



# The nitrated fatty acid, 10-nitrooleate inhibits the neutrophil chemotaxis via peroxisome proliferator-activated receptor gamma in CLP-induced sepsis in mice

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## ABSTRACT

The inhibition of polymorphonuclear neutrophils' (PMNs) migration to the source of injury is among the most prominent aspects of immunosuppression following sepsis, although the precise mechanisms involved remain unclear and multifaceted. Increasing evidence connects this immunosuppression to nitric oxide (NO), as NO production is a classic feature of inflammation probably through neutrophil activation and migration. Nitrated fatty acids (NFA) such as 10-nitrooleate (OA-NO<sub>2</sub>), nitrolinoleic acid etc. produced endogenously by the non-enzymatic reaction of NO with unsaturated fatty acids, are found to be potent activators of the transcription factor, peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). Upregulation of PPAR $\gamma$  during immunosuppression and the subsequent inhibition of neutrophil migration in sepsis have been reported. However, the interplay of OA-NO<sub>2</sub>, NO and PPAR $\gamma$  in polymicrobial-induced immunosuppression has not been established. Hence to understand this, we have studied the role of OA-NO<sub>2</sub> in blood PMNs migration, the effects of iNOS inhibitor on PMNs migration and PPAR $\gamma$  activity in cecal ligation and puncture (CLP)-induced sepsis in mice. We found increased expression of PPAR $\gamma$  and its DNA-binding activity in the lungs and blood PMNs from CLP mice. CLP or OA-NO<sub>2</sub> treatment inhibited PMNs' migration in response to fMLP stimulation. Pharmacological inhibition of iNOS resulted in decreased PPAR $\gamma$  DNA-binding activity with a concomitant increase in the migration of PMNs to the site of infection. OA-NO<sub>2</sub> treatment also inhibited the production of inflammatory cytokines (TNF $\alpha$  and IL-1 $\beta$ ) secretion from PMNs stimulated with lipopolysaccharide. We have also established that, OA-NO<sub>2</sub> mediated inhibition of PMNs migration in vivo and ex vivo are regulated through PPAR $\gamma$ -dependent pathway. This study further highlights the fact that the activation of PPAR $\gamma$  by the NFA has a pivotal role in PMNs' migration and immunosuppression.

## 1. Introduction

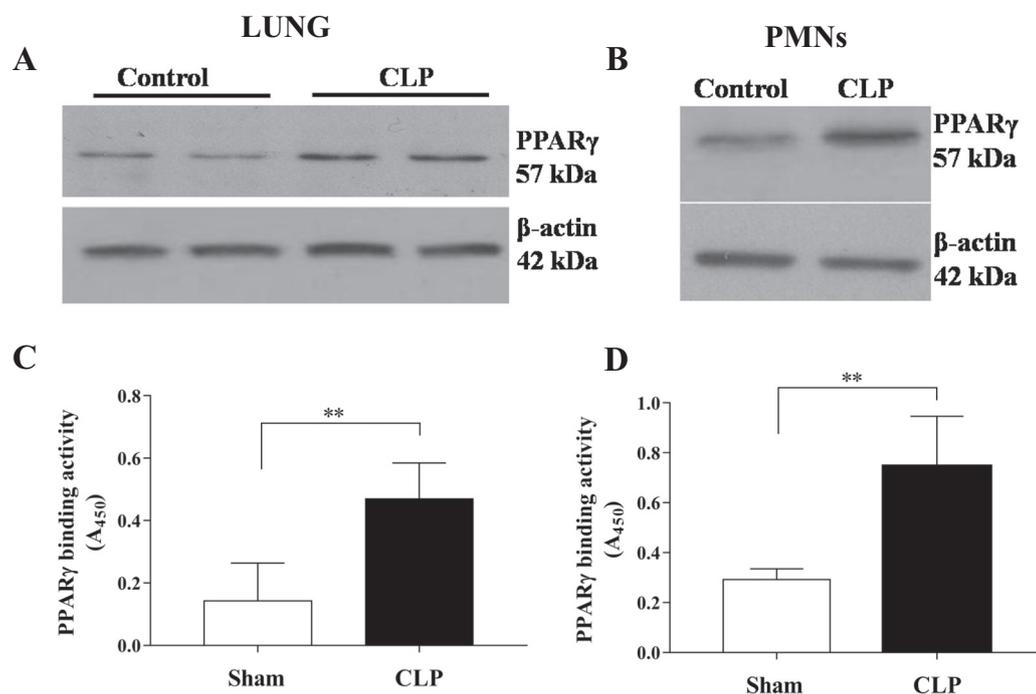
Polymorphonuclear neutrophils (PMNs) play a major role in containing the continuous onslaught of pathogens and as a result, even a slight impairment of their function can cause fatal clinical conditions such as sepsis [1]. PMNs also play a key role in resolving inflammation via Annexin A1, lipid mediators, IL-10 secretion, apoptosis etc. [2]. Even though PMNs play critical roles in innate immunity and represent the predominant cellular component at acute inflammation sites, they

