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Pin1

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The TLR7/8 Agonist CL097 Primes N-Formyl-Methionyl-Leucyl-Phenylalanine-Stimulated NADPH Oxidase Activation in Human Neutrophils: Critical Role of p47phox Phosphorylation and the Proline Isomerase Pin1

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Superoxide anion production by the neutrophil NADPH oxidase plays a key role in host defense; however, excessive superoxide production is believed to participate to inflammatory reactions. Neutrophils express several TLR that recognize a variety of microbial motifs or agonists. The interaction between TLR and their agonists is believed to help neutrophils to recognize and eliminate the pathogen. However, the effects of some TLR agonists on the NADPH oxidase activation and the mechanisms controlling these effects have not been elucidated. In this study, we show that the TLR7/8 agonist CL097 by itself did not induce NADPH oxidase activation in human neutrophils, but induced a dramatic increase of fMLF-stimulated activation. Interestingly, CL097 induced cytochrome b558 translocation to the plasma membrane and the phosphorylation of the NADPH oxidase cytosolic component p47phox on Ser345 and Ser315. Phosphorylation of Ser328 was decreased by a protein kinase C inhibitor. Genistein, a broad-range protein tyrosine kinase inhibitor, inhibited the phosphorylation of these serines. Our results also show that CL097 induced proline isomerase 1 (Pin1) activation and that juglone, a Pin1 inhibitor, inhibited CL097-mediated priming of fMLF-induced p47phox phosphorylation and superoxide production. These results show that the TLR7/8 agonist CL097 induces hyperactivation of the NADPH oxidase by stimulating the phosphorylation of p47phox on selective sites in human neutrophils and suggest that p38 MAPK, ERK1/2, protein kinase C, and Pin1 control this process. 


P olymorphonuclear neutrophils (neutrophils), the most abundant circulating leukocytes, are essential for host defense against pathogens (1). Upon infection, neutrophils migrate toward the infection site attracted by chemotactic factors such as the complement fraction C5a, the fMLF peptide, IL-8, platelet-activating factor, or leukotriene B4 (2). Once at the infection site, neutrophils recognize pathogen-associated molecular patterns via pattern recognition receptors such as TLR, which are transmembrane receptors that play an important role in innate immune recognition of pathogens (3, 4). Neutrophils will then rapidly phagocytose the Ab- and complement-opsonized pathogen. Phagocytosis is accompanied by neutrophil activation, resulting in the release of proteolytic enzymes and antimicrobial peptides, and the massive production of reactive oxygen species (ROS) (5).

The TLR family of receptors is composed of up to 10 members in humans (TLR1–10) and 12 in mice (6). TLR have a common structure with an extracellular recognition domain and an intracellular Toll/IL-1R (TIR) domain (4). Human neutrophils express all TLR except TLR3 (7). TLR recognize a variety of pathogen-derived agents and molecules such as triacyl lipopeptides, peptidoglycans, zymosan, lipoteichoic acid, LPS, flagellin, and demethylated CpG motifs of ssDNA (8–12). TLR7 and -8 are located in the intracellular endosomal compartment, where they recognize ss RNA and imidazoquinolones (6, 13). Upon activation, TLR1, -2, and -6 recruit two adaptor proteins, TIRAP and MyD88. TLR4 recruits TRAM, MyD88, TIR domain-containing adaptor inducing IFN-β-related adaptor molecule, and TIR domain-containing adaptor inducing IFN-β, whereas TLR5, -7, -8, and -9 recruit only MyD88. These recruits lead to activation of the IL-1R-associated kinase and that of downstream protein kinases, which control specific cellular functions such as cytokine production (14) and inhibition of apoptosis (15).

