as neutrophils and monocytes, play a vital role in host defense, innate immunity and inflammation.^{1,2} Upon phagocytosis of microbes, they produce high levels of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide by a process called the respiratory burst.^{3,4} The enzyme system responsible for superoxide production in phagocytic cells is a multicomponent enzyme called the phagocyte NADPH oxidase.⁵⁻⁷ This enzyme complex includes the membrane-bound cytochrome b₅₅₈ (composed of p22phox and gp91phox/NOX2) and cytosolic proteins (p47phox, p67phox, p40phox and Rac1/2).⁵⁻⁷ In resting cells, the components are segregated between cytosol and membranes, but upon activation, the cytosolic components translocate to the membrane to form the catalytically active oxidase complex.^{8,9} This activation is associated with the phosphorylation of p47phox, p67phox, p40phox, p22phox and gp91phox and with Rac1/2 activation.^{10,11}

ROS production is required for host defense, as illustrated by the genetic immunodeficiency disease called chronic granulomatous diseases (CGD) in which phagocytes do not produce ROS and affected patients have more fatal infections than healthy individuals.¹²⁻¹⁶ CGD is a rare disease found in 1 of 200,000 to 250,000 individuals. It is caused by a gene mutation in one of the NADPH oxidase components, the most frequent CGD form being caused by mutations in the gp91phox/CYBB gene (65%), followed by mutation in the p47phox/NCF1 gene (20%), the p22phox/CYBA gene (less than 5%), the p67phox/NCF2 gene (less than 5%), the p40phox/NCF4 and Rac2/NCF3 genes (both forms less than 5%).¹²⁻¹⁶

In addition to host defense, ROS production can participate to tissue injury and excessive inflammation if not well controlled.^{10,17-19} Thus, activation of the phagocyte NADPH oxidase must be

tightly regulated to ensure that ROS are produced only when and where required. One mechanism by which the NADPH oxidase is regulated is the phosphorylation of its organizer subunit, p47phox^{10,11}. Stimulation of neutrophils by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLF) or the PKC activator phorbol myristate acetate (PMA) induces NADPH oxidase activation via p47phox phosphorylation.²⁰⁻²³ This phosphorylation occurs on several serine residues localized in the C-terminal p47phox sequence (Ser 303-379), and phosphorylation of ~~some~~ sites specific serines is required for NADPH oxidase activation, as shown by site-directed mutagenesis and transfection of EBV-transformed B-lymphocytes.^{22,23} In contrast to activating agents, priming agents such as the proinflammatory cytokines, TNF α and GM-CSF, and the TLR agonist, LPS, do not stimulate ROS production and only induce partial p47phox phosphorylation on Ser345.²⁴⁻²⁷ Phosphorylated Ser345 has been shown to be a docking site for the proline isomerase Pin1, which induces p47phox conformational changes and facilitates p47phox phosphorylation by various protein kinases.²⁷⁻²⁹

In resting cells, p47phox can be found in a free form or in a complex with p67phox and p40phox³⁰⁻³². The interaction between p47phox and p67phox is mediated via binding of SH3 domains to a polyproline region.³³⁻³⁵ We sought to investigate the role of p67phox in p47phox phosphorylation using site-specific anti-phospho-p47phox antibodies and neutrophils from p67phox^{-/-}-CGD patients. We observed that fMLF and PMA induced phosphorylation of Ser304, Ser315, Ser320 and Ser328 of the p47phox subunit in human neutrophils but phosphorylation of all sites was dramatically impaired in neutrophils from p67phox-deficient CGD patients.

Methods

Materials

Phosphate Buffered Saline (PBS), Hanks Buffered Saline salt (HBSS), PMA, fMLF, diisopropylfluorophosphate (DFP), reduced glutathione, proteases and kinases inhibitors were from Sigma Chemical (St. Louis, MO, USA). Dextran, and Ficoll were from GE-Healthcare. Reagents for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blotting were purchased from Bio-Rad (Richmond, CA, USA). Polyclonal phospho-specific antibodies were produced by our group as previously described^{23,28}. The anti-p47phox, anti-p67phox and anti-gp91phox were from Santa Cruz Biotechnologies (CA, USA). Peroxidase-labelled goat anti-rabbit antibody was purchased from Jackson laboratories (CA, USA). Blotto dry milk and Luminol reagents for protein detection were purchased from Santa Cruz Biotechnologies (CA, USA).

Ethics statement

Neutrophils were isolated from the venous blood of healthy volunteers and from two unrelated p67phox-deficient CGD patients after written informed consent was obtained. The study was approved by the institutional review boards (IRBs) and ethics committee of INSERM (EFS convention number: 2018010827). All procedures were conducted in accordance with the 1975 declaration of Helsinki, as revised in 2013. Data collection and analyses were performed anonymously.

Preparation of human neutrophils

Human neutrophils from venous blood, were obtained by Dextran sedimentation and Ficoll centrifugation.^{36,37} Neutrophils were 96% pure and 99% viable. The cells (5×10^7 /ml) were suspended in PBS and treated with the serine protease inhibitor DFP (2.7 mM) for 20 min at 4°C, then washed and resuspended in HBSS buffer containing 1.2 mM Ca⁺⁺, 1.2 mM Mg⁺⁺ and 5 mM D-glucose.

Neutrophil stimulation and lysis

For the phosphorylation experiments, neutrophils resuspended at 15×10^6 cells/0.4 ml of Hanks buffer were incubated for 10 min at 37 °C prior to stimulation with PMA or fMLF for the indicated times and concentrations. The reaction was stopped by adding 5X modified Laemmli sample buffer^{37,38} containing 5 mmol/L Na_3VO_4 , 2.5 mmol/L p-NPP, 10 mmol/L NaF, 5 mmol/L EDTA, 5 mmol/L EGTA, 20 $\mu\text{g/L}$ leupeptin, 20 $\mu\text{g/L}$ pepstatin and 20 $\mu\text{g/L}$ aprotinin. Samples were then incubated for 2 min in boiling water (100°C) and stored at -80°C until use.

EBV-transformed B lymphocytes preparation and stimulation

Peripheral blood from healthy donors and p67phox-deficient CGD patients was subjected to fractionation by Ficoll centrifugation. Mononuclear cells were collected, washed twice, and cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin in the presence of supernatants from the EBV producing cell line, B95-8²³. Three weeks later, EBV-transformed B lymphocytes (EBV-L) had proliferated and represented more than 90% of the cultured cells as determined by flow cytometry. For the phosphorylation experiments, EBV-transformed lymphocytes were stimulated with PMA and prepared as described above for neutrophils.

ROS production assay

ROS production was measured by the luminol and horse radish peroxidase (HRP)-enhanced chemiluminescence method³⁹. Neutrophils (0.5×10^6 cells) or EBV-L (2×10^6 cells) were resuspended in

500 μ L HBSS containing 10 μ M luminol and 10 U HRP, preheated at 37°C in the thermostated chamber of the luminometer (Biolumat LB937; Berthold) for 10 min, and then stimulated with different concentrations of fMLF or PMA and chemiluminescence was recorded. In the presence of HRP, chemiluminescence was completely inhibited by SOD as we previously showed³⁹, indicating that this method detects superoxide anion production.

COSphox cells

The COSphox stable cell lines, which had been transfected with the NADPH oxidase components (gp91phox, p22phox, p47phox and p67phox) or without p67phox, were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μ g/ml streptomycin and other antibiotics for selection as detailed before⁴⁰. For siRNA knockdown, COSphox cell line expressing gp91phox, p22phox, p47phox and p67phox were transiently transfected with either p67phox siRNAs (sc-36163, Santa Cruz, CA, USA) or scramble siRNA control (sc-37007), using the siRNA transfection reagent and protocol recommended by the manufacturer. Forty-eight hours after transfection, the effect of siRNA-mediated knockdown was determined using Western blotting with anti-p67^{phox} antibody. Two million cells were resuspended in HBSS, preheated and then treated with PMA (200 ng/ml) for 30 min at 37 °C, lysed and proteins denatured.

Expression of recombinant p47phox, p67phox and p40phox

For recombinant protein expression, the pGEX-6P1 plasmids containing cDNA encoding glutathione *S*-transferase (GST)-p47phox, GST-p67phox or pET-p40phox were transformed into

BL21 *Escherichia coli*, and the cells were cultured overnight in 100 ml of bacterial culture medium containing ampicillin (100 µg/ml) at 37°C. The overnight culture was then diluted 10-fold and allowed to grow until the optical density reached 0.8 at 600 nm. Expression of the proteins was induced by incubation with isopropyl-β-D-thiogalactoside (20 µM) for 3 hours at 37°C. Cells were pelleted by centrifugation at 2500xg for 25 min, lysed by sonication in lysis buffer (50 mM NaCl; 5 mM MgCl₂; 50 mM Tris-HCl, pH 7.5; 1 mM DTT; 1% Triton X-100; and a mixture of protease inhibitors), and centrifuged at 10,000xg for 20 min at 4°C. The soluble GST-p47phox, GST-p67phox and His-Tagged-p40phox were affinity-purified by incubation of the supernatant with glutathione-Sepharose 4B beads or nickel affinity chromatography, respectively, as previously described³⁶.

In vitro phosphorylation of p47phox by PKC isoforms

Phosphorylation by PKC was performed as previously described^{36,41}. Briefly, the reaction mixture contained 1 µg p47phox recombinant protein in the absence or presence of p67phox and p40phox, and 25 ng purified PKC in 40 mM HEPES, 10 mM MgCl₂, 1 mM DTT, 5 µg/ml diacylglycerol, 150 µg/ml phosphatidylserine, 3 mM CaCl₂, and 50 µM ATP (containing 1 µCi[γ-³²P]-ATP) in a total volume of 50 µl. The reactions were incubated for 30 min at 30°C and terminated at the indicated times by addition of 10 µl of 5X Laemmli sample buffer and denaturation at 100°C.

Electrophoresis and blotting

Heat-denatured cell samples were sonicated twice for 5 sec to break down DNA and centrifuged at 2000xg for 8 min. The samples (1 million cell-equivalent) were subjected to 10% SDS-PAGE using standard techniques^{37,38,42}. The separated proteins were transferred to nitrocellulose membranes, which were blocked with 5% milk in Tris-buffered saline containing Tween 20 (TBS-T) for 1 hour. After

blocking, the membranes were probed with the anti-phospho-Ser304-p47phox (1:2000), anti-phospho-Ser315-p47phox (1:2000), anti-phospho-Ser320-p47phox (1:2000), anti-phospho-Ser328-p47phox (1:2000), anti-phospho-Ser345-p47phox (1:10,000) and anti-p47phox (1:5000) antibodies for 1 hour at room temperature or overnight at 4°C. Membranes were then washed and incubated with HRP-labeled goat anti-rabbit antibody (1:10,000) for 1 hour at room temperature. The anti-gp91phox and anti-p67phox antibodies were used at 1:5000 and 1:2000, respectively. The protein bands were revealed using enhanced chemiluminescence reagents. Quantification of protein bands was performed by densitometry using Image J software analysis (Wayne Rasband, National Institute of Health, USA). Phosphorylated p47phox was quantified after correction for the total amount of p47phox protein.

Statistical analysis

All results are expressed as means \pm SD. Data were analysed with GraphPad Prism 7 software (GraphPad Software, San Diego, CA). Differences between groups were analysed by one-way ANOVA test with Tukey's Multiple Comparison post-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ values were considered as significant.

Results

FMLF induced a rapid and transient phosphorylation of p47phox on Ser304, 315, 320 and 328 in human neutrophils

To investigate the possible role of p67phox in the phosphorylation of p47phox, we used neutrophils from very rare CGD patients who are deficient for p67phox (<1 case per 5,000,000 individuals), and very sensitive site-specific anti-phospho-p47phox antibodies that we developed in our laboratory^{23,28}.