are unable to resolve inflammation or clear pathogens during severe forms of sepsis, implying that PMNs' functions are apparently restricted [3]. It has been shown that the abilities of PMNs including cellular signalling, gene expression, migration, antimicrobial activity and forming neutrophil extracellular traps are probably dysregulated in sepsis [4,5]. However, increasing evidence points to a role for nitric oxide (NO) [6], as the production of NO is a classic feature of inflammation and may therefore be an appropriate inducer of a delayed, over-compensatory immunosuppressive response. Several studies have

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**Fig. 1.** Cecal ligation and puncture (CLP)-subjected mice lung and polymorphonuclear neutrophils (PMNs) show increased expression of the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) protein and DNA-binding activity. PPAR $\gamma$  protein expression was analysed by immunoblotting. A) Representative immunoblot of total lung protein collected from sham (control) and CLP-subjected mice, and B) Representative immunoblot of total PMNs protein from sham (control) and CLP-subjected mice. The blot shown here is representative of three separate experiments. The PPAR $\gamma$  transcription factor-binding activity to its DNA response elements was performed, as described in the methods, using lung and PMNs' nuclear protein extracts by the ELISA based assay. PPAR $\gamma$ -DNA binding activity of lung (C), and PMNs (D) nuclear protein extract from sham and CLP mice. Data are shown as mean  $\pm$  SEM. (\*\*p < 0.01 vs. Sham; n = 5 mice/group).

demonstrated the inhibitory effects of NO on neutrophil activation and migration [7–10]. Serum nitrate concentrations have also been reported to be elevated during sepsis [11].

Nitrated fatty acids (NFA; OA-NO<sub>2</sub>, LNO<sub>2</sub>) which are products of non-enzymatic reaction of NO with naturally occurring unsaturated fatty acids [12,13], have been identified as one of the largest single pools of biologically active NO derivatives in human plasma [14]. While the biological role of NFAs is not clear, initial *in vitro* studies have shown them to be potent suppressors of inflammatory responses [15,16]. The majority of NFAs' known effects are mediated by the activation of the transcription factor, peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) [17]. PPAR $\gamma$  has been shown to play a central role in many of the inflammatory pathways and helps in limiting the inflammation and its resolution [18–22]. Specifically, we [23] and others [24] have shown that the activation of PPAR $\gamma$  inhibits neutrophil chemotaxis. Numerous evidences support the fact that the activation of PPAR $\gamma$  inhibits neutrophil activity and inflammation during sepsis [25]. We accordingly hypothesize that NO inhibits neutrophil activation and migration following severe infection by forming NFAs, with the consequent activation of PPAR $\gamma$ . Inducible nitric oxide synthase (iNOS) is an enzyme that plays a vital role in the synthesis of NO in physiological conditions [26]. Earlier studies conducted elsewhere show that the inhibition of iNOS by the selective inhibitor aminoguanidine (AG), ameliorated sepsis in CLP rats [27]. There are no reports on NFAs' role with respect to iNOS and PPAR $\gamma$  involvement in sepsis, however. We have chosen a well-established animal model of sepsis called CLP induced sepsis, as the most feasible experimental approach [28] to study the role of OA-NO<sub>2</sub>, AG, and PPAR $\gamma$  activation in the sepsis-induced mechanism of PMNs' chemotaxis. CLP induced sepsis consequently causes lung injury [29]. Hence, the main focus of this study is lungs.