Neutrophil ROS production is controlled by the NADPH oxidase enzyme complex (also called NOX2) (16, 17). This multicomponent enzyme is dormant in resting cells and can be activated by various stimuli (18). In the activated form, the NADPH oxidase complex mediates the transfer of electrons from cytosolic NADPH to oxygen to produce superoxide anion (16). Superoxide is the precursor of other toxic ROS, such as hydrogen peroxide (H2O2) and hypochlorous acid (HOCl), which are involved in microbial killing (19). The NADPH oxidase consists of a membrane-bound flavocytochrome b558 (composed of p22phox and gp91phox/NOX2) and four cytosolic subunits: p47phox, p67phox, p40phox, and Rac1/2 (20, 21). Activation of the NADPH oxidase is initiated by
p47phox phosphorylation and its migration with that of the other cytosolic components to the membrane, where they associate with the membrane-bound components to assemble the catalytically active oxidase (20). Agonists such as the bacterial peptide fMLF and the pharmacological protein kinase C (PKC) activator PMA at micromolar concentrations strongly induce p47phox phosphorylation on several serines and NADPH oxidase activation (20). Proinflammatory agents such as cytokines (GM-CSF, TNF-α, and IL-8) alone induce a very weak oxidative response by neutrophils but strongly enhance ROS release upon exposure to a secondary stimulus such as fMLF (18). This priming effect at the infection site could facilitate the rapid elimination of the pathogen; however, excessive and uncontrolled priming could participate to the inflammatory reaction and thus be self-destructive (22, 23).

The priming effect has been shown to be controlled by the partial phosphorylation of p47phox (24–26), the activation of the proline isoserine 1 (Pin1) (27), and the translocation of the flavocytochrome b588 to the plasma membranes (28, 29).

Among the TLR agonists, LPS and zymosan have been described to exert a priming effect on IL-1β-induced ROS production (18). However, the effect of CL097, a TLR7/8 agonist, on the neutrophil ROS production and its activation pathways are unknown. In this study, we show that CL097 acts as a priming agent of the neutrophil ROS production. CL097 induced p47phox phosphorylation on specific sites and activation of Pin1, which could play a critical role in this priming effect. The TLR7/8–p47phox–Pin1 axis could be targeted to limit excessive ROS production at inflammatory sites.

Methods and Materials

Reagents

CL097 and other TLR agonists were from InvivoGen (San Diego, CA). PMA, fMLF, cytochrome c, protein kinase inhibitors, protease and phosphatase inhibitors, endotoxin-free buffers, and salt solutions were from Sigma Chemical (St. Louis, MO), SDS-PAGE (NaDodSO4-PAGE) and Western blotting reagents were purchased from Bio-Rad (Richmond, CA). Polyclonal anti-TLR7 and anti-TLR8 Abs were from Abcam (Cambridge, U.K.). The rabbit polyclonal Abs against phospho-sites p47phox and p47phox have been described elsewhere (27).

Neutrophil and monocyte preparation

Circulating neutrophils were isolated from healthy volunteers by Polymorphprep (Axis Shield, Dundee, Scotland) gradient centrifugation (26, 27). The neutrophil band was collected, and the cells were washed in PBS and counted. Neutrophils were 96% pure and 99% viable. Monocytes were purified by Ficol gradient centrifugation and depletion of lymphocytes by the Untouched Monocytes Isolation Kit following the corresponding instructions (Miltenyi Biotec and Invitrogen).

Superoxide anion production assay

Neutrophils were treated with CL097 at 37°C for 30 min in HBSS. Thereafter, extracellular superoxide production was measured in response to fMLF (10 M, 1 min) by the superoxide dismutase-inhibitable ferricytochrome c reduction assay (24, 25).

Intracellular superoxide anion production was measured with a flow cytometric assay using hydroethidine (HE) as previously described (15). HE diffuses into cells and, during the neutrophil oxidative burst, nonfluorescent intracellular HE is oxidized by superoxide anion to highly fluorescent ethidium that is trapped in the nucleus by intercalation into DNA. Whole-blood samples (500 μl) were loaded for 15 min with HE (1.5 μg/ml) at 37°C and then incubated with PBS, CL097 (5 μg/ml), or TNF-α (20 ng/ml) for 30 min. TNF-α was used as a positive control; samples were then treated with PBS or 10 M fMLF for 5 min. RBCs were lysed, and after one wash, WBCs were resuspended in 1% paraformaldehyde-PBS and immediately analyzed by flow cytometry as described below.

Measurement of ROS production by luminol-amplified chemiluminescence

Neutrophils (5 × 105) were suspended in 0.5 ml HBSS containing 10 μM luminol at 37°C with or without different concentrations of CL097 for 30 min, and then the cells were stimulated with 10 M fMLF. Chemiluminescence was recorded with a luminometer (LB937; Berthold-Biolumat).

