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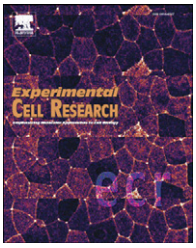

Research Article

# Expression of TREM-1 is inhibited by $PGD_2$ and $PGJ_2$ in macrophages☆

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ABSTRACT

TREM-1 is a superimmunoglobulin receptor present on neutrophils and monocytes, which plays an important role in the amplification of inflammation. The natural ligands for TREM-1 have not been identified; however, Toll-like receptor ligands are known to induce the expression of TREM-1. Blockade of TREM-1 has shown to improve survival in animal models of sepsis. In the present studies, we investigated the role of lipid mediators in the expression of TREM-1. In a macrophage cell line, we show that the expression of TREM-1 in response to LPS and bacteria *Pseudomonas aeruginosa* is inhibited by $PGD_2$ and cyclopentanone prostaglandins $PGJ_2$ and 15-d$PGJ_2$. The inhibition of TREM-1 by these prostaglandins is independent of the $PGD_2$ receptors and PPARγ but occurs by activation of Nrf2 and inhibition of NF-κB. Our data suggest a novel mechanism by which these prostaglandins exhibit anti-inflammatory effects and a new therapeutic approach to inhibition of TREM-1.



## Introduction

Triggering receptor expressed on myeloid cells 1 (TREM-1) is a member of the superimmunoglobulin family that is expressed selectively on macrophage and neutrophils [1,2]. TREM superfamily of receptors includes activating receptors, namely TREM-1 and TREM-2 [3,4], which modulate the inflammatory response. TREM-1 activation amplifies the Toll-like receptor-initiated responses to invading pathogens allowing the secretion of proinflammatory chemokines and cytokines [5–7]. The ligand for TREM-1 has not been identified; however, we and others have shown that TREM-1 expression is induced in response to bacteria and microbial products [8,9]. In mouse models of sepsis, blockade of TREM-1 has shown to improve survival [10–12].

Prostanoids, including prostaglandins and thromboxanes, are produced when arachidonic acid is released from the plasma membrane by phospholipases and metabolized by cyclooxygenases [13,14]. Prostanoid formation occurs when cyclooxygenase oxygenates arachidonate converting it to $PGG_2$, which is then reduced to $PGH_2$. $PGH_2$, in turn, is converted to five primary active

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metabolites, $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, or thromboxane $A_2$ via distinct synthases [14,15]. $PGD_2$, $PGJ_2$, and 15-d$PGJ_2$ metabolites are naturally occurring derivatives of prostaglandins, characterized by the presence of a cyclopentanone ring system. Several *in vitro* and *in vivo* studies suggested that these PGs may exert anti-inflammatory and other physiological effects via $PGD_2$ receptors (DP1 and DP2) [14–19] and through interaction with intracellular peroxisome proliferator-activated receptor γ (PPARγ) [20,21].

Prostaglandins modulate a variety of pathophysiological responses including innate immune response. In a model of *Pseudomonas aeruginosa* lung infection, we have shown that inhibition of COX-2 enhances bacterial clearance [22], whereas overexpression of COX-2 in the lung inhibits the clearance of bacteria [23]. The mechanisms by which prostaglandins modulate immune response have not been completely defined. $PGE_2$ production is enhanced in the lung during bacterial pneumonia and studies by Aronoff et al. [24] have suggested that PGE2 is immunosuppressive. These investigators have shown that PGE2 inhibits phagocytosis and the generation of ROS through the NADPH oxidase system which is a mechanism by which $PGE_2$ is immunosuppressive in infections [25].