## 2. Materials and methods

### 2.1. Cecal ligation puncture (CLP) and polymorphonuclear neutrophils (PMNs) isolation from blood

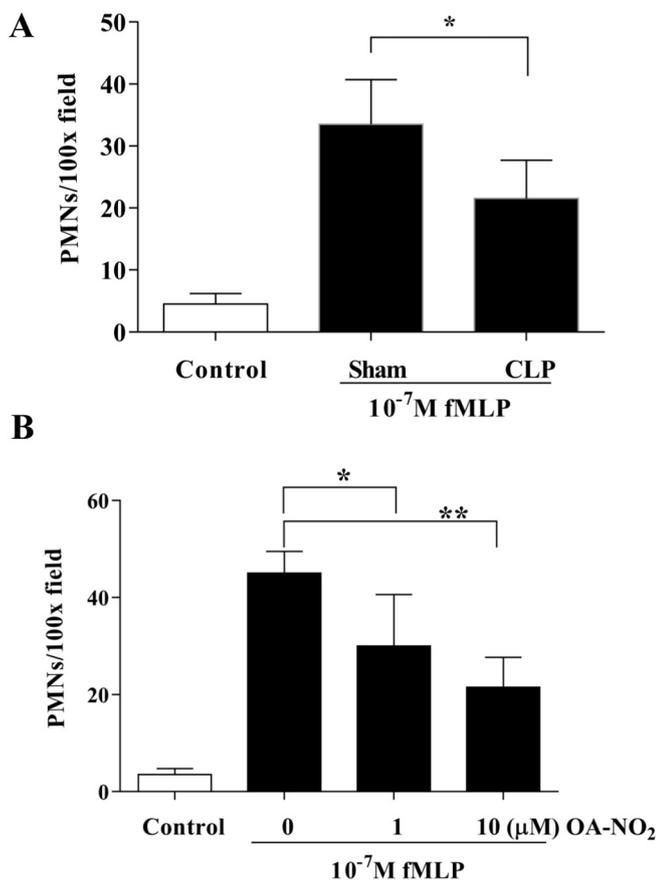
CLP and PMNs isolation were carried out according to our earlier protocols [23]. Female C57BL/6 mice (8 weeks old) were anaesthetized by an intraperitoneal injection of ketamine and xylazine mixture. The

peritoneal cavity of the anaesthetized animal was cut open to expose the cecum, which was then ligated using a 3–0 silk suture. After ligation, an 18-gauge needle was used to create a through-and-through puncture wound. After the completion of the surgery, 1 ml of sterile normal saline was administered subcutaneously to replenish lost fluids. The sham-operated control mice underwent the same procedure but were not ligated or punctured. In a separate experiment, mice were treated with the iNOS inhibitor, AG (90 mg/kg subcutaneously), or the PPAR $\gamma$  antagonist GW9662 (1 mg/kg, intraperitoneal), 30 min before CLP surgery to study the PMNs' chemotaxis and PPAR $\gamma$  DNA-binding activity. In another set of experiments, peritoneal cavity cells were collected after 6 h of CLP surgery by washing the cavity with 3 ml of PBS. The number of PMNs in the cavity was measured, based on their morphological appearance in a cell smear preparation on a glass slide, followed by staining with the Kwik-Diff Kit (Thermo Fisher Scientific, Pittsburgh, PA). All the animal protocols were reviewed and approved by the institutional animal ethics committee of Yogi Vemana University, according to CPCSEA guidelines.

Mouse blood was obtained by cardiac puncture and murine peripheral blood neutrophils isolated by the Ficoll-Hypaque gradient centrifugation and sedimentation in 5% dextran [23]. This method yields approximately  $1.7 \times 10^6$  cells/mouse, with PMNs representing > 90% of the cells isolated. Monocytes which are rich source of cytokines, typically make up < 2% of mouse WBC count [30]. PMNs purity was assessed by differential counting by Wright-Giemsa staining as described previously [31] and monocyte contamination was found to be negligible (< 0.1%). Once isolated, PMNs were re-cultured in RPMI 1640 supplemented with 10% (v/v) FBS (Sigma) and 1% (v/v) penicillin-streptomycin. The cells were maintained at 37 °C in humidified incubators supplemented with 5% CO<sub>2</sub>.