Determination of CD11b-positive and 7D5-positive cells at the neutrophil surface

Whole-blood samples (500 μl) from healthy donors were incubated at 37°C for 30 min with PBS (resting), CL097 (5 μg/ml), or TNF-α (20 ng/ml). Samples were then incubated with fMLF (10 M) or PBS (negative control) for another 5 min. A total of 100 μl each sample was then stained with 10 μl PE-conjugated anti-human CD11b mAb (BD Biosciences, San Jose, CA) or 10 μl FITC-conjugated anti-human flavocytochrome b558 mAb 7D5 (MBL Medical and Biological Laboratories, Naka-Ku Nagoya, Japan) for 30 min at room temperature in the dark. Red cells were lysed with BD FACS Lysing Solution (BD Biosciences), and white cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry. Nonspecific Ab binding was determined on cells incubated with the same concentration of an irrelevant Ab of the same isotype.

Flow cytometry

Forward and side scatter were used to identify the neutrophil population and to gate out other cells and debris in a FACSCanto II (BD Biosciences). The purity of the gated cells was assessed by using monoclonal anti-CD15 Abs (BD Biosciences). The mean fluorescent intensity (MFI) of ethidium, CD11b-positive cells, and 7D5-positive cells was then determined in the neutrophil populations. Five thousand events per sample were analyzed, and all results were obtained with a constant photomultiplier gain value. Results were expressed as MFI.

Western blotting analysis

Neutrophils (15 × 106) in HBSS (400 μl) were treated with CL097, PMA, or TNF-α for the indicated times and at the indicated concentrations at 37°C with mild shaking. The reaction was stopped by adding 5× concentrated Laemmli sample buffer (30) containing 5 mmol/l Na3VO4, 2.5 mmol/l p-nitrophenyl phosphate, 10 mmol/l NaF, 5 mmol/l EDTA, 5 mmol/l EGTA, 20 μg/ml leupeptin, 20 μg/ml pepstatin, and 20 μg/ml aprotinin. Samples were then incubated for 2 min in boiling water (100°C) and stored at −80°C until use. Neutrophils lysates were sonicated and subjected to 10% SDS-PAGE (equivalent of 1 × 106 cells/well) using standard techniques (30).

The separated proteins were transferred to nitrocellulose (31), which was blocked with 5% milk in TBS containing Tween 20 for 1 h. After blocking, the membranes were probed with the appropriate Abs (i.e., anti-phospho-Ser15–p47phox [1:2000], anti-phospho–Ser328–p47phox [1:2000], anti-phospho–Ser345–p47phox [1:2000], anti-phospho–Ser328–p70 SRC [1:2000], anti-phospho–Thr241–PIN1 [1:2000], anti-phospho–Thr260–PIN1 [1:2000], and anti-phospho–ERK1 [1:2000], followed by incubation with HRP-labeled goat anti-rabbit Ab (1:5000). For the anti-TLR7 and -TLR8 blots, resting neutrophils and monocytes were lysed and subjected to SDS-PAGE and Western blot using anti-TLR7 (1:500) and -TLR8 (1:500) Abs. The protein bands were revealed using ECL (Santa Cruz Biotechnology, Santa Cruz, CA). The intensity of phosphorylated p47phox and p47phox bands was quantified by densitometry using the Image J analysis program (National Institutes of Health). Phosphorylated intensities were corrected for the corresponding amounts of p47phox present on the membrane.

Pin1 activity assay

Pin1 activity was measured using a previously described technique, with some modifications (27). Briefly, neutrophils were lysed by sonication (twice for 10 s at 4°C) in lysis buffer containing 50 mM HEPES, 100 mM NaCl, 0.25% CHAPS, 5 mM NaF, 1 mM β-glycerophosphate, and 1 mM EGTA. The assay mixture consisted of 93 μM HEPES buffer (50 mM HEPES [pH 7.8], 100 mM NaCl, 2 mM DTT, and 0.04 mg/ml BSA), 5 μl cell lysate (equivalent 106), and 2 μl (20 ng/ml) trypsin solution (Sigma-Aldrich). The reaction was started by adding 50 μl (720 μM) of the peptide Trp-Phe-Tyr-Ser(PO3H2)-Pro-Arg-pNA (NeoMPSance), and the p-nitroaniline released was continuously followed for 4 min by spectrophotometry at 390 nm.