The effects of $PGD_2$ on immune cell function are less well characterized [26]. In a mouse model of *P. aeruginosa* infection, we have shown that overexpression of $PGD_2$ enhanced the clearance of *P. aeruginosa*, whereas clearance of bacteria was impaired in the PGD synthase knockout mice suggesting that $PGD_2$ is immunomodulatory [27]. The mechanisms by which $PGD_2$ mediates immunomodulatory effects are not fully understood. Cyclopentanone prostaglandins are known to affect cell signaling through modulation of transcription factors which could play a seminal role in immune cells [28,29]. These effects are dose-dependent [30]. In a recent study, Yoon et al. [31] showed that the expression of TLR2 in microglial cells is regulated by prostaglandin $PGD_2$, 15-d$PGJ_2$, and 15-d$PGD_2$. These studies suggest that prostaglandins may have a broader role in modulating the signaling pathways beginning from cell surface receptors.

In the present study, we questioned if the expression of TREM-1 in macrophages is modulated by $PGD_2$ and cyclopentanone prostaglandins $PGJ_2$ and 15-d$PGJ_2$. We found that in macrophages, the expression of TREM-1 in response to LPS in inhibited by $PGD_2$, $PGJ_2$, and 15-d $PGJ_2$. We also investigated the mechanisms by which these prostaglandins modulate the expression of TREM-1. In macrophages, we have shown that the expression of TREM-1 is independent of $PGD_2$ receptors or PPARγ; however, the effects by $PGJ_2$ by receptor-independent mechanisms.

## Materials and methods

### Materials

$PGD_2$, $PGE_2$, $PGJ_2$, 15-d$PGJ_2$, BW245C, and 15(*R*)-methyl $PGD_2$ were from Cayman Chemical (Ann Arbor, MI).

### Cell lines and cell culture

A murine macrophage cell line RAW 264.7 (American Type Culture Collection) was maintained in DMEM supplemented with 10% FBS (HyClone). The tissue culture medium was supplemented with penicillin (100 U/ml)/streptomycin (100 μg/ml; Invitrogen Life Technologies). Cells were passaged once or twice weekly.

*Bone marrow-derived macrophages (BMDMs)*

Briefly, mice were killed by asphyxiation with $CO_2$. Cellular material was aspirated from femurs and spun at 400 $\times g$ at 4 °C for 5 min. Cells were then resuspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 10% L929 cell-conditioned medium (LCM). The cells were allowed to mature into phenotypic macrophages by incubation in the presence of LCM for 5 days before the experiments were done. Total cell counts were determined with a grid hemocytometer, and $2\times10^6$ cells were plated per well for experiments.

*TREM-1 luciferase reporter cell lines*

Stable transfection of TREM-1 promoter-luciferase construct was made as follows: a DNA fragment containing 1.3-kb-long murine TREM-1 promoter sequence was cloned into BglII and KpnI sites of pGL4.17v plasmid (Promega, Madison, WI). The cloned TREM-1 promoter sequence was confirmed by sequencing and was prepared by Endo-free Maxiprep kit (Qiagen). For stable transfection of the plasmid, RAW 264.7 ($0.5\times10^6$ cells) were transfected with a plasmid by GenePORTER 2 (Gene Therapy Systems, Inc., San Diego, CA), as specified by the manufacturer. The transfected cells were incubated for 14–16 hours in a 37 °C, $CO_2$ incubator, and were passaged once before adding G418 (800 mg/ml; Sigma, St. Louis, MO) to select the stably transfected cells. Successful stable cell lines were chosen after assessing TREM-1 transcriptional activity by luciferase assay following LPS treatment (100 ng/ml) for 5 h.

### RNA extraction

Total cellular RNA was extracted using the Trizol® RNA extraction Kit (Invitrogen) according to the manufacturer's protocol. The RNA samples were treated with DNase I (Qiagen) and stored at −80 °C until used. The RNA quality was examined using gel electrophoresis and using $A_{280}/A_{260}$ ratio (SMARTspec, Bio-Rad). The cDNA was synthesized from 1 μg of total RNA by using SuperScript first-strand synthesis system (Fermentas). Following a denaturation step of 5 min at 70 °C, RNA was reverse-transcribed to single-stranded cDNA using oligo(dT) primers . The reverse transcription reaction was performed in a total volume of 20 μl containing 0.2 mM of each dNTP, 40 U of MMuLV reverse transcriptase and 20 U of RiboLock Ribonuclease inhibitor at 37 °C for 60 min and 70 °C for 10 min.