### 2.2. Measurement of PMNs' chemotaxis

PMNs' chemotaxis was assayed using a 12-well Boyden chemotaxis chamber (Neuroprobe, Cabin John, MD) as previously described [32]. Chemotaxis was expressed as the number of PMNs per high-power (100 $\times$ ) field (hpf) migrating through a 3- $\mu$ m polycarbonate membrane filter. Migration was induced by  $10^{-7}$  M fMLP with or without 10-nitrooleate (OA-NO<sub>2</sub>, Cayman chemical; 1 and 10  $\mu$ M) pre-treatment or



**Fig. 2.** PMNs from cecal ligation and puncture (CLP)-subjected mice and 10-nitrooleate treated PMNs show decreased chemotaxis to chemotactic peptide fMLP. A) Cells were isolated from control, sham-operated and CLP-mice and stimulated with  $10^{-7}$  M fMLP for the assessment of chemotaxis ( $*p < 0.05$  vs. Sham). B) Normal (healthy) mice blood PMNs were isolated and treated with 10-nitrooleate (OA-NO<sub>2</sub>, 1 and 10  $\mu$ M) for 30 min and stimulated with  $10^{-7}$  M fMLP for the assessment of chemotaxis. The data represented here are mean  $\pm$  SEM PMNs count per high-powered field at 100 $\times$  magnification. Results are expressed as the average of the three different experiments performed in triplicates. ( $*p < 0.05$  vs. (0) DMSO;  $**p < 0.01$  vs. (0) DMSO).

Hank's balanced salt solution as a control. After incubation, the polycarbonate membrane was removed from the Boyden chamber and placed under 100 $\times$  magnification to count the migrated PMNs. To establish that the OA-NO<sub>2</sub> mediated inhibition of chemotaxis is due to PPAR $\gamma$ , another set of experiment was conducted wherein PMNs were treated with the PPAR $\gamma$  antagonist, GW9662 (10  $\mu$ M), 30 min before the OA-NO<sub>2</sub> treatment, which was followed by chemotaxis assay.

### 2.3. PPAR $\gamma$ DNA-binding activity assay and ELISA

After the CLP, mice lung or blood PMNs' nuclear proteins were extracted using a nuclear protein extraction kit (Active Motif, Carlsbad, CA). Protein concentrations were determined using the BCA protein assay kit (Pierce). To measure PPAR $\gamma$  activity, an ELISA-based PPAR $\gamma$  transcription factor assay kit (TransAM; 40196; Active Motif) was used according to the manufacturer's directions. Briefly, equal amounts (20  $\mu$ g) of nuclear extracts were loaded into wells containing immobilized double-stranded DNA incorporating the consensus PPAR response element. After washing, the bound PPAR $\gamma$  was detected using a specific primary and secondary antibody that provide a sensitive colorimetric readout [33].

The levels of inflammatory cytokines from the PMNs' culture supernatant was measured using commercial kits (TNF $\alpha$ , Cat # KMC3011

and IL-1 $\beta$ , Cat# EM2IL6; Thermo Fisher Scientific, PA) following the manufacturer's protocols described earlier [34]. The degree of modulation of these cytokines by OA-NO<sub>2</sub> was compared to that of cells activated with one  $\mu$ g/ml lipopolysaccharide (LPS, prepared from *Escherichia coli* O111:B4, Sigma Aldrich, India) for 4 h. LPS dose for stimulation was set according to Sohn, Paape [35].

### 2.4. Statistical analysis

Data were presented as mean  $\pm$  SEM. The significance of the statistical difference between the means was analysed using the Student's *t*-test (single comparisons) or one-way ANOVA with the post-hoc Tukey test (multiple comparisons) using GraphPad Prism 7.0 (San Diego, CA).  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Induction of polymicrobial infection in mice by CLP increases PPAR $\gamma$ expression and activity