We first determined the optimal kinetics and concentrations of two different agonists, the bacterial peptide fMLF and the PKC activator PMA using neutrophils from healthy donors. Neutrophils were denatured in a 5X modified Laemmli sample buffer, sonicated and centrifuged at low speed, and the supernatants were used for SDS-PAGE and Western-blotting as previously described³⁷. Under those conditions, p47phox was fully solubilized and only detected in the supernatant (Supplemental Figure 1). Results show that fMLF (at 1 μ M) induced the phosphorylation of p47phox on Ser304, 315, 320, and 328 very rapidly as it was detected as soon as 5 sec, the shortest time at which we could stimulate and stop the reaction (Figure 1A). Phosphorylated p47phox from different experiments was quantified and corrected for the total amount of p47phox protein by densitometry as described in the Methods. The mean of these experiments (Supplemental Figure 2) shows that fMLF-induced p47phox phosphorylation on Ser304, 315, 320, and 328 reached a peak at 10 sec, followed by a decline, likely due to dephosphorylation as the total amount of p47phox did not change over time. Ser345, a site known to be phosphorylated in TNF α -, GM-CSF- or LPS-treated neutrophils^{26,27}, was already phosphorylated in non-stimulated neutrophils and fMLF barely increased its phosphorylation. The four phosphorylated serines were almost completely dephosphorylated after a 90 second-stimulation. Interestingly, the kinetics of fMLF-induced p47phox-phosphorylation precede those of the NADPH oxidase activation (Figure 1B). fMLF-induced phosphorylation of Ser304, 315, 320 and 328 was concentration-dependent (Figure 1C and Supplemental Figure 3), starting between 10^{-9} and 10^{-8} M, and reaching its maximal effect at 10^{-6} M, which is the optimal fMLF concentration to induce NADPH oxidase activation (Figure 1D).

PMA induced a less rapid but more sustained phosphorylation of p47phox on Ser304, 315, 320, 328 than fMLF in human neutrophils

PMA is a known strong activator of the NADPH oxidase and an inducer of p47phox phosphorylation in human neutrophils. PMA-induced neutrophil activation occurs by direct-activation of PKC⁴³. To evaluate the PMA optimal concentration and time-course, we stimulated healthy neutrophils between 1 and 12 min at concentrations ranging from 10 to 200 ng/mL. We show that PMA (at 200 ng/ml) induced the time-dependent phosphorylation of p47phox on Ser304, 315, 320 and 328 (Figure 2A). PMA induced only a moderate increase in the phosphorylation of Ser345. Phosphorylated p47phox from different experiments was quantified by densitometry and corrected for total p47phox amount as described in the Methods. The mean of these experiments (Supplemental Figure 4) shows that PMA-induced phosphorylation occurs after a 2-min lag time, then increased and plateaued over time. In contrast to what we observed with fMLF, dephosphorylation was not observed within the 12-min time frame tested. Interestingly, the kinetics of PMA-induced p47phox-phosphorylation parallel the kinetics of NADPH oxidase activation (Figure 2B). PMA-induced phosphorylation of Ser304, 315, 320 and 328 was concentration-dependent (Figure 2C and Supplemental Figure 5), starting at 10-50 ng/ml and reaching its maximal effect at 100 ng/ml and higher, which are the optimal concentrations to induce NADPH oxidase activation (Figure 2D).

Phosphorylation of p47phox on Ser304, 315, 320, 328 and 345 in neutrophils and EBV-transformed lymphocytes from p67phox-CGD deficient patients is dramatically reduced

To investigate the possible role of p67phox in the phosphorylation of p47phox, we isolated neutrophils from a unique cohort of two CGD patients deficient for p67phox. The two patients are not related, and they have the same p67phox/NCF2 gene mutation [c.364+1G>A]¹², which results in the absence of the p67phox protein (Figure 3A). We first confirmed that neutrophils from these patients did not produce ROS under resting conditions and after stimulation with PMA and fMLF (Supplemental Figure 6). Furthermore, Western blot analysis confirmed that the p67phox protein was absent (Figure 3A). In addition, the p40phox subunit was barely expressed (12.5%), while g91phox, p47phox and p22phox

were all normally expressed. Interestingly, when we investigated p47phox phosphorylation in resting and fMLF- and PMA-stimulated neutrophils, we observed that the phosphorylation of p47phox on Ser304, Ser315, Ser320 and Ser328 was dramatically reduced in neutrophils from p67phox^{-/-}-CGD patients compared to controls (Figure 3B and Supplemental Figure 7). These results were similar in neutrophils from both CGD patients. Given the rarity of p67phox deficiency¹²⁻¹⁶, we used EBV-transformed lymphocytes (EBV-L) to confirm these results. Western blot analysis of EBV-L showed that the p67phox protein was completely absent in p67phox^{-/-}-patient-derived EBV-L compared to control while gp91phox and p47phox were normally expressed (Figure 4A). As observed in neutrophils from p67phox^{-/-}-CGD patients, PMA-induced phosphorylation of p47phox on Ser304, Ser315 and Ser328 in EBV-L was dramatically reduced (Figure 4B). Interestingly, the phosphorylation of Ser345 in the absence or presence of PMA was also reduced in p67phox^{-/-}-patient-derived EBV-L. Unexpectedly, Ser320 was not phosphorylated in PMA-stimulated EBV-L. Altogether, these data suggest that p67phox is required for optimal phosphorylation of p47phox in neutrophils and EBV-L.

Knockdown of p67phox expression or its absence in COSphox cells impaired phosphorylation of p47phox

To confirm the results obtained with neutrophils and EBV-L, we used an siRNA approach to inhibit p67phox expression in COSphox cells. Results show that the siRNA against p67phox dramatically decreased the expression of the protein in these cells (Figure 5A), which resulted in the significant decrease of PMA-stimulated p47phox phosphorylation on Ser304, 315 and 328 (Figure 5A and 5B). Interestingly, Ser320 and 345 showed only a marginal increase in p47phox phosphorylation, and absence of p67phox had no effect on their phosphorylation. To confirm the siRNA data, we used COSphox cells with or without p67phox expression. As expected, the phosphorylation of p47phox was lower in cells expressing only gp91phox, p22phox and p47phox compared to cells also expressing

p67phox (Figure 5C and 5D).

The lack of p67phox does not alter the phosphorylation of proteins unrelated to the NADPH oxidase and NADPH oxidase-derived ROS are not involved in p47phox phosphorylation in normal neutrophils

To check if the absence of p67phox specifically affects the phosphorylation of p47phox, we analyzed the phosphorylation p38MAPKinase and ERK1/2, which are not part of the NADPH oxidase complex, and showed that their phosphorylation was not affected in neutrophils from p67phox^{-/-}-CGD patients (Supplemental Figure 8). As neutrophils from p67phox^{-/-} CGD patients do not express the p67phox protein and do not produce ROS, the impairment of p47phox phosphorylation could be due either to the absence of p67phox or the lack of ROS. To investigate whether ROS produced by the NADPH oxidase are involved in p47phox phosphorylation, we isolated neutrophils from healthy donors and treated them with the NADPH oxidase inhibitor DPI, or the superoxide and H₂O₂ catabolizing enzymes, SOD and catalase, in the presence or absence of PMA. Results showed that DPI completely inhibited ROS production and that SOD+catalase had a strong antioxidant effect (Supplemental Figure 9); however, their addition failed to impair PMA-induced phosphorylation of p47phox (Supplemental Figure 9). Similar results were obtained with fMLF as the stimulus (data not shown). Altogether, these results demonstrate that NADPH oxidase-derived ROS are not involved in p47phox phosphorylation and suggest that the decrease in p47phox phosphorylation observed in p67phox^{-/-} cells is not due to the absence of ROS, but rather to the absence of p67phox or associated proteins.

Recombinant p67phox and p40phox enhance the phosphorylation of p47phox by PKC isoforms *in vitro*

To investigate whether p67phox could enhance the p47phox phosphorylation, we performed *in vitro* phosphorylation experiments using the PKC isoforms found to be expressed in neutrophils in the presence or absence of p67phox. Results showed that recombinant p47phox alone was weakly phosphorylated by PKC α , β , δ and ζ ; however, the addition of recombinant p67phox enhanced p47phox phosphorylation (Figure 6). As p40phox was also depleted in p67phox^{-/-} neutrophils, we performed the same *in vitro* phosphorylation experiments in the absence or presence of p40phox. In the presence of p40phox, p47phox phosphorylation by PKC α , β , δ and ζ , was also mildly enhanced; however, when both p40phox and p67phox were present, p47phox phosphorylation was strongly increased (Figure 6A and 6B). These results suggest that both p67phox and p40phox are required for optimal p47phox phosphorylation on Ser304, Ser315, Ser320 and Ser328. In addition, we observed that the effect of p67phox on p47phox phosphorylation was dose-dependent (Figure 7). Interestingly, heat-denaturation of p67phox resulted in reduction of its enhancing effect on PKC-mediated phosphorylation of p47phox, suggesting that the native p67phox is required (Figure 7). Although we only show here the phosphorylation of Ser328, the same results were obtained for the phosphorylation of the other serines (data not shown). In addition, bovine serum albumin, a protein unrelated to the NADPH oxidase and with the same molecular weight as p67phox, had no effect on the phosphorylation of p47phox (Supplemental Figure 10).

Discussion

Superoxide production by the phagocyte NADPH oxidase is a tightly regulated process, which requires translocation of the cytosolic subunits to the membrane, leading to enzyme activation^{8,10}. Among the different processes required for NADPH oxidase activation and ROS production, p47phox

phosphorylation is a key step in intact cells^{10,11}. In the current study, we determined the optimal conditions for the phosphorylation of p47phox in neutrophils stimulated by fMLF and PMA, using phosphosite-specific antibodies and investigated the role of p67phox in this process. Stimulation with fMLF and PMA induced the phosphorylation of p47phox on Ser304, Ser315, Ser320 and Ser328, although with different time course. In contrast, Ser345 phosphorylation appeared to be already present at steady-state and was only slightly increased by both stimuli. Interestingly, we observed a strong reduction of p47phox phosphorylation in neutrophils from p67phox^{-/-}-CGD patients. Furthermore, *in vitro* studies showed that the addition of recombinant p67phox alone or to a lesser extent that of p40phox alone, enhanced p47phox phosphorylation on Ser304, Ser315, Ser320, and Ser328, an effect that was further enhanced when both were combined.

It has been known for some time that p47phox is phosphorylated on several serines localized in the carboxy-terminal region²⁰⁻²². The initial studies on p47phox phosphorylation in intact neutrophils were performed using [³²P]-labeled orthophosphoric acid^{20,21}. This technique is cumbersome and requires high amounts of radioactive materials, a large number of cells, and a specific antibody capable of immunoprecipitating p47phox. In addition, this technique does not give any information about the individual phosphorylated sites, unless used in tandem with site-directed mutagenesis and two-dimensional phosphopeptide mapping, two very time-consuming techniques. To allow the study of p47phox phosphorylation in cell lysates without the need of radioactivity and immunoprecipitation, we have developed anti-p47phox antibodies highly specific for each serine phosphorylated upon stimulation. These antibodies allow to investigate p47phox phosphorylation by Western blotting using only few cells, which is an advantage in the case of children with rare diseases.

In this study, we have optimized the experimental protocol to allow for the dynamic capture of p47phox phosphorylation in fMLF- and PMA-stimulated neutrophils. Phosphorylation of Ser304, Ser315, Ser320 and Ser328 from fMLF-stimulated neutrophils reached a peak after only 10 sec, and

decreasing over time. This fast and transient phosphorylation of p47phox is consistent with the fast and transient activation of fMLF-induced ROS production by neutrophils. However, the stimulatory effect of PMA on the phosphorylation of Ser304, Ser315, Ser320 and Ser328 occurred more slowly and was sustained for up to 20 min (data not shown). Both agonists induced little or no increase in the phosphorylation of Ser345 over that at steady state.

In resting cells, p47phox is in the cytosol either in a free form or in a complex with p67phox^{30,31}. Indeed p47phox has 2 SH3 domains and a proline-rich region while p67phox has 2 SH3 domains, with one p67phox-SH3 domain interacting with the p47phox proline-rich sequence³²⁻³⁵. In this study, we had a unique cohort of p67^{-/-}phox-CGD patients, which allowed us to demonstrate for the first time that the absence of p67phox in neutrophils results in a decrease of p47phox phosphorylation. These data were confirmed in patient-derived EBV-transformed lymphocytes and in COSphox cells lacking p67phox, suggesting that p67phox, or an associated protein, is required for optimal p47phox phosphorylation. The decrease of p47phox phosphorylation was less pronounced in COSphox cells than in neutrophils and EBV-L possibly because COSphox cells do not express the same protein kinases and the same PKC isoforms as neutrophils^{44,45}. Indeed, PKC β II and PKC δ , two major PKC isoforms in neutrophils, are not expressed in COSphox cells. Investigation into whether the phosphorylation of p47phox by other PKC isoforms or other kinases does not require p67phox would be of interest in the future.