Statistical analysis

All results are expressed as means ± SEM. Significant differences (p < 0.05) were identified with Student t tests and one-way ANOVA followed by Tukey’s post hoc test using Prism 4.0 software (GraphPad).

Results

CL097 induces priming of fMLF-induced superoxide and ROS production by human neutrophils

The effect of CL097, a TLR7/8 agonist, on neutrophil superoxide and ROS production is not known. We used the cytochrome c
reduction assay to detect the extracellular superoxide anion production and the luminol-amplified chemiluminescence assay to detect the intracellular and extracellular (total) ROS production. In initial experiments, CL097 alone (0.25–20 μg/ml) did not induce extracellular superoxide anion production or total ROS production by human neutrophils (data not shown). To determine if CL097 affected the fMLF-induced superoxide production, cells were then incubated in the absence or presence of CL097 (0.25–20 μg/ml) for 30 min and then stimulated with fMLF (10^{-7} M). Results show that CL097 induced a clear increase of fMLF-induced superoxide (Fig. 1A) and ROS (Fig. 1B) production by neutrophils with a maximum response within the 2.5–5 μg/ml concentration range, followed by a decreased response at concentrations >5 μg/ml. The CL097 effect was time dependent, as treatment for 30 min with 5 μg/ml produced the maximum effect on extracellular superoxide production (Fig. 1C), and treatment for 15 min produced the maximum effect on intracellular and extracellular ROS production (Fig. 1D). Using hydroethidine and flow cytometry analysis that detects intracellular superoxide anion production, we confirmed that CL097 alone had no effect on intracellular superoxide anion production by blood neutrophils but enhanced fMLF-induced intracellular production (Fig. 1E). These data show that CL097, a known TLR7/8 agonist, induces priming of fMLF-induced NADPH oxidase activation in human neutrophils. TLR7/8 expression in neutrophils was confirmed by SDS-PAGE and Western blotting using commercially available anti-TLR7 or anti-TLR8 Abs and human monocytes as control (Fig. 1F).

**CL097 induces cytochrome b558 translocation at the plasma membranes**

Priming of neutrophil superoxide production is controlled by translocation of the cytochrome b558 from the intracellular granules to the plasma membrane and by partial p47phox phosphorylation (18). We thus first examined whether the CL097-induced priming of superoxide production was associated with cytochrome b558 translocation using a specific anti-cytochrome

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** CL097 induces priming of fMLF-stimulated superoxide and ROS production by human neutrophils. Neutrophils were pretreated with different concentrations of CL097 (0.5–10 μg/ml) for 30 min at 37°C before stimulation with fMLF (10^{-7} M), and superoxide production was measured using the SOD-inhibitable cytochrome c reduction assay (100% corresponds to 4.5 nmol produced per 1 million cells at 10^{-7} M fMLF) (A) or luminol-amplified chemiluminescence (B). CL097 (5 μg/ml) was incubated with neutrophils for 5, 15, 30, and 45 min before stimulation with fMLF (10^{-7} M) and superoxide anion measurement (C) or luminol-amplified chemiluminescence detection (D). Whole blood was incubated with HE and CL097 (5 μg/ml) for 30 min before stimulation with fMLF (10^{-7} M) for 5 min, and MFI was measured in neutrophils by flow cytometry (control nontreated cells [ctl]) (E). Neutrophils and monocytes (1 × 10^6 cells) were lysed and proteins analyzed by SDS-PAGE and Western blot using an anti-TLR7 or anti-TLR8 Ab (F). Results represent means ± SEM (n = 4, **p < 0.01).
b558 Ab (FITC-7D5 mAb) recognizing the extracellular cytochrome b558 epitopes and flow cytometry. The anti-CD11b Ab was used as a degranulation marker. Results show that CL097 alone induced cytochrome b558 and CD11b translocation to the plasma membrane to the same extent as TNF-α and fMLF (Fig. 2). Moreover, an additive effect of CL097 and fMLF on cytochrome b558 translocation was found.