### Quantitative real-time polymerase chain reaction

The resulting cDNA template was subjected to quantitative real-time PCR (qrtPCR) real-time using Taqman-based Applied Biosystems gene expression assays Mm00451738_m1 (Ref Seq: NP_067381.1) for TREM-1 (*Mus musculus*) and Mouse ACTB (4352933-0803021) for endogenous control gene actin (FAM-MGB probe dye labeled) and the TaqMan Fast Universal PCR Master Mix (ABI). PCR was conducted using Applied Biosystems (ABI Prism 7900HT) according to the manufacturer's instructions. For quantitative PCR analysis, each sample was run in triplicates. Each run included a no-template control to test for contamination of assay reagents. Real-time After a 94 °C denaturation for 10 min, the reactions were cycled 40 times with a 94 °C denaturation for 15 s and a 60 °C annealing for 1 min. Three types of controls aimed

at detecting genomic DNA contamination in the RNA sample or during the RT or quantitative PCR reactions were always included: a RT mixture without reverse transcriptase, a RT mixture including the enzyme but no RNA, negative control (reaction mixture without cDNA template). Data were collected and analyzed using OneStep Software (ABI). Relative quantification was performed using the comparative threshold (CT) method after determining the CT values for reference (actin) and target (TREM-1) genes in each sample sets according to the $2^{-\Delta\Delta Ct}$ method (ΔΔCT, Delta Delta CT). Changes in mRNA expression level were calculated after normalization to actin. The ΔΔCT method provides a relative quantification ratio according to calibrator that allows statistical comparisons of gene expression among samples. Values of fold changes in the control sample versus the posttreatment samples represent averages from triplicate measurements. Changes in gene expression were reported as fold changes relative to controls.

### *Semiquantitative RT–PCR*

RT–PCR was conducted using Peltier Thermal Cycler (MJ Research) according to the manufacturer's instructions. Reactions were performed in a 25-μl volume with 0.2 mM dNTPs, 2.5 μl of reaction buffer, 0.1 μM primers, 2.5 mM $MgCl_2$, 1.25 U of *Taq* polymerase (all reagents were from Fermentas), and 4 μl of cDNA. Actin was chosen as housekeeping gene for relative quantification to normalize target gene expression. The following primers were used for RT–PCR amplification: TREM-1 forward (5′-CGG AAT TCGAGC TTG AAG GAT GAG GAA GGC-3′), TREM-1 reverse (5′-AAT CCA GAG TCT GTC ACT TGA AGG TCA GTC-3′), actin forward (5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′), and β-actin reverse (5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′). PCR was performed under the conditions of an initial 1 min at 94 °C, followed by 30 cycles for β-actin or 30 cycles for TREM-1 (5 s at 94 °C, 30 s at 58 °C, and 90 s at 72 °C), with a final 7 min at 72 °C. PCR products were visualized by gel electrophoresis in 2% agarose after ethidium bromide staining. They were then imaged using Gel Doc 2000 (Bio-Rad Laboratories).

### *Transient transfection and luciferase assay*

RAW 264.7 cells $5\times10^6$ were cotransfected with Nrf2 SiRNA (Santa Cruz) and NF-κB reporter luciferase plasmid with Lipofectamine 2000 (Invitrogen). Cells were allowed to recover for 24 h before treatment. Reporter gene activity was measured with the Luciferase kit (Promega) 8 h after LPS and $PGJ_2$ treatments.