We demonstrated that the effect of p67phox was not observed when cells were deprived of ROS, either by SOD/catalase combination or by pharmacological inhibition of the NADPH oxidase by DPI, suggesting that p67phox is most likely responsible for the increase in p47phox phosphorylation. This critical finding was not reported in previous studies on p67phox^{-/-}-CGD patients using ³²P-labeling⁴⁶, probably because this technique does not allow to detect each phosphorylated serine but rather reflects the overall phosphorylation. The anti-p47phox antibodies against each phosphorylated site are ~~more~~

sensitive and easier to use and may allow to uncover new mechanisms for p47phox phosphorylation. Interestingly, in line with our observation, previous studies reported that phosphorylation of p47phox was also altered in neutrophils from gp91phox-deficient CGD patients^{47,48}.

We observed that the p40phox subunit was also dramatically depleted in neutrophils from p67phox^{-/-} CGD patients, as previously described^{32,49}. *In vitro* phosphorylation experiments clearly showed that both p67phox and p40phox alone or in combination increased p47phox phosphorylation by PKC isoforms. In addition, we observed that when p67phox was denatured, its effects on p47phox phosphorylation were lost. The decreased phosphorylation of p47phox could be observed in the combined absence of p67phox and p40phox, either in p67phox^{-/-} neutrophils, or in p67phox siRNA-treated COSphox cells that do not express p40phox. These results suggest that a p47phox/p67phox/p40phox interaction may favor p47phox phosphorylation. Alternatively, a protein associated with p67-phox and p47phox may be involved; thus, the mechanism of this impairment should be studied in more detail in future studies. In line with our observations, p67phox and p40phox were shown to enhance NADPH oxidase activation in a cell-free activation system.⁵⁰⁻⁵⁴

In summary, this study characterized the phosphorylation of p47phox in fMLF- and PMA-stimulated neutrophils on Ser304, Ser315, Ser320 and Ser328 by using anti-phospho-p47phox antibodies and showed that p67phox and p40phox are novel regulators of p47phox-phosphorylation in human neutrophils. This study and the developed tools may facilitate investigations on the role of phosphorylation in NADPH oxidase activation.

Acknowledgments

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Authorship

SAB designed and conducted the experiments, and wrote the manuscript; VM; MHN; CP, YL, SL and TB conducted experiments and wrote the manuscript; MAGP wrote the manuscript; RDY and PMCD designed experiments and wrote the manuscript; JEB designed and supervised experiments and wrote the manuscript.

Conflict of Interest

Neither author has a conflict-of-interest related to this work.

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Figure legends

Figure 1. Kinetics and concentration-dependent effect of fMLF on p47phox phosphorylation and ROS production in human neutrophils. (A) Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with fMLF ($1 \mu\text{M}$) for different times and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti-phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody; (B) Neutrophils ($0.5 \times 10^6/500 \mu\text{l}$) were incubated in HBSS for 5 min in the presence of $10 \mu\text{M}$ luminol at 37°C , stimulated with fMLF ($1 \mu\text{M}$) and ROS production was measured by luminol-amplified chemiluminescence over time; (C) Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with different concentrations of fMLF for 10 sec and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using the same antibodies as in (A); (D) ROS production of neutrophils ($0.5 \times 10^6/500 \mu\text{l}$ HBSS) was measured as in (B). Experiments were repeated three times. ($n = 3$, mean \pm SEM).

Figure 2. Kinetics and concentration-dependent effect of PMA on p47phox phosphorylation and ROS production in human neutrophils. (A) Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with PMA (200 ng/ml) for different times and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti- phospho-Ser304, -phospho-Ser315, -phospho-

Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody. (B) Neutrophils ($0.5 \times 10^6/500 \mu\text{l}$) were incubated in HBSS for 5 min in the presence of $10 \mu\text{M}$ luminol at 37°C then stimulated by with PMA (100 ng/ml) and ROS production was measured by luminol-amplified chemiluminescence over time; (C) Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with different concentrations of PMA during 10 min and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using the same antibodies as in (A); (D) ROS production of neutrophils ($0.5 \times 10^6/500 \mu\text{l}$ in HBSS) was measured as in (B). Experiments were repeated three times ($n = 3$, mean \pm SEM).

Figure 3. Phosphorylation of p47phox in neutrophils from p67phox-deficient patients and from healthy donors. (A) Neutrophils from healthy donors (PMN-Control) and p67phox-deficient patients (PMN-CGD(p67^{-/-})) ($15 \times 10^6/400 \mu\text{l}$) were incubated with fMLF ($1 \mu\text{M}$) or PMA (200 ng/ml) for 10 sec and 10 min, respectively, and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using anti-gp91phox, anti-p67phox, anti-p47phox, anti-p40phox and anti-p22phox antibodies; (B) Neutrophils from healthy donors (PMN-Control) and p67phox-deficient patients (PMN-CGD(p67^{-/-})) ($15 \times 10^6/400 \mu\text{l}$) were incubated with fMLF ($1 \mu\text{M}$) or PMA (200 ng/ml) for 10 sec and 10 min, respectively, and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti- phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody. Experiments were repeated twice in duplicates.

Figure 4. Phosphorylation of p47phox in EBV-transformed B lymphocytes from p67phox-deficient patients and from healthy donors. (A) EBV-transformed B lymphocytes from healthy donors (EBV-L) and p67phox-deficient patients (EBV-L-p67phox^{-/-}) ($15 \times 10^6/400 \mu\text{l}$) were stimulated by with PMA (200 ng/ml) for 10 min and the reaction was stopped by denaturation with 100 μl Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using anti-gp91phox, anti-p67phox and anti-p47phox antibodies; (B) EBV-transformed B lymphocytes from healthy donors (EBV-L) and p67phox-deficient patients (EBV-L-p67phox^{-/-}) ($15 \times 10^6/400 \mu\text{l}$) were stimulated with PMA (200 ng/ml) for 10 min and the reaction was stopped by denaturation with 100 μl Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti- phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody. Experiments were repeated three times.

Figure 5. p67phox knockdown reduces the phosphorylation of p47phox and its expression increases this process in COSphox cell lines. (A) COSphox cells (expressing gp91phox, p22phox, p47phox and p67phox) were transiently transfected with siRNA p67phox or scrambled siRNA as control. Forty-eight hours after transfection, cell homogenates were prepared and subjected to Western blotting using anti-p47phox and anti-p67phox antibodies to verify the effect of siRNA-mediated knockdown (A; bottom panels) and with different anti-phospho-p47phox antibodies as indicated (A; top panels). Images shown are representative Western blots from 3 independent experiments. (B) The NIH Image J software was used for densitometry analysis and the results expressed as mean +/- SEM

are shown (n=3; * p < 0.05). (C) COS-7 cells were either transiently transfected with gp91phox cDNA in pEF-PGKpac and p22phox/p47phox/p67phox cDNA in pRK5 or transiently transfected with only gp91phox cDNA in pEF-PGKpac and p22phox/p47phox cDNA in pRK5. Twenty-four hours after transfection, the cells were stimulated with vehicle or PMA (200 ng/ml final) for 30 min. Cell homogenates were prepared and subjected to Western blotting using different anti-phospho-p47phox antibodies, as indicated. (D) Images shown are representative Western blots from 3 independent experiments. The NIH Image J software was used for densitometry analysis (n=3 ; * p < 0.05).

Figure 6. Phosphorylation of recombinant p47phox by purified PKC isoforms *in vitro* and effect of p67phox and p40phox. Recombinant p47phox protein was incubated without or with p67phox or p40phox or p67phox+p40phox for 10 min; PKC α , PKC β , PKC δ or PKC ζ were added for 5 min and the reaction was started by adding ATP, followed by incubation at 30°C for 15 min. The reactions were terminated by addition of Laemmli 5X sample buffer and denaturation at 100°C. Proteins were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti-phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328 antibodies and anti-p47phox antibody. Experiments were repeated three times.

Figure 7. Concentration-dependent effect of p67phox on p47phox phosphorylation by PKC isoforms *in vitro* and effect of denatured p67phox. Recombinant p47phox protein was incubated without or with different concentrations of p67phox or with heat-denatured p67phox for 10 min; PKC α , PKC β , PKC δ or PKC ζ were added, incubated for 5 min and the reaction was started by adding

ATP, followed by incubation at 30°C for 15 min. The reactions were terminated by the addition Laemmli of 5X sample buffer and denaturation at 100°C. Proteins were subjected to SDS-PAGE and Western-blotting using various anti-phospho-p47phox antibodies. Results with anti-phospho-Ser328 antibody are shown. Experiments were repeated three times.

Figure 1.

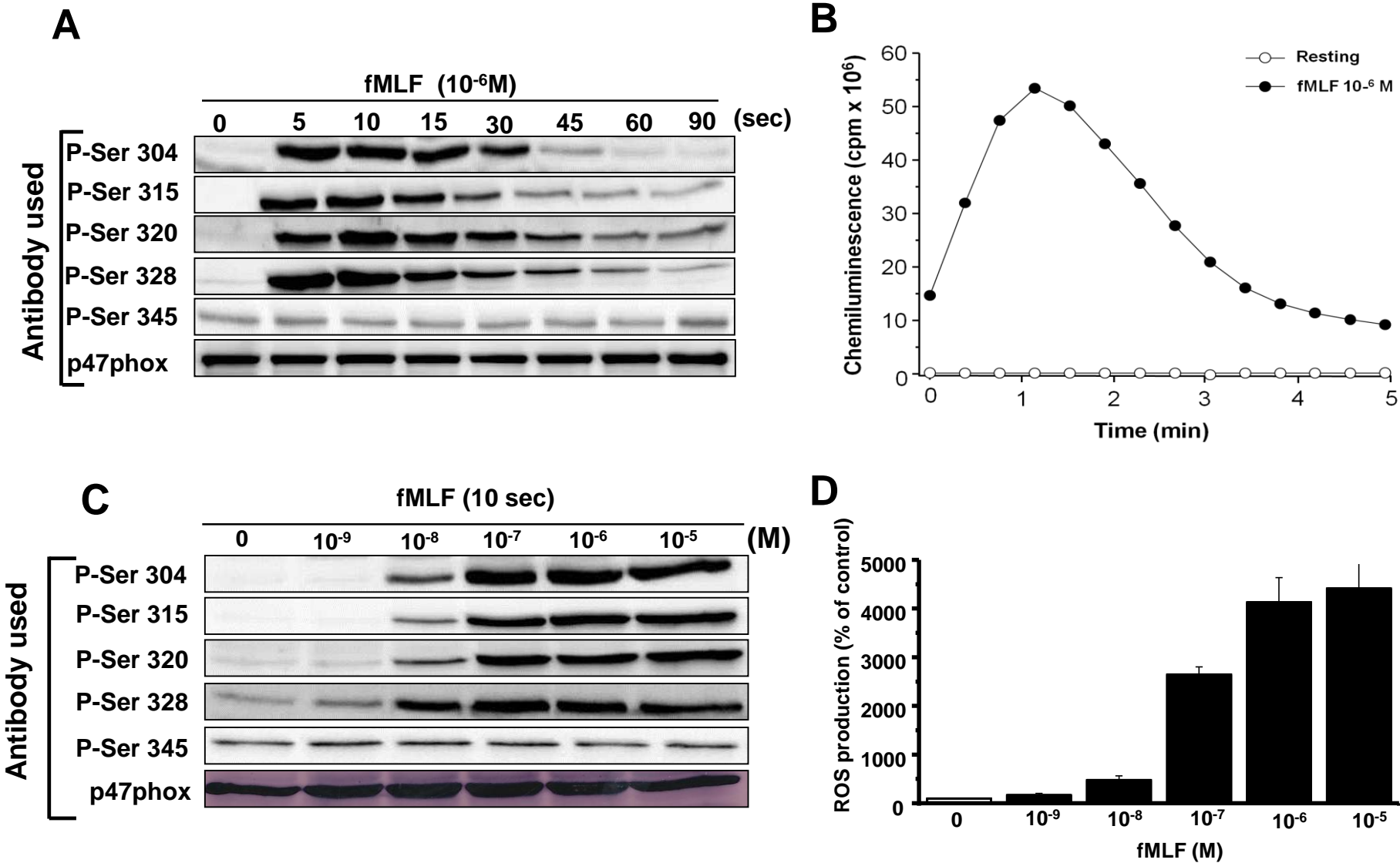


Figure 2.