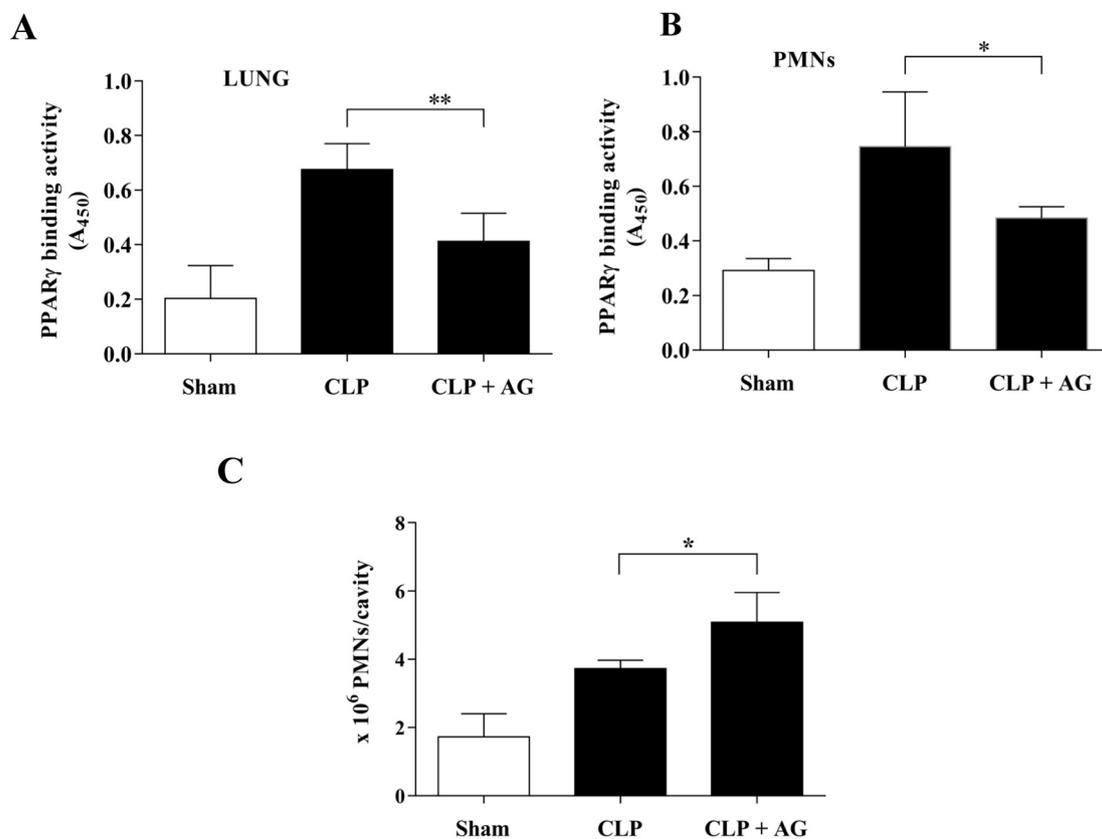
Lung tissue and PMNs were isolated 12 h post CLP. Lung protein was subjected to immunoblotting and analysed for PPAR $\gamma$  expression. Lung PPAR $\gamma$  protein expression was significantly increased in CLP-subject mice when compared to sham operated controls (Fig. 1A). Further analysis of PMNs isolated from CLP mice shows increased expression of PPAR $\gamma$  compared to control PMNs (Fig. 1B) which is consistent with total lung protein and suggesting that polymicrobial infection is partly dependent on PPAR $\gamma$ . Next, we wanted to ascertain whether the increased expression of PPAR $\gamma$  has any role in its binding to the PPAR response elements of DNA. To study this, we isolated nuclear protein from the lung and PMNs which were subjected to the ELISA-based PPAR $\gamma$  transcription factor DNA-binding assay. Our results revealed that increased PPAR $\gamma$  binding activity was observed in lung and PMNs isolated from CLP mice, compared to sham-operated mice (Fig. 1C, D).

### 3.2. CLP or 10-nitrooleate (OA-NO<sub>2</sub>) treatment reduces PMNs' chemotaxis to fMLP

PMNs isolated from CLP mice were subjected to fMLP-induced chemotaxis. A reduced chemotaxis of PMNs was observed in the CLP-subjected mice compared to the sham-operated PMNs (Fig. 2A), whereas the OA-NO<sub>2</sub> treatment dose-dependently reduced the chemotaxis of PMNs isolated from the normal mice (Fig. 2B), suggesting that endogenously produced OA-NO<sub>2</sub> might be activating PPAR $\gamma$  and further inhibiting the PMNs' chemotaxis in vivo.

### 3.3. Pharmacological inhibition of iNOS reverses the CLP effect on PPAR $\gamma$ activity and PMNs' chemotaxis

The inhibition of iNOS in mice was carried out by administering AG (90 mg/kg subcutaneously) 30 min before CLP surgery and after 6 h of surgery, blood PMNs were isolated and subjected to a chemotaxis assay. Total lung and isolated PMNs' PPAR $\gamma$  activity was measured in nuclear extracts, as mentioned above, by the ELISA based assay. In AG treated mice, lung and PMNs' PPAR $\gamma$  binding activity to DNA was significantly reduced when compared to the control mice (Fig. 3A, B). We also measured the number of PMNs present in the peritoneum of the CLP and control mice. The AG-treated CLP-mice showed increased infection site recruitment of PMNs and increased PMNs' chemotaxis to fMLP compared to the untreated CLP-mice (Fig. 3C, D). This suggests that the inhibition of iNOS, in turn, inhibits the endogenous production of the PPAR $\gamma$  ligands, which we hypothesize as OA-NO<sub>2</sub> that activates PPAR $\gamma$  during the inflammation.