**CL097 induces the phosphorylation of p47phox on specific sites and enhances fMLF-induced p47phox phosphorylation**

Activation of the neutrophil NADPH oxidase by fMLF or PMA is accompanied by the phosphorylation of the regulatory subunit of NADPH oxidase, p47phox, on serines 303–379 in the C-terminal region (20). In contrast, priming of neutrophils with TNF-α or GM-CSF results in the phosphorylation of p47phox on a single site, Ser345 (26). Thus, we examined whether CL097-induced priming of superoxide production was associated with p47phox phosphorylation using specific anti–phospho-Ser Abs developed in our laboratory (27). Interestingly, Western blotting analysis indicated that CL097 selectively induced the phosphorylation of p47phox on three serines: Ser315, Ser328, and Ser345 (Fig. 3), which are also phosphorylated in PMA-stimulated neutrophils along with Ser303 and Ser320, as expected, whereas TNF-α induced the phosphorylation of Ser345 only. The CL097-induced p47phox phosphorylation was time dependent (Fig. 4), with a time scale similar to that of the CL097-induced priming of superoxide anion production.

The peptide fMLF (10^−7 M) induces a very weak phosphorylation of the same serines as PMA (27). We tested the effect of CL097 on fMLF-induced p47phox phosphorylation by treating purified neutrophils with CL097 or fMLF, alone or sequentially, and analyzing the phosphorylation of p47phox on specific serines by Western blotting. Priming with CL097 followed by fMLF stimulation markedly increased p47phox phosphorylation on Ser315 and Ser328, showing that phosphorylation of these sites is primed by the TLR7/8 agonist (Fig. 5). Surprisingly, Ser345 appears to be maximally phosphorylated by CL097, and fMLF did not further enhance its phosphorylation. Ser303 and Ser320 were not phosphorylated under these conditions.

**FIGURE 3.** CL097 induces phosphorylation of p47phox on specific sites in a concentration-dependent manner. (A) Neutrophils (15 × 10^6 cells/ml) were incubated with various concentrations of CL097 (0.5, 2.5, 5, and 10 μg/ml) for 30 min or with PMA (100 ng/ml) for 10 min or TNF-α (20 ng/ml) for 20 min. Cells were lysed, and proteins from 1 × 10^6 cells were analyzed by SDS-PAGE and immunoblotting with anti–phospho-Ser315 Ab, anti–phospho-Ser328, anti–phospho-Ser330/334, anti–phospho-Ser315, anti–phospho-Ser330/334, or anti-p47phox Ab (p47phox). (B) Western blots from different experiments were scanned and phosphorylated, total p47phox were quantified by densitometry, and the intensity of phosphorylated p47phox was corrected for the protein amount of p47phox. Results are expressed as mean ± SEM (n = 4, *p < 0.05, **p < 0.01).

**FIGURE 4.** CL097 induces phosphorylation of p47phox on specific sites in a time-dependent manner. (A) Neutrophils (15 × 10^6 cells/ml) were incubated with CL097 (5 μg/ml) for the indicated times (0, 15, 30, 45, and 60 min). Cells were lysed, and proteins from 1.5 × 10^6 cells were analyzed by SDS-PAGE and immunoblotting with anti–phospho-Ser315, anti–phospho-Ser328, anti–phospho-Ser330/334, anti–phospho-Ser315, anti–phospho-Ser330/334 Abs, or anti-p47phox Ab (p47phox). (B) Western blots from different experiments were scanned; phosphorylated and total p47phox were quantified by densitometry, and the intensity of phosphorylated p47phox was corrected for the amount of p47phox. Results are expressed as mean ± SEM (n = 4, **p < 0.01).
CL097 induces the phosphorylation of ERK1/2, p38 MAPK, and PKCβ, and inhibitors of these protein kinases inhibit CL097-induced p47phox phosphorylation and NADPH oxidase hyperactivation

As CL097 induced p47phox phosphorylation on Ser^315, Ser^328, and Ser^345, we investigated the pathways that may be involved. We first tested if CL097 was able to activate the major protein kinases known to be involved in p47phox phosphorylation in human neutrophils. Indeed, a kinetic study (0–45 min) showed that CL097 alone induced the phosphorylation of ERK1/2, p38 MAPK, and PKCβ, with a time course similar to that of p47phox phosphorylation on Ser^315, Ser^328, and Ser^345 (Fig. 6).