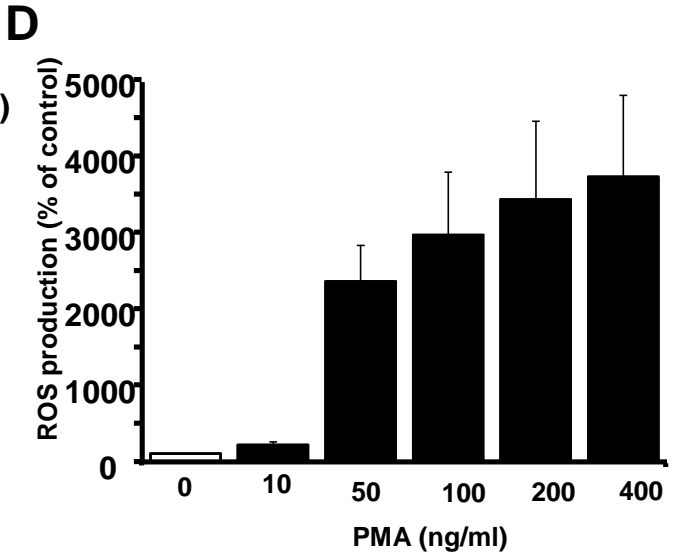
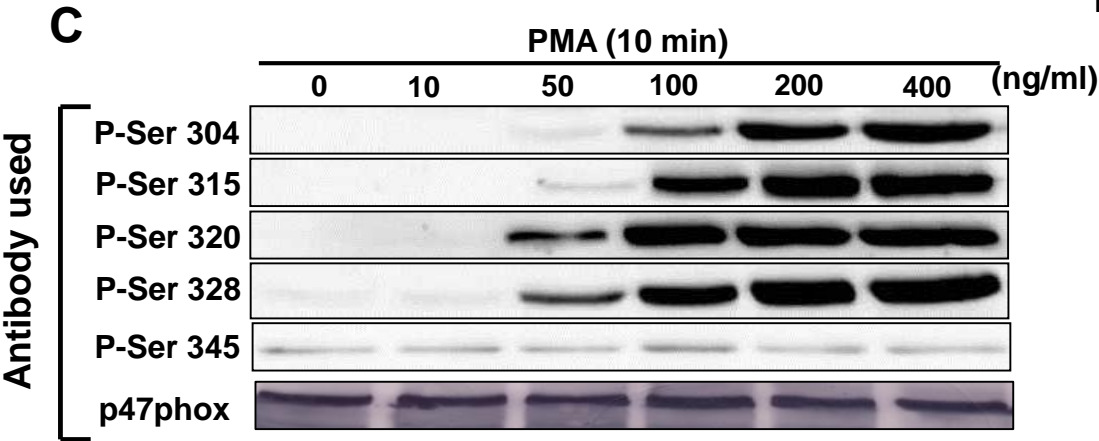
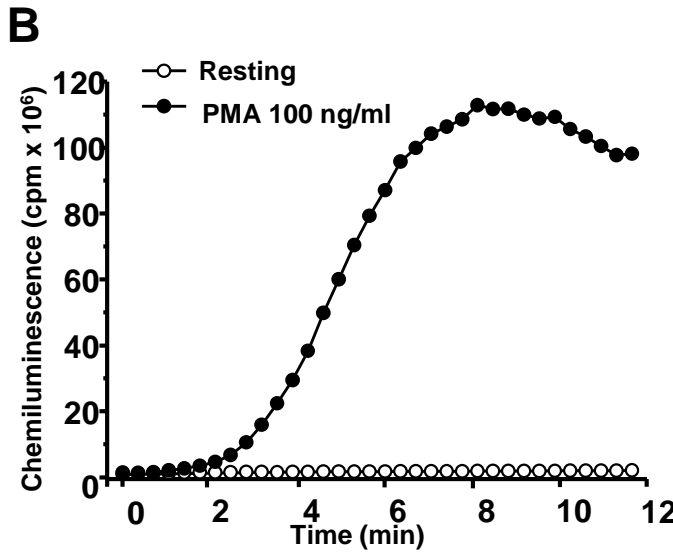
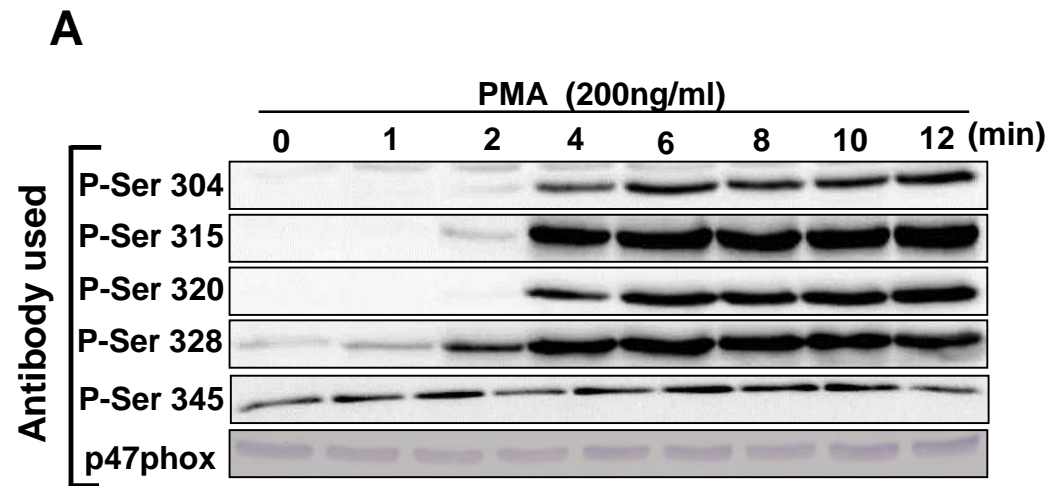


Figure 3.

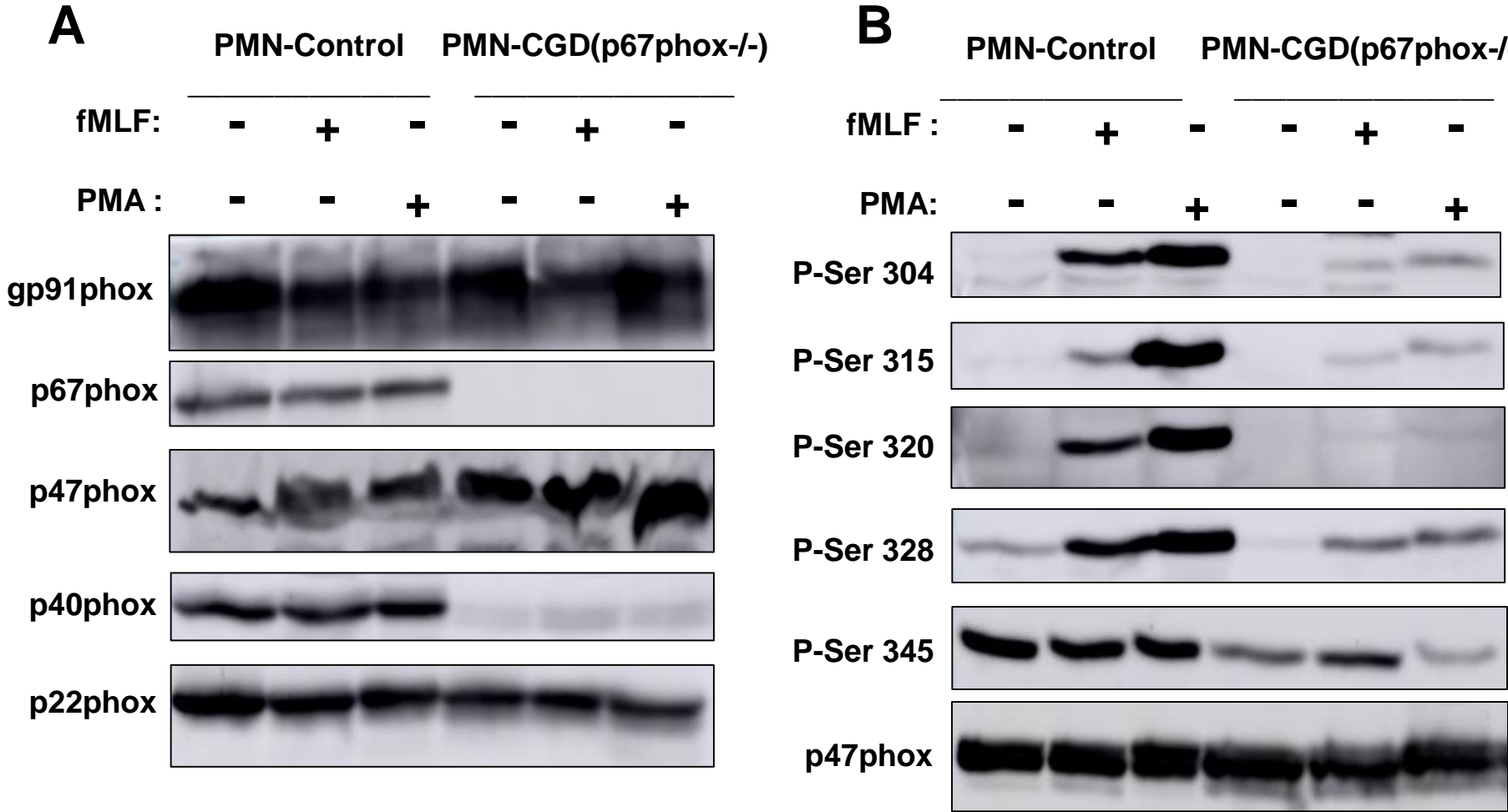
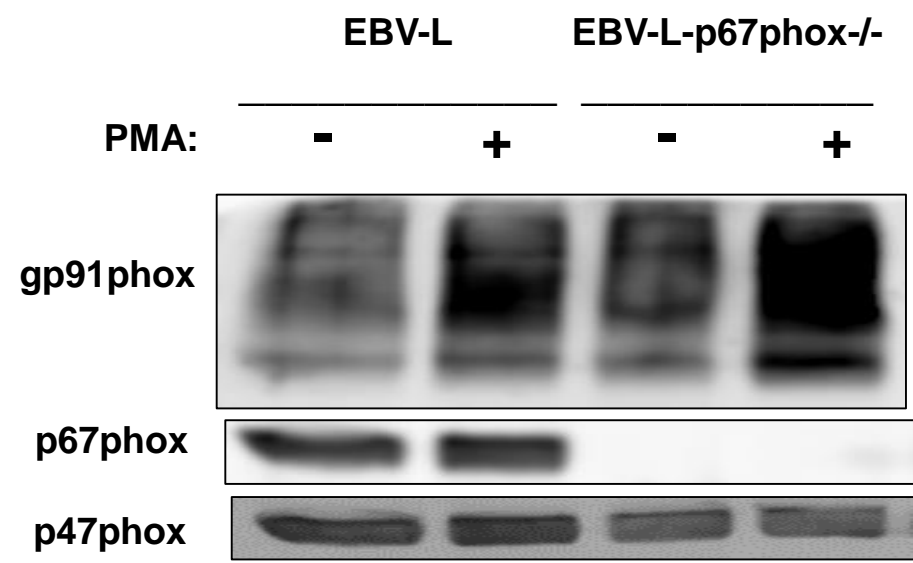


Figure 4.