### *Flow cytometric analysis*

A suspension of $1\times10^6$ cells/ml was prepared in phosphate-buffered saline containing 0.1% sodium azide, 10 mg/ml bovine serum albumin, and 200 μg/ml normal mouse $IgG_1$ (sc-3877, Santa Cruz), and incubated on ice for 20 min. Subsequent staining with either phycoerythrin-conjugated rat $IgG_{2A}$ MoAbs anti-mTREM-1 (FAB1187P, R&D) or Rat $IgG_{2A}$ isotypic controls (IC006P, R&D) was performed on ice for 30 min. The stained cells were washed, resuspended, and analyzed for unicolour immunofluorescence by flow cytometry (CyAn™ ADP High-Performance Flow Cytometer). The gate that contained macrophages was verified, and a minimum of $10^4$ cells were analyzed for each sample. Results were processed using expo for summit analysis software V 4.3.

### *Western blotting*

BMDM ($1\times10^6$ cells) cell lysate was dissolved in sample buffer (350 mM Tris (pH 6.8), 10% SDS, 30% glycerol, 600 mM DTT, and 0.05% bromphenol blue), loaded onto 10% SDS–PAGE gel. Nuclear and cytoplasmic extracts were prepared using Nuclear and Cytoplasmic Extraction Reagents (Active Motif) according to the manufacturer's instructions. Protein content was determined with a Biorad protein assay (BIORAD). Equal amounts of protein (20 μg) were analyzed by 10% SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked (5% nonfat dry milk in TBS/0.1% Tween 20 (TBST), 1 h RT) and incubated overnight at 4 °C with anti-p65, TREM-1, Nrf2, IκBα, or β-actin antibodies (Santa Cruz). Membranes were washed with TBST, incubated with peroxidase-conjugated secondary Abs (1 h, room temperature in TBST), washed, and developed using the ECL chemiluminescence detection reagent (Pierce).

### *Statistical analysis*

Results are expressed as the mean ± SD. Statistical analysis was performed using the paired Student *t* test and one-way ANOVA using Graphpad Instat, and $p<0.05$ was considered to indicate significance.

## Results

### *$PGD_2$, $PGJ_2$ and 15 deoxy$PGJ_2$ inhibit the expression of TREM-1 in macrophages*

Both prostaglandin $D_2$ ($PGD_2$) and its dehydration end product 15-deoxy-Delta-prostaglandin $J_2$ (15-d$PGJ_2$) play an important role in regulating inflammatory response. $PGD_2$ and 15 d-$PGJ_2$ have variable biological effects and can be pro or anti-inflammatory [13,20,21]. We investigated the effects of these prostaglandins on the expression of TREM-1 message in macrophage cell lines in response to LPS. RAW cells were treated with $PGD_2$, and 15-deoxy$PGJ_2$ in concentration of 0.1, 1, and 10 μM in the presence and absence of LPS (100 ng/ml). RNA was extracted and TREM-1 message was determined by RT and real-time RT–PCR 4 hours after treatment with LPS. In our previous work, we have shown that TREM-1 message is expressed by 4 hours after treatment with LPS [9]. There was no difference in expression of TREM-1 noted with 0.1 and 1 μM $PGD_2$ and 15-d-$PGJ_2$ (data not shown). However, the expression of TREM-1 message in response to LPS was inhibited when cells were treated with 10 μM prostaglandins as detected by RT and real-time RT–PCR (Figs. 1A and B). We confirmed that the cells were viable when treated with LPS and 10 μM prostaglandins (data not shown).

We then performed experiments to investigate the effects of prostaglandins on the expression of TREM-1 protein in response to LPS. RAW cells were treated with 10 μM $PGD_2$, $PGJ_2$, and 15-d $PGJ_2$ in the presence and absence of LPS (100 ng/ml). Cells were harvested at 12 and 24 hours. Protein expression of TREM-1 was determined by FACS and confocal analysis. The expression of TREM-1 was induced in response to LPS which was inhibited by $PGD_2$ and 15-d$PGJ_2$ as detected by FACS analysis at 12 (data not shown) and 24 hours (Fig. 2A). Further confirmation of inhibition of TREM-1 was obtained by performing Western blotting for