As Ser^345 is located in a sequence recognized and phosphorylated by MAPK (-PXSP), and Ser^315 and Ser^328 are known PKC phosphorylation sites, we tested the effects of kinase inhibitors on p47phox phosphorylation (i.e., PD98059, which inhibits MEK1/2 [the upstream activator of ERK1/2], SB203580, a p38 MAPK inhibitor, GF109203X, a PKC inhibitor, and genistein, a broad range protein tyrosine kinase inhibitor). Results show that pretreatment of neutrophils with SB203580, PD98059, or genistein resulted in the inhibition of CL097-induced p47phox phosphorylation on Ser^345, whereas GF109203X had no effect (Fig. 7A, 7C). Interestingly, phosphorylation of p47phox on Ser^315 and Ser^328, which are not located within an MAPK phosphorylation site, was also inhibited by SB203580 and PD98059 (Fig. 7B, 7C); GF109203X inhibited Ser^328 phosphorylation but not Ser^315 phosphorylation, and genistein inhibited phosphorylation of both sites. These results show that CL097 induces activation of several kinases that participate to the phosphorylation of three critical serines in the C-terminal portion of p47phox.

To investigate whether p38 MAPK, ERK1/2, PKC, and tyrosine kinases are involved in CL097-induced priming of NADPH oxidase, we tested their inhibitors on this process. Interestingly, results show that SB203580, the p38 MAPK inhibitor, did not affect fMLF-induced superoxide anion production but inhibited CL097-induced priming (Fig. 7D). PD98059, GF109203X, and genistein inhibited both fMLF-induced superoxide anion and CL097-induced priming (Fig. 7D).
The proline isomerase Pin1 controls CL097-induced priming of NADPH oxidase by regulating p47phox phosphorylation

We have previously shown that Pin1 is essential for TNF-α-induced priming of superoxide production by human neutrophils as it binds to phosphorylated Ser345 p47phox to facilitate the phosphorylation on other sites such as Ser315 and Ser328 (27). Considering that CL097 strongly induced the phosphorylation of Ser345, we investigated the role of Pin1 in CL097-induced priming. As previously reported, basal Pin1 activity was observed in resting neutrophils (27). Incubation of neutrophils with CL097 resulted in the stimulation of Pin1 activity (Fig. 8A), which was inhibited by the selective Pin1 inhibitor juglone. To test the role of Pin1 in CL097-induced priming of ROS production, neutrophils were incubated in the absence or presence of juglone (250 nM) for 30 min, before fMLF (10⁻⁷ M) stimulation and measurement of superoxide production with the cytochrome c reduction assay. Juglone did not affect fMLF-induced superoxide production (Fig. 8B). As expected, CL097 primed fMLF-induced superoxide anion production by neutrophils in the absence of juglone. Pretreatment of neutrophils with juglone completely abrogated the priming effect of CL097 (Fig. 8B). This effect was confirmed by using chemiluminescence technique (data not shown). Juglone did not affect neutrophil viability at the concentrations tested (data not shown).

To further understand the role of Pin1 in CL097-induced priming of NADPH oxidase activation, we tested the effect of juglone on p47phox phosphorylation. We found that juglone inhibited the markedly increased phosphorylation of Ser345 induced by CL097 (Fig. 9), and the Pin1 selective inhibitor juglone inhibited this phosphorylation in a dose-dependent manner (data not shown). Taken together, these results suggest that Pin1 regulates CL097-induced phosphorylation of Ser315 and Ser328 and CL097-induced priming of fMLF-stimulated ROS production by human neutrophils.