A



B

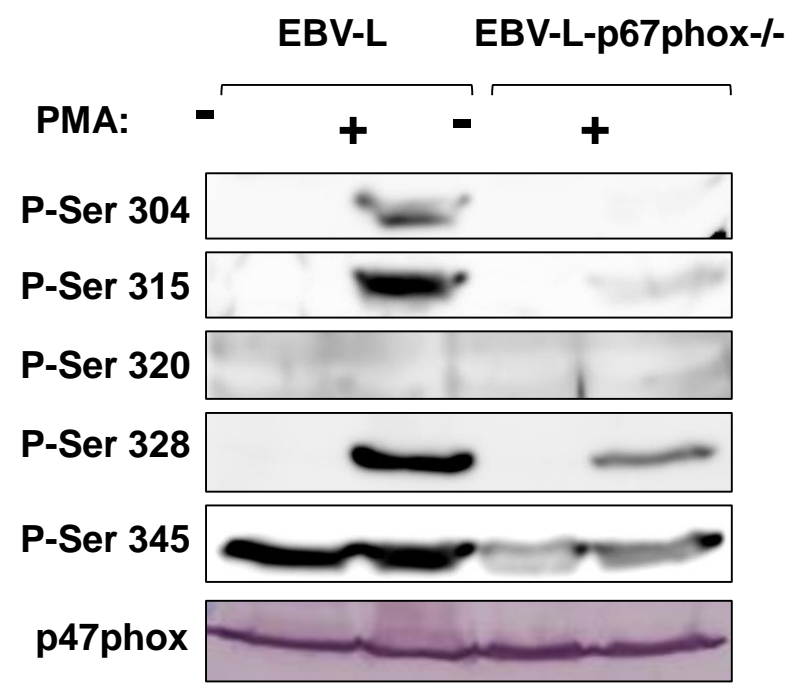
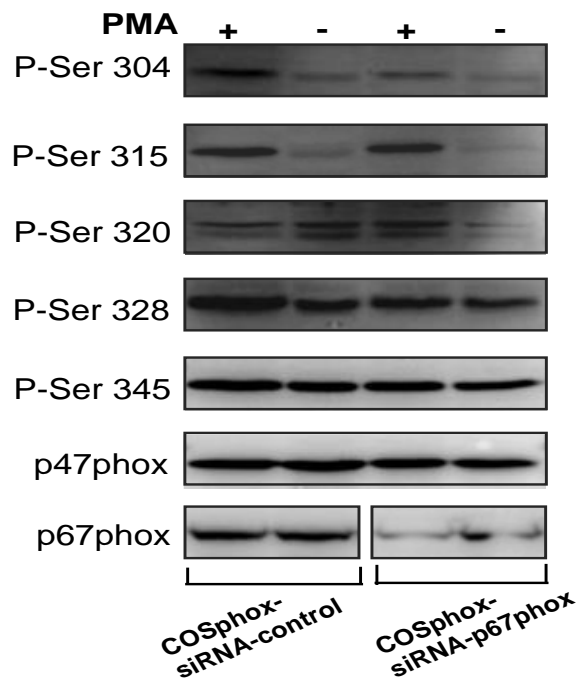
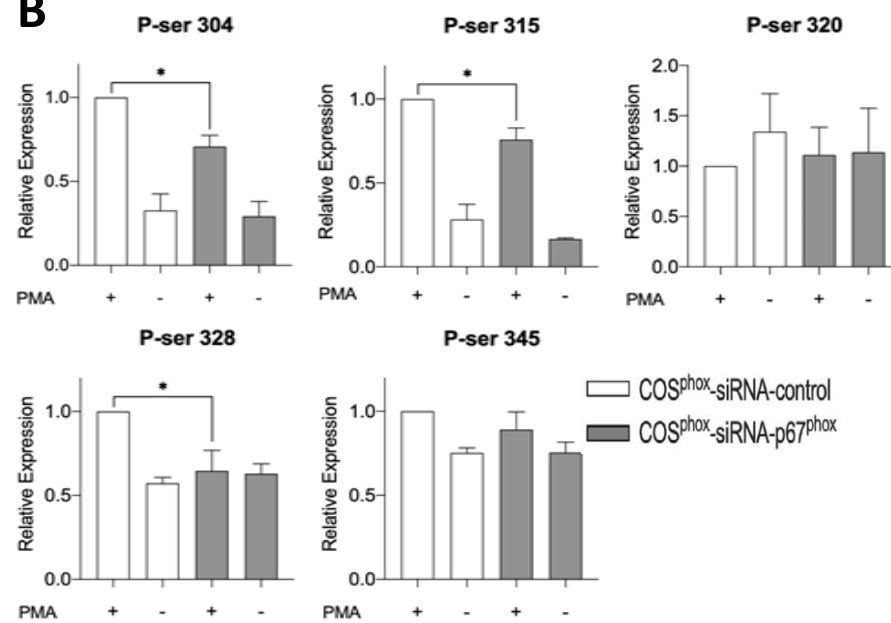


Figure 5

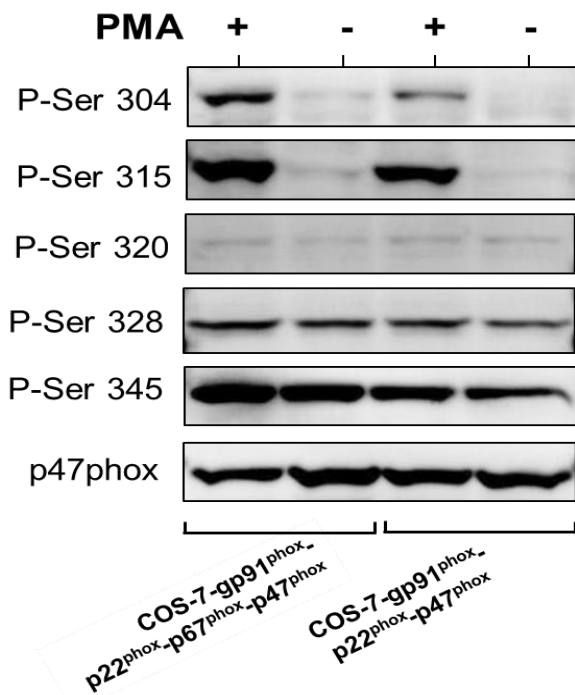
A



B



C



D

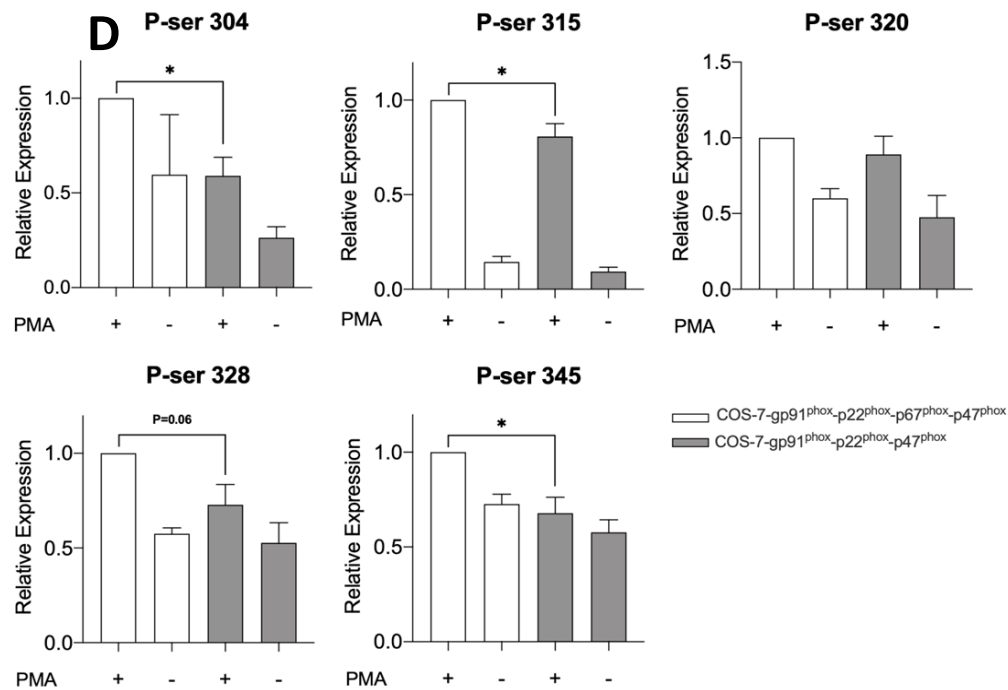


Figure 6.

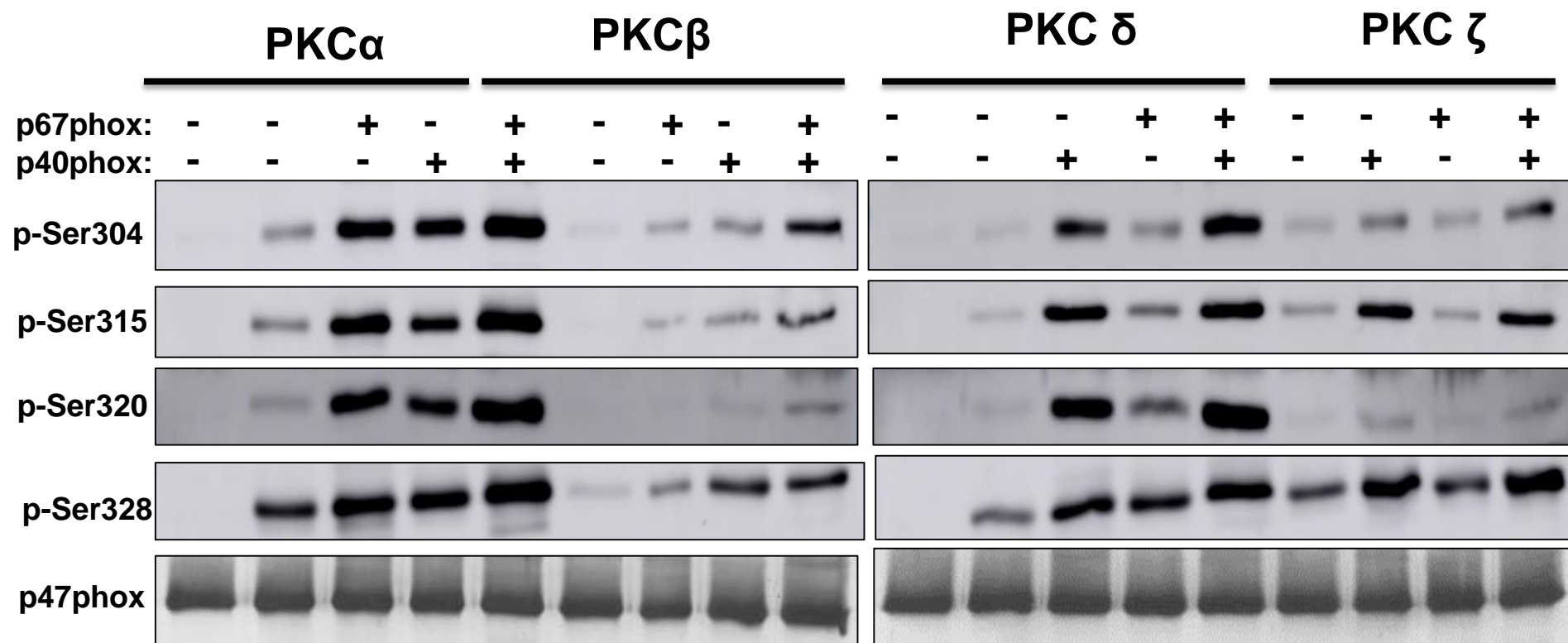
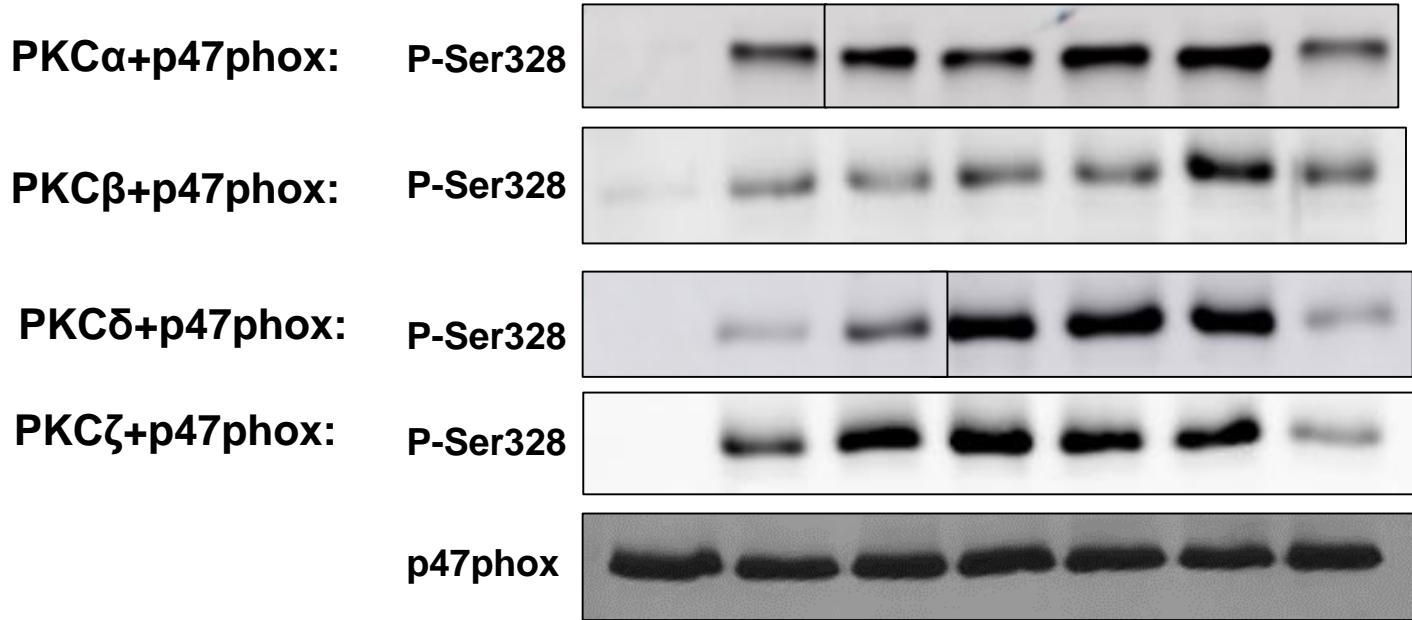