**Fig. 3.** Pharmacological inhibition of iNOS reverses CLP effects on PPAR $\gamma$  activity and PMNs' chemotaxis. Animals were pre-treated with aminoguanidine (AG) (90 mg/kg subcutaneously) 30 min before CLP surgery, and after 12 h of surgery, lung tissue and PMNs were collected for the PPAR $\gamma$  transcription factor-binding activity. A) PPAR $\gamma$  transcription factor-binding activity to its DNA response elements was performed in nuclear extracts of lung and B) PMNs. Data are shown as mean  $\pm$  SEM (\* $p$  < 0.05 vs. CLP; \*\* $p$  < 0.01 vs. CLP;  $n$  = 5 mice/group). C) In a separate experiment, peritoneal cavity fluid was collected after 6 h of surgery and the total PMNs' count was performed to assess infection site recruitment. The results are expressed as the average of the three different experiments performed. Data are shown as mean  $\pm$  SEM. (\* $p$  < 0.05 vs. CLP).

### 3.4. CLP-induced reduction in PMNs chemotaxis is PPAR $\gamma$ dependent

To establish whether OA-NO<sub>2</sub> actions are PPAR $\gamma$  dependent, the PMNs isolated from normal mice blood were pre-treated with GW9662, a potent PPAR $\gamma$  antagonist, for 30 min before the OA-NO<sub>2</sub> treatment. Thereafter, the cells were subjected to a chemotaxis assay. Treatment with GW9662 significantly reversed the effects of OA-NO<sub>2</sub> on PMNs' chemotaxis, suggesting that the actions of OA-NO<sub>2</sub> are PPAR $\gamma$  dependent (Fig. 4A). Further, we enumerated the PMNs from GW9662 pre-treated CLP-mice peritoneum and found increased infection site recruitment of PMNs compared to untreated CLP mice, suggesting that chemotaxis is PPAR $\gamma$  dependent (Fig. 4B).

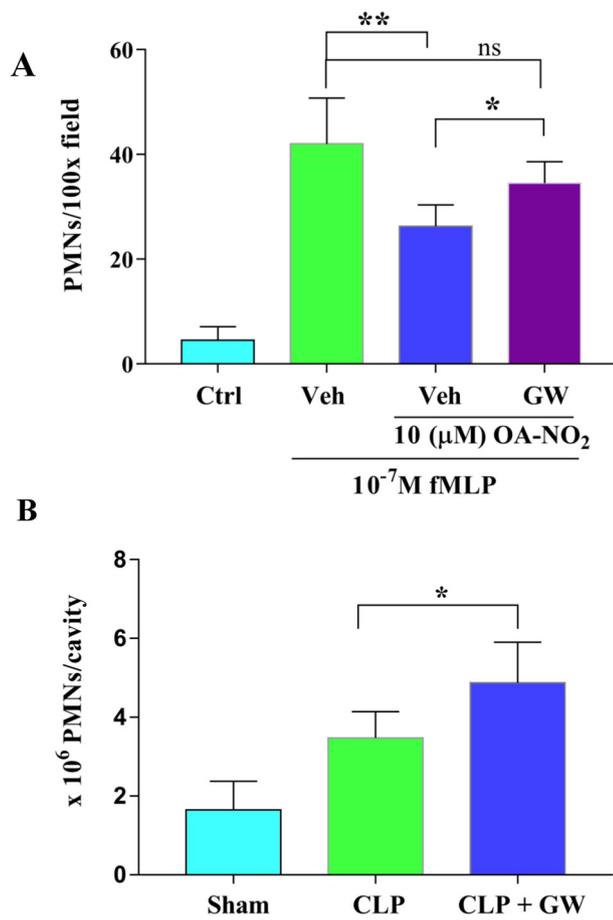
### 3.5. OA-NO<sub>2</sub> decreases the production of inflammatory markers

Neutrophils are key cells involved in the control of polymicrobial sepsis [36]. In an attempt to determine whether OA-NO<sub>2</sub> blocks the production of proinflammatory cytokines (TNF $\alpha$  and IL-1 $\beta$ ), mouse PMNs were first pre-treated with or without OA-NO<sub>2</sub> (1 and 10  $\mu$ M) for 30 min and then the cells were activated by 1  $\mu$ g/ml LPS. After 4 h of incubation, cell culture supernatant was collected and used to measure the TNF $\alpha$  and IL-1 $\beta$  cytokines by means of ELISA. LPS-treated cells secreted a significant amount of the cytokines mentioned above compared to the control cells (no stimuli). Interestingly, OA-NO<sub>2</sub> pre-treated cells showed significantly reduced amounts of proinflammatory cytokines compared to the untreated control cells (Fig. 5).