Discussion

In the current study, we have shown that CL097, a known TLR7/8 agonist, induces NADPH oxidase priming, resulting in an increase...
The phosphorylation of Ser345, and PKC is involved in the phosphorylation of Ser315, Ser316, and Ser320. Ser315 is phosphorylated by a different, yet uncharacterized, protein kinase. These pathways could be controlled by a common upstream protein tyrosine kinase, which was inhibited by genistein. Moreover, our results show that CL097 induces phosphorylation of the cytosolic component p47phox on Ser315, Ser320, and Ser328, but not that of Ser345. Our results also suggest that p38 MAPK and ERK1/2 are necessary for the phosphorylation of Ser320, and PKC is involved in the phosphorylation of Ser328. Ser315 is phosphorylated by a different, yet unidentified, protein kinase. These pathways could be controlled by a common upstream protein tyrosine kinase, which was inhibited by genistein. Moreover, our results show that CL097 induces phosphorylation of PI3K and fMLF-stimulated phosphorylation of p47phox on Ser315 and Ser328 and of NADPH oxidase hyper-activation.

TLR7 and TLR8 are phylogenetically and structurally very close (4). Several studies have previously examined the expression of TLR7 and TLR8 in human neutrophils. Although the expression of TLR8 was clearly demonstrated at the mRNA and protein levels (32–38), some studies have reported the expression of TLR7 in human neutrophils (32, 33), whereas other studies did not detect its expression (37, 38). In this study, although commercial Abs do not have high affinity, we detected TLR7 and TLR8 in human neutrophils and monocytes. TLR7 and TLR8 are located in the intracellular endosomes (4); further studies will be conducted to localize TLR7/8 in human neutrophils.

Although single-stranded viral RNA is the natural ligand of TLR7 and TLR8, the imidazoquinoline resiquimod (R848) is recognized as a potent synthetic agonist of TLR7/8 (33). CL097 is a highly water-soluble derivative of R848, which also acts on TLR7/8 (39). The effects of CL097 on neutrophil functions have not been previously studied; however, R848 was shown to induce several neutrophil functions such as cytokine production (33, 37, 38), degranulation (33, 38), chemotaxis (33), phagocytosis (33), survival (40), and enhanced leukotriene B4 and platelet-activating factor biosynthesis (35). The effect of R848 on neutrophil ROS production is not clear, as some studies have reported that R848 can by itself induce a weak NADPH oxidase activation in neutrophils (32, 38), and one study reported that R848 induces priming of neutrophil ROS production (33). The results reported in this study show that CL097, as R848, primes the fMLF-stimulated ROS production. The priming effect was obtained even at 0.25 μM, and kinetics studies showed that CL097-induced priming of ROS production as measured by luminol-amplified chemiluminescence precedes CL097-induced priming of superoxide production as measured by cytochrome c reduction assay. As luminol-amplified chemiluminescence detects intracellular and extracellular ROS, and cytochrome c reduction assay detects only extracellular superoxide, these data suggest that CL097 induced priming of intracellular NADPH oxidase before the priming of plasma membrane NADPH oxidase. However, the pathways induced by TLR7/8 agonists in human neutrophils and how they regulate NADPH oxidase activation were unknown until now.

NADPH oxidase activation and regulation is controlled by the phosphorylation of its cytosolic component p47phox on Ser315 and Ser328 (20). It is clear that stimulation of neutrophils by high concentrations of the chemotactic peptide fMLF or by the PKC agonist PMA induces complete phosphorylation of p47phox (20). Treatment of neutrophils with priming agents such as the proinflammatory cytokines (TNF-α and GM-CSF) induces partial phosphorylation of p47phox on Ser345, an MAPK phosphorylation site (26). Phosphorylation of p47phox on Ser345 is a critical mechanism in GM-CSF- and TNF-α–induced priming of fMLF-induced ROS production by neutrophils, as it enhances fMLF-induced p47phox phosphorylation on other sites (Ser315, Ser320, and Ser328) (27). Interestingly, in this study, we show that the TLR7/8 agonist CL097 induced the phosphorylation of p47phox on Ser345, Ser328, and Ser315 and primed fMLF-induced phosphorylation of Ser328 and Ser315, but not Ser345. CL097 alone induced the phosphorylation of p38 MAPK and ERK1/2 in human neutrophils. Interestingly, activation of these two MAPK by TLR7/8 agonists has also been observed in other cell types, such as monocytes, lymphocytes, and dendritic cells, where it is involved in the nuclear translocation of the transcription factor NF-κB, which controls the expression of proinflammatory cytokine genes (39, 40). In eosinophils, stimulation by R848...
(TLR7/8 agonist) led to p38 MAPK activation only (32). To investigate the role of the MAPK in these phosphorylations, we used the p38 MAPK inhibitor SB203580 and the MEK1/2 inhibitor PD98059. Both inhibitors prevented not only CL097-induced p47phox phosphorylation on Ser345 but also the phosphorylation of Ser315 and Ser328. It is noteworthy that Ser345 is located in a proline-directed sequence recognized by MAPK, but Ser315 and Ser328 are not. These results suggest that p38 MAPK and ERK1/2 are required for the phosphorylation of p47phox on Ser345 and that this initial phosphorylation could regulate the sequential phosphorylation on Ser315 and Ser328. We also show that GFI09203X, a PKC inhibitor, inhibited the CL097-induced phosphorylation of Ser328 but not the phosphorylation of Ser315. Genistein, a broad-range protein tyrosine kinase inhibitor, inhibited phosphorylation of all serines. These results suggest that engagement of TLR7/8 in human neutrophils induces several pathways involved in p47phox phosphorylation; p38 MAPK and ERK1/2 are required for the phosphorylation of Ser345. PKC is involved in Ser328 phosphorylation, a third different yet not identified pathway is involved in Ser315 phosphorylation, and an upstream protein tyrosine kinase controls these pathways. Interestingly, only SB203580, the p38 MAPK inhibitor, did not affect fMLF-induced NADPH oxidase activation but inhibited CL097-induced NADPH oxidase priming.