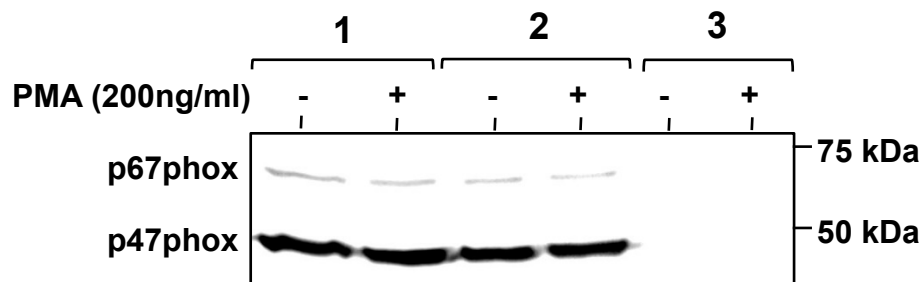
Figure 7.

PKC (1 ng/ μ L):	-	+	+	+	+	+	+
p67phox (μ g/mL):	-	-	8,5	17	34	51	-
Heat-inactivated-p67phox (μ g/mL):	-	-	-	-	-	-	34



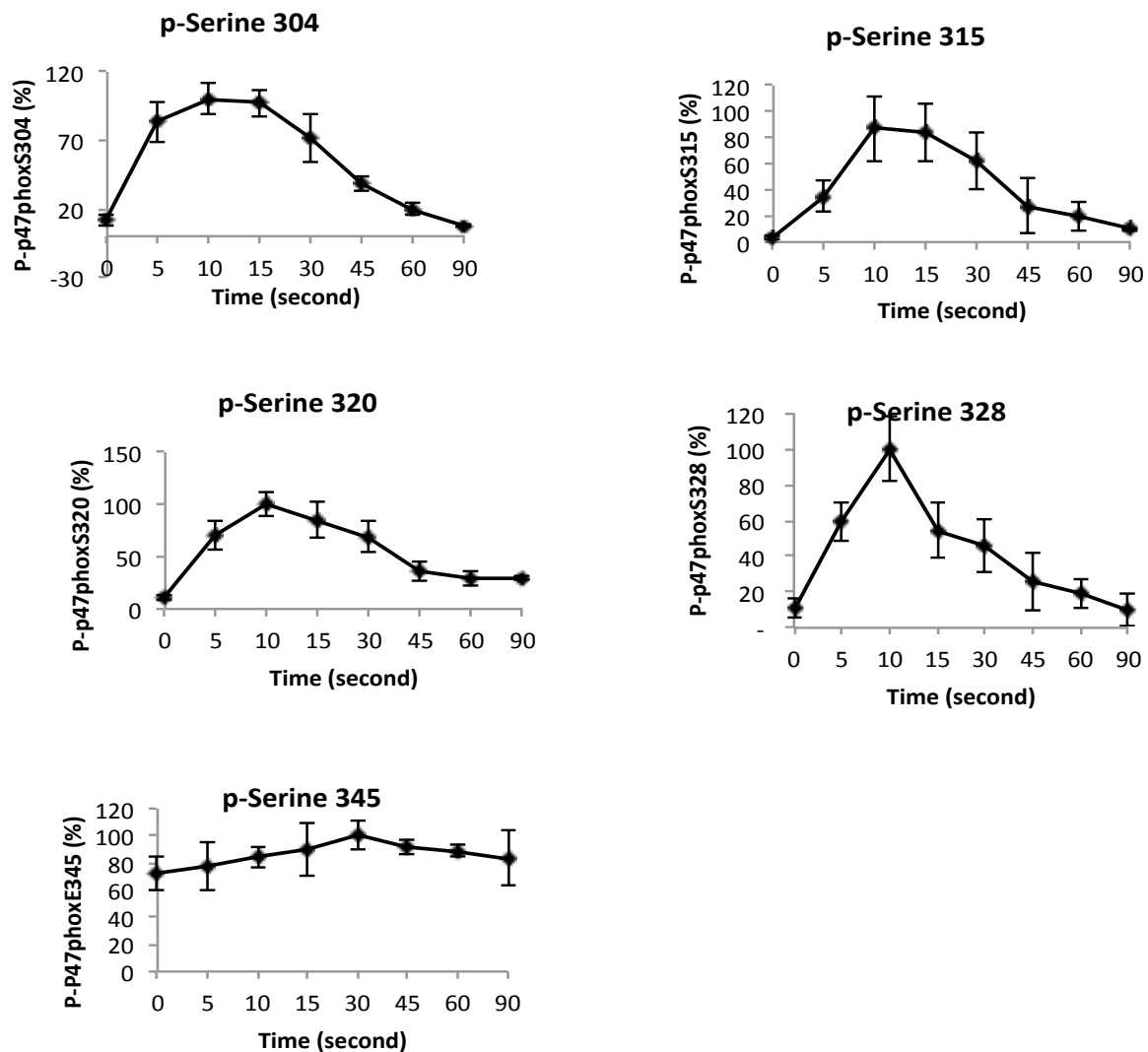
Supplemental data:**Impaired p47phox phosphorylation in neutrophils from p67phox-deficient chronic granulomatous disease patients**

Sahra Amel Belambri^{1,3}, Viviana Marzailoli¹, Margarita Hurtado-Nedelec^{1,2}, Coralie Pintard¹, Shiyu Liang⁴, Yezhou Liu⁴, Tarek Boussetta¹, Marie-Anne Gougerot-Pocidallo^{1,2}, Richard D. Ye⁴, Pham My-Chan Dang¹ and Jamel El-Benna¹

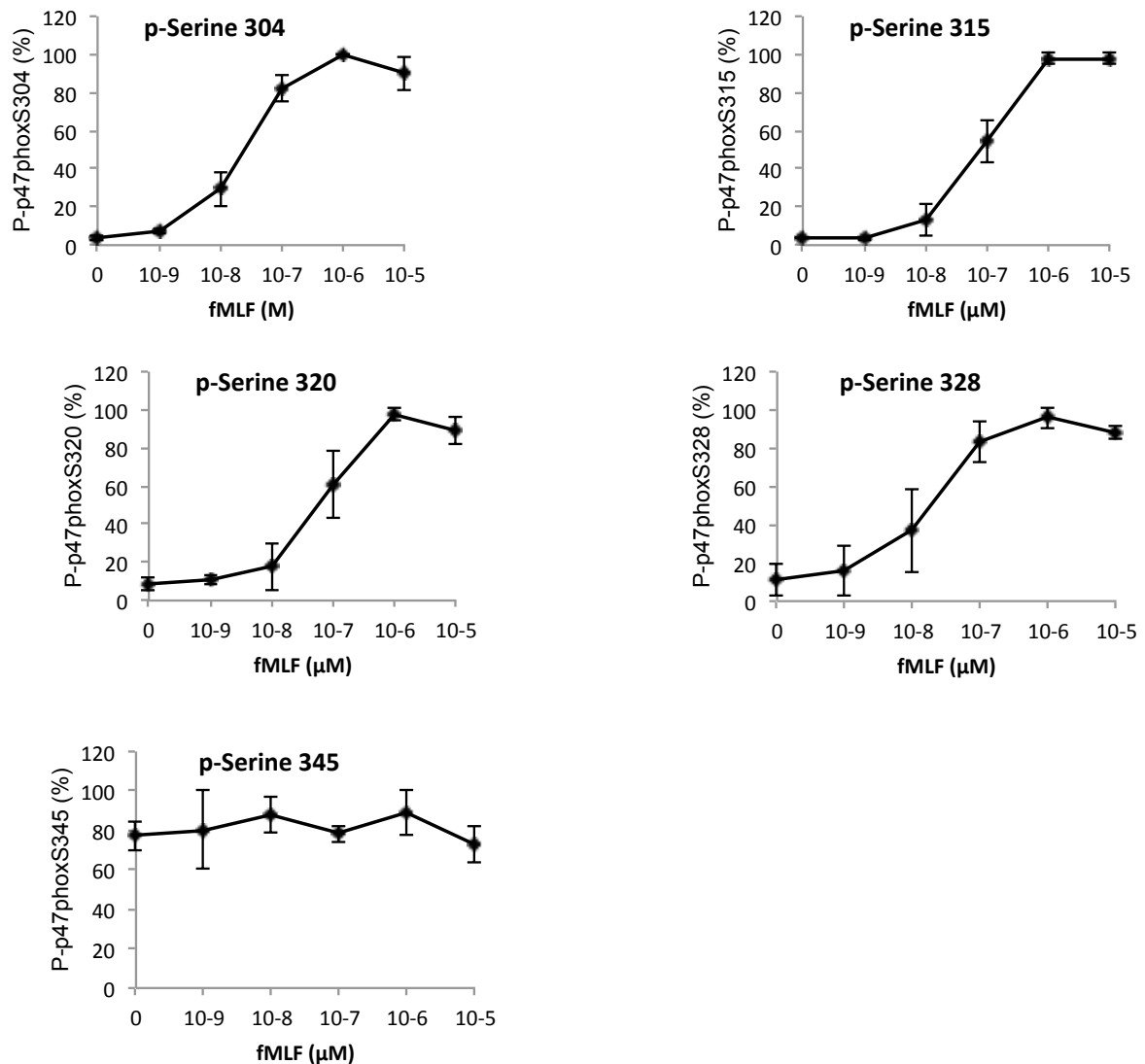


- 1 : Denatured neutrophils without centrifugation
- 2 : Denatured neutrophils supernatants after centrifugation
- 3 : Denatured neutrophils pellets after centrifugation