## 4. Discussion

This study shows that the inhibition of iNOS by AG decreases the DNA-binding ability of PPAR $\gamma$  and increases the migration of PMNs to the site of infection in CLP-induced sepsis. Studies conducted earlier showed that NO inhibits neutrophil chemotaxis and the inhibition of iNOS-mediated NO production by AG restores the chemotaxis activity [37]. Although research has been carried out showing the interaction between iNOS and PPAR $\gamma$  in various conditions such as seizures [38], diabetic nephropathy [39], depression [40] and type 2 diabetes [41,42], this is the first report wherein AG treatment has been shown to directly inhibit PPAR $\gamma$  DNA-binding ability in CLP-mice. This suggests that the inhibition of iNOS, in turn, suppresses the endogenous production of PPAR $\gamma$  activators such as the NFAs. The endogenous formation of OA-NO<sub>2</sub> in a murine model of focal cardiac ischemia-reperfusion has been documented [12,43]. However, the precise change in the NO or OA-NO<sub>2</sub> levels was not measured in this study. In an earlier study, the pre-treatment of neutrophils with an endogenous PPAR $\gamma$  agonist, 15-Deoxy- $\Delta$ -12,14-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), inhibited neutrophil chemotaxis by suppressing the ICAM-1 expression [23]. The possibilities of other PPAR $\gamma$  ligands, produced during the inflammatory conditions at nanomolar concentrations required for the activation of PPAR $\gamma$ , have not been reported.

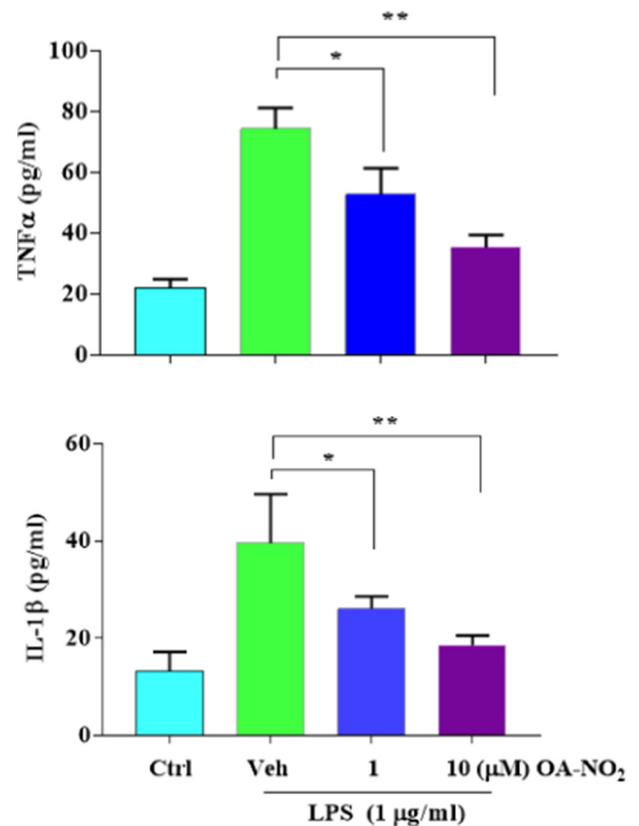
PPAR $\gamma$  activators exert a wide range of effects on the leukocytes that play important roles in innate immunity. More specifically, PPAR $\gamma$  activators dose-dependently inhibit eosinophil and monocyte chemotaxis in vitro [44,45] as well as that of PMNs' migration in vivo following the administration of PPAR $\gamma$  ligand [46]. Our results show that the increased expression of PPAR $\gamma$  protein and its DNA-binding activity in



**Fig. 4.** CLP-induced inhibition of PMNs chemotaxis is PPAR $\gamma$ -dependent. A) Normal blood PMNs were pre-treated with or without the PPAR $\gamma$  antagonist GW9662 (GW, 10  $\mu$ M), for 30 min, followed by the 10-nitrooleate (OA-NO<sub>2</sub>-10  $\mu$ M) treatment and then stimulated with 10<sup>-7</sup> M fMLP for the assessment of chemotaxis. Ctrl, (unstimulated cells, \*p < 0.05 vs. OA-NO<sub>2</sub>; \*\*p < 0.01 vs. Veh (DMSO); ns-not significant). B) Animals were pre-treated with or without the PPAR $\gamma$  antagonist, GW (1 mg/kg, intraperitoneal), 30 min before CLP surgery and after 6 h of surgery, the PMNs in the peritoneal cavity were determined. Data are shown as mean  $\pm$  SEM. (\*p < 0.05 vs. CLP, n = 5 mice/group).