We have previously shown that the proline isomerase Pin1 plays a role in p47phox phosphorylation (27). In this study, we show that TLR7/8 induces Pin1 activation in human neutrophils and that inhibition of Pin1 activity by juglone resulted in the inhibition of CL097-induced priming of fMLF-stimulated p47phox phosphorylation and ROS production by neutrophils. The initial phosphorylation of p47phox on Ser345 results in recruitment of the activated Pin1 (27), which in turn will induce conformational changes of p47phox to facilitate phosphorylation of Ser315 and Ser328 by the other kinases. Inhibiting the phosphorylation of Ser345 by MAPK will prevent Pin1 recruitment, thereby resulting in inhibition of Ser315 and Ser328 phosphorylation. This could explain the inhibitory effect of SB203580 and PD98059 on Ser315 and Ser328 phosphorylation. Similarly, inhibiting the activity of Pin1 with juglone will prevent recruitment of activated Pin1 by phosphorylated Ser345-p47phox, and phosphorylation of Ser315 and Ser328. Thus, these data suggest that Pin1 is a critical factor in TLR7/8-induced hyperactivation of neutrophil NADPH oxidase and excessive ROS generation.

We also found that CL097 induced a significant cytochrome b558 translocation to the plasma membrane. This effect was comparable to the fMLF and TNF-α-induced translocation and could account for CL097-induced NADPH oxidase hyperactivation. It is known that p38 MAPK is involved in TNF-α- and LPS-induced cytochrome b558 translocation (29). The pathways involved in CL097-induced cytochrome b558 translocation are under investigation in our laboratory.

The single-stranded viral RNA is the natural ligand of TLR7 and TLR8 (33). Our results suggest that ssRNA could induce neutrophil NADPH oxidase hyperactivation during viral infection, and TLR7/8 may mediate viral infection-induced inflammation. Recently, Wang et al. (41) have shown that influenza virus induced TLR7/8-mediated neutrophil cytokine production, and other studies reported that TLR7/8 agonists induced neuroinflammation (39) and skin inflammation accompanied by neutrophil infiltration in mice (42). TLR7/8-mediated NADPH oxidase hyperactivation and excessive ROS production could participate in these inflammatory reactions. Inhibitors of p38 MAPK and Pin1 by inhibiting excessive ROS production might have anti-inflammatory effects while preserving the physiological ability of the bacterial N-formyl peptides to activate neutrophils.

The results presented in this manuscript clearly show that the TLR7/8 agonist CL097 induced priming of neutrophil NADPH oxidase activation and p47phox phosphorylation on selective sites (Ser345, Ser315, and Ser328). The results also show that CL097 induced Pin1 activation in human neutrophils and suggest that Pin1 could act as an amplifier of p47phox phosphorylation and ROS production at inflammatory sites.
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Disclosures

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