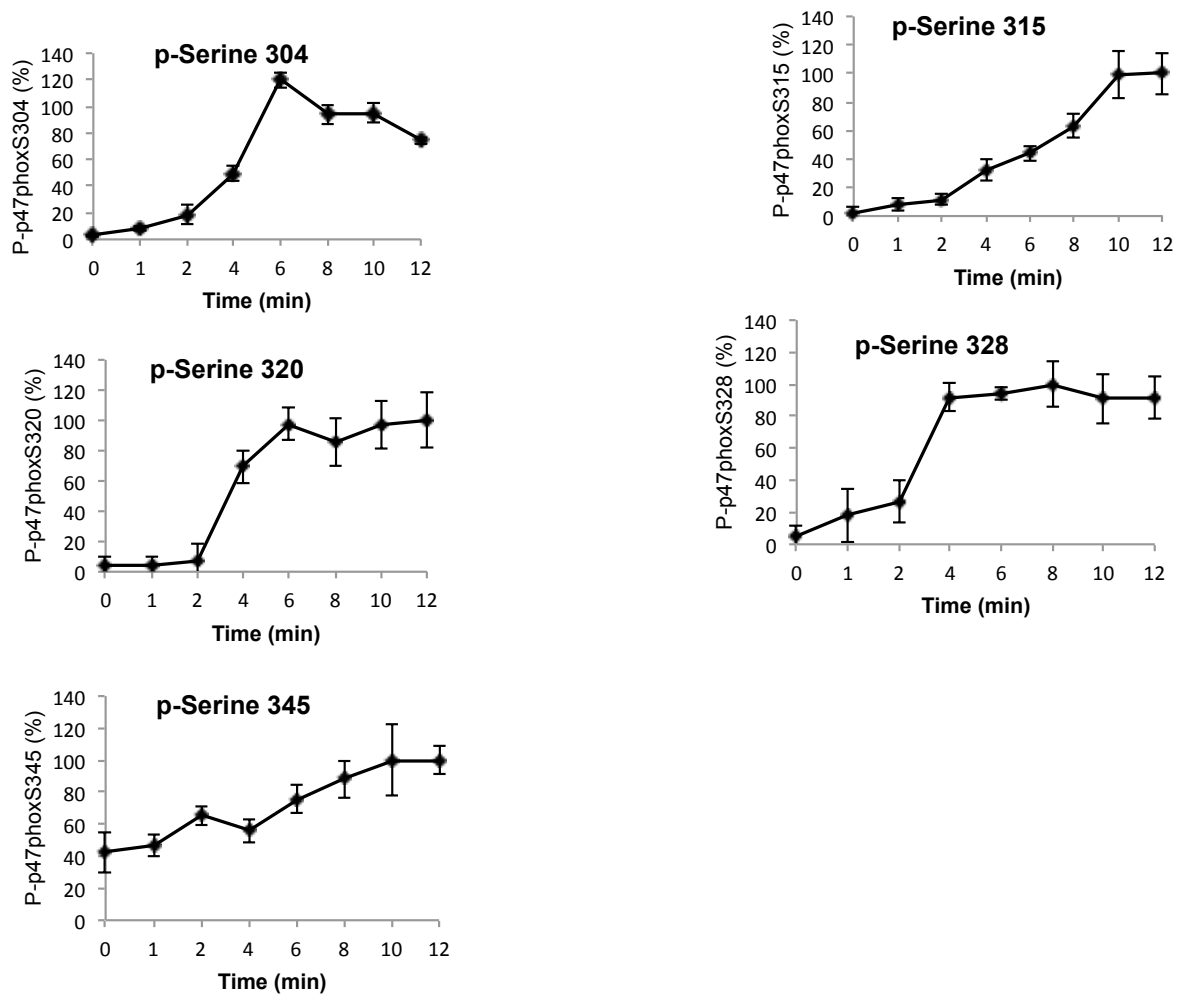
Supplemental Figure 1. p47phox is only present in the supernatant of denatured and sonicated neutrophils. Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were stimulated with PMA (200 ng/ml) for 10 min and the reaction was stopped by denaturation in Laemmli sample buffer and centrifuged at $2000 \times g$ for 10 min. Supernatants and pellets were subjected to SDS-PAGE and Western-blotting using an anti-p47phox antibody. Experiments were repeated 3 times.



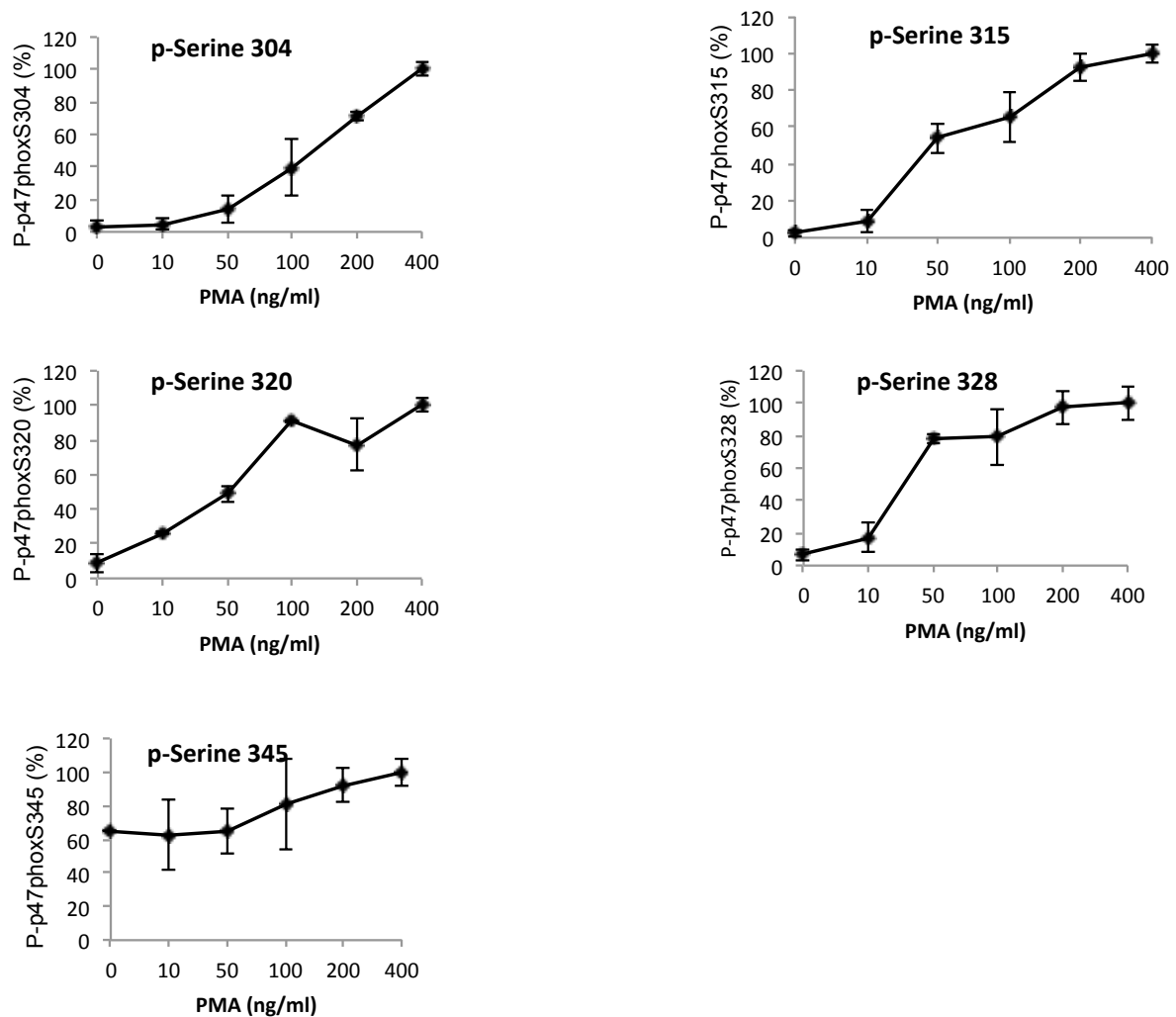
Supplemental Figure 2. Kinetics of fMLF-induced p47phox phosphorylation in human neutrophils. Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with fMLF ($1 \mu\text{M}$) for different times and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti-phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody. Phosphorylated p47phox from three experiments was quantified by densitometry and corrected for the total amount of p47phox protein as described in the Methods (mean \pm SEM; $n = 3$).



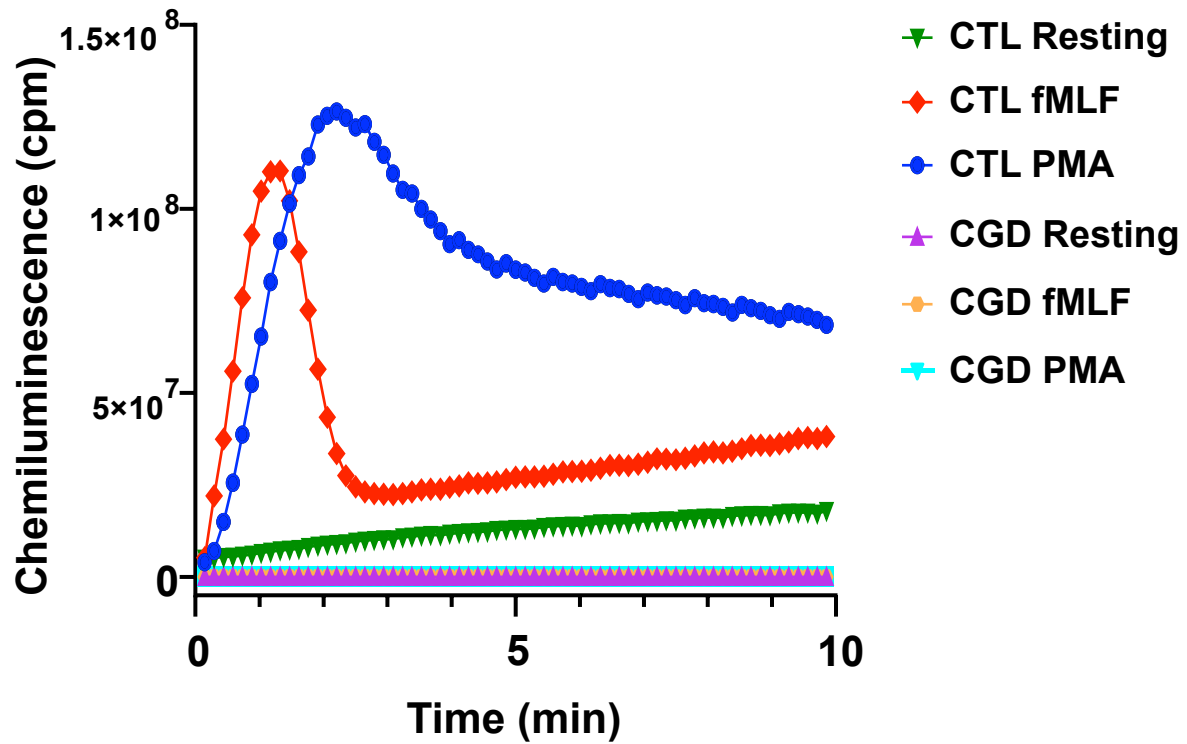
Supplemental Figure 3. Concentration-dependent effect of fMLF on p47phox phosphorylation in human neutrophils. Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with different concentrations of fMLF for 10 sec and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti-phospho-Ser304, phospho-Ser315, phospho-Ser320, phospho-Ser328, phospho-Ser-345 antibodies and anti-p47phox antibody. Phosphorylated p47phox from three experiments was quantified by densitometry and corrected for the total amount of p47phox protein as described in the Methods (mean \pm SEM, $n = 3$).