CLP mice lung and PMNs are in agreement with earlier studies [23]. In our study, ex vivo, OA-NO<sub>2</sub> dose-dependently inhibited PMNs' chemotaxis in response to fMLP, which was similar to the CLP-induced inhibition of neutrophil chemotaxis to the infection site in mice and are corroborative with a previous report using two different types of PPAR $\gamma$  ligands, 15d-PGJ<sub>2</sub> or troglitazone [23]. NFAs bind to a PPAR $\gamma$  ligand-binding site that is different from synthetic thiazolidinediones (TZD). Hence, the functional outcomes may be different and likely to be better than that of the TZD [47].

On the other hand, we showed that PMNs' chemotaxis inhibition was restored to control levels upon pre-treatment of the PMNs with the PPAR $\gamma$  antagonist, GW9662. This clearly shows that the OA-NO<sub>2</sub>-mediated inhibition of PMNs' chemotaxis is mediated through PPAR $\gamma$ , as OA-NO<sub>2</sub> is one of the well-known endogenous PPAR $\gamma$  ligands [48]. However, OA-NO<sub>2</sub>-mediated inhibition of inflammation was considered to be the end result of both PPAR $\gamma$ -dependent and independent pathways [49]. In another study, PPAR $\gamma$  present in endothelial cells was found to suppress inflammation caused by endotoxemia and injury [50]. LPS-induced lung inflammation and injury had been exacerbated in endothelial cell-specific PPAR $\gamma$  knockout mice (KO), and anti-inflammatory action of OA-NO<sub>2</sub> was nullified in LPS-induced epithelial cells isolated from the PPAR $\gamma$  KO mice, thus corroborating our results



**Fig. 5.** The PPAR $\gamma$  agonist, 10-nitrooleate, inhibits the secretion of inflammatory cytokines from PMNs. Mice blood PMNs were collected and cultured in the presence of 10-nitrooleate (OA-NO<sub>2</sub>) and stimulated with lipopolysaccharide (LPS). The secretion of the inflammatory cytokines (TNF $\alpha$  and IL-1 $\beta$ ) from the PMNs culture supernatant was measured by ELISA as described in the methods. Data are shown as mean  $\pm$  SEM. (\*p < 0.05 vs. Veh (DMSO-LPS); \*\*p < 0.01 vs. Veh (DMSO-LPS)).

that OA-NO<sub>2</sub> activity is indeed PPAR $\gamma$  dependent.

It has been well established that the activation of PPAR $\gamma$  decreases inflammatory responses in a variety of disease models [51,52]. In the present study, for the first time, an endogenous PPAR $\gamma$  ligand, OA-NO<sub>2</sub>, downregulated the expression of the inflammatory cytokines, TNF $\alpha$  and IL-1 $\beta$ , in PMNs stimulated with LPS. Earlier studies showed that OA-NO<sub>2</sub> downregulated the p65 and NF- $\kappa$ B expression [53]. The OA-NO<sub>2</sub>-mediated suppression of NF- $\kappa$ B not only reduces the activity of PMNs but also that of alveolar macrophages [54]. This is in accordance with numerous other studies conducted elsewhere [55–57]. NFAs are well established drug candidates for chronic inflammatory and fibrotic diseases [58]. They are endogenously produced and readily found in plasma, cell membranes and tissues [59]. However, their dose setting, time of administration etc. need to be optimised. Further studies are warranted for a clear understanding of the role of NFA in the resolution of inflammation and in immunosuppression.

In conclusion, our study shows that the inhibition of iNOS may have beneficial effects on the outcome of sepsis. The end products of iNOS enzyme's activity and NFA play a crucial role in inhibiting the migration of PMNs and such inhibition is partly PPAR $\gamma$ -mediated. Our study suggests that pathways that inhibit PPAR $\gamma$  activation may result in increased PMNs' chemotaxis to the site of infection, and subsequently improve host immunity in sepsis.

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