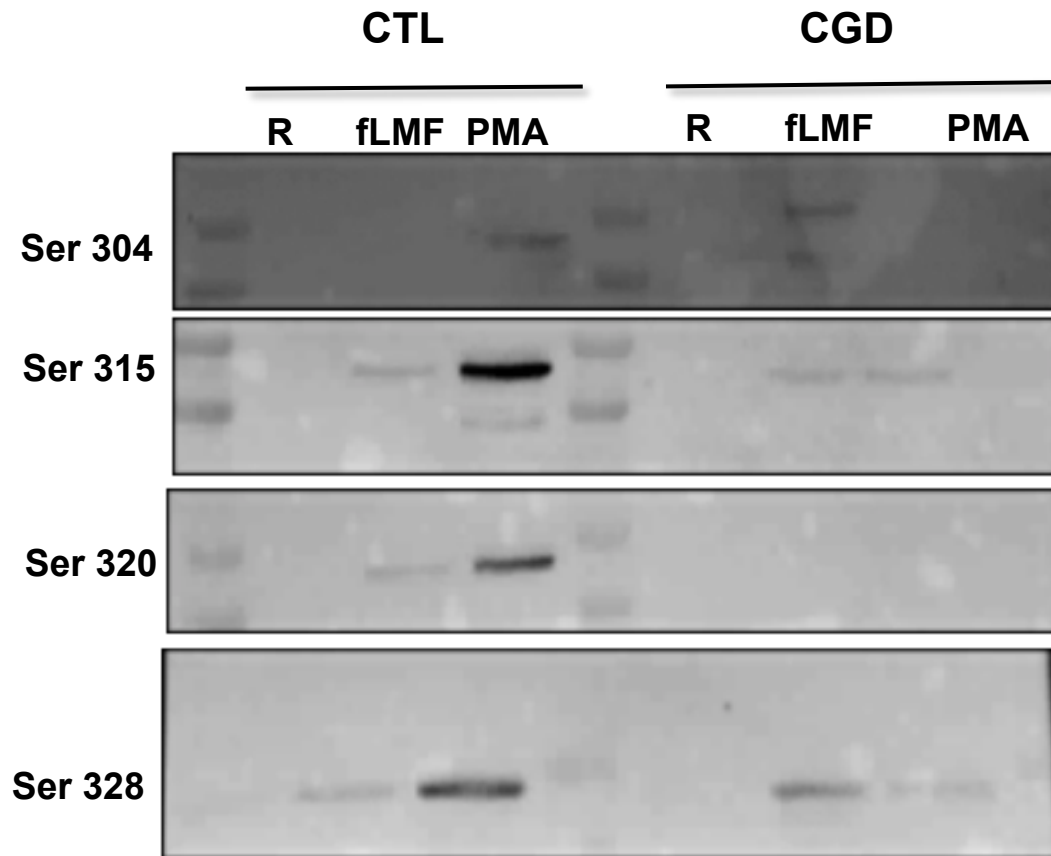
Supplemental Figure 4. Kinetics of PMA-induced p47phox phosphorylation in human neutrophils. Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with PMA (200 ng/ml) for different times and the reaction was stopped by denaturation with 100 μl hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti- phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody. Phosphorylated p47phox from three experiments was quantified by densitometry and corrected for the total amount of p47phox protein as described in the Methods (mean \pm SEM, n = 3).



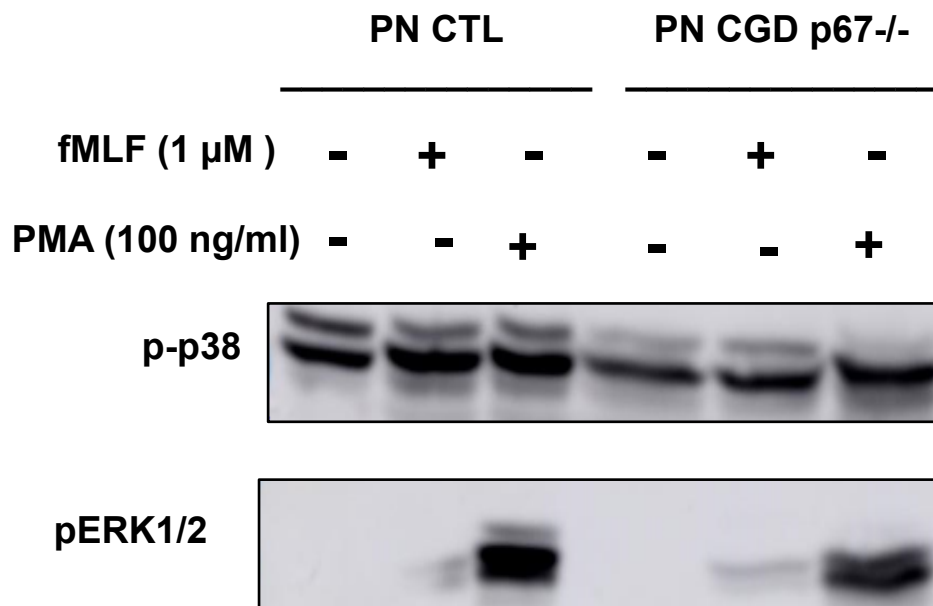
Supplemental Figure 5. Concentration dependent effect of PMA on p47phox phosphorylation in human neutrophils. Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated with different concentrations of PMA for 10 min and the reaction was stopped by denaturation with 100 μl hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti-phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody. Experiments repeated three times. Phosphorylated p47phox from three experiments was quantified by densitometry and corrected for the total amount of p47phox protein as described in the Methods (mean \pm SEM, n = 3).



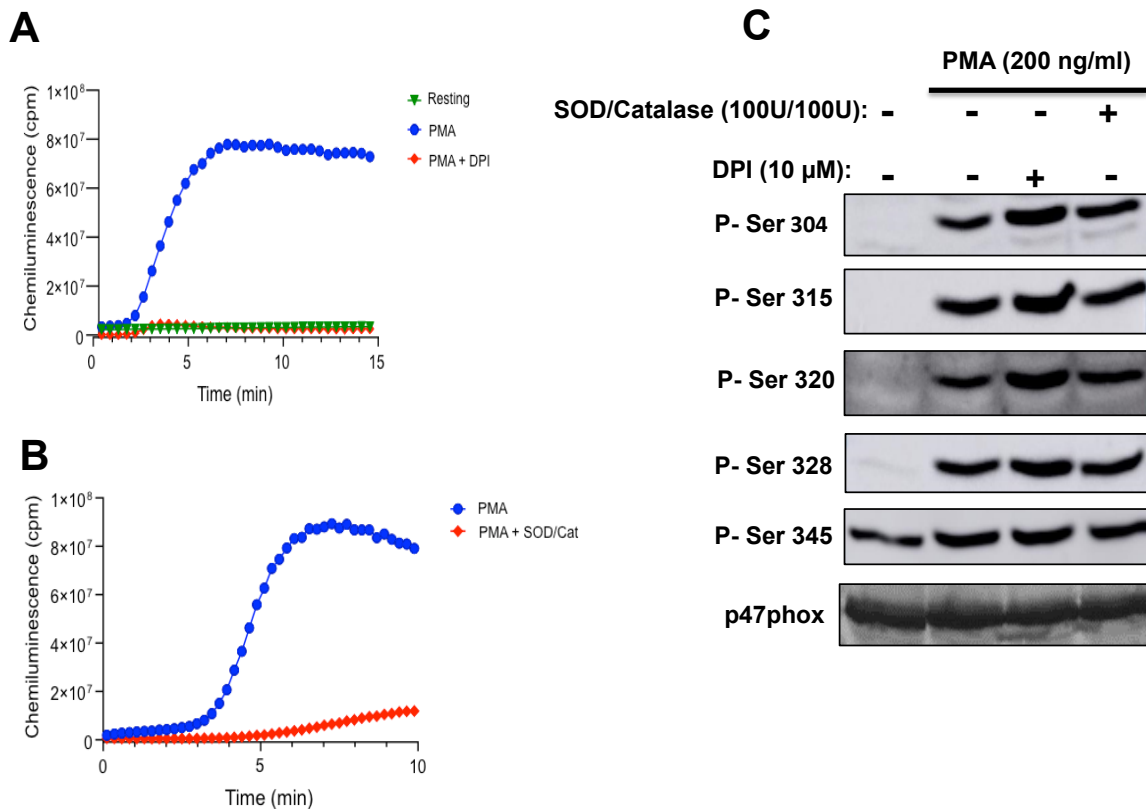
Supplemental Figure 6. Kinetics of ROS production in neutrophils from p67phox-deficient patients and from healthy donors. Neutrophils from healthy donors and from p67phox-deficient patients ($15 \times 10^6/500 \mu\text{l}$) were incubated with fMLF ($1 \mu\text{M}$) or PMA (200 ng/ml) in the presence of luminol and ROS production was measured by luminol-amplified chemiluminescence.



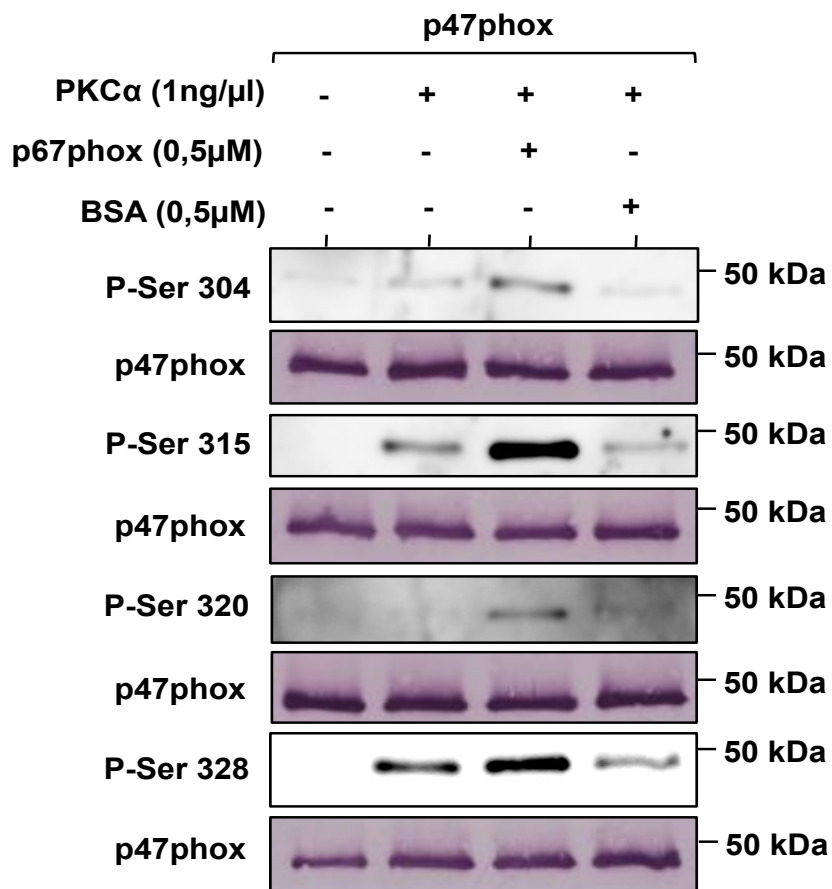
Supplemental Figure 7. Phosphorylation of p47phox in neutrophils from a second p67phox-deficient patient and from a healthy donor. Neutrophils from healthy donors (CTL for Control) and p67phox-deficient patients (CGD) ($5 \times 10^6/200 \mu\text{l}$) were incubated with fLMF ($1 \mu\text{M}$) or PMA (200 ng/ml) for 10 sec and 10 min, respectively and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti-phospho-Ser304, phospho-Ser315, phospho-Ser320, phospho-Ser328, antibody.



Supplemental Figure 8. Phosphorylation of p38MAPKinase and ERK1/2 in neutrophils from p67phox-deficient patients and from healthy donors. Neutrophils from healthy donors and p67phox-deficient patients ($15 \times 10^6/400 \mu\text{l}$) were stimulated with fMLF or PMA (200 ng/ml) and the reaction was stopped by denaturation with 100 μl Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using anti-phospho-p38MAPKinase or anti-phospho-ERK1/2. Experiments were repeated three times.



Supplemental Figure 9. Effect of DPI and SOD+Catalase on PMA-induced p47phox phosphorylation in human neutrophils. (A) Human neutrophils ($0.5 \times 10^6/500 \mu\text{l}$) were incubated for 15 min without or with DPI ($10 \mu\text{M}$) or (B) SOD (100U)+Catalase (100U), then stimulated with PMA (200 ng/ml). ROS production was measured by luminol-amplified chemiluminescence. (C) Human neutrophils ($15 \times 10^6/400 \mu\text{l}$) were incubated for 15 min without or with DPI ($10 \mu\text{M}$) or SOD (100U)+Catalase (100U) then stimulated with PMA (200 ng/ml) for 10 min and the reaction was stopped by denaturation with $100 \mu\text{l}$ hot Laemmli sample buffer (5X). Homogenates were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies: anti- phospho-Ser304, -phospho-Ser315, -phospho-Ser320, -phospho-Ser328, -phospho-Ser-345 antibodies and anti-p47phox antibody. The experiments were repeated three times.



Supplemental Figure 10. Effect of p67phox and BSA on p47phox phosphorylation by PKC α *in vitro*. Recombinant p47phox protein was incubated without or with recombinant p67phox or BSA for 10 min, PKC α was added for 5 min and the reaction was started by adding ATP for 15 min at 30°C. The reactions were terminated by addition of Laemmli 5X sample buffer and denaturation at 100°C. Proteins were subjected to SDS-PAGE and Western-blotting using different anti-phospho-p47phox antibodies. Experiments were repeated three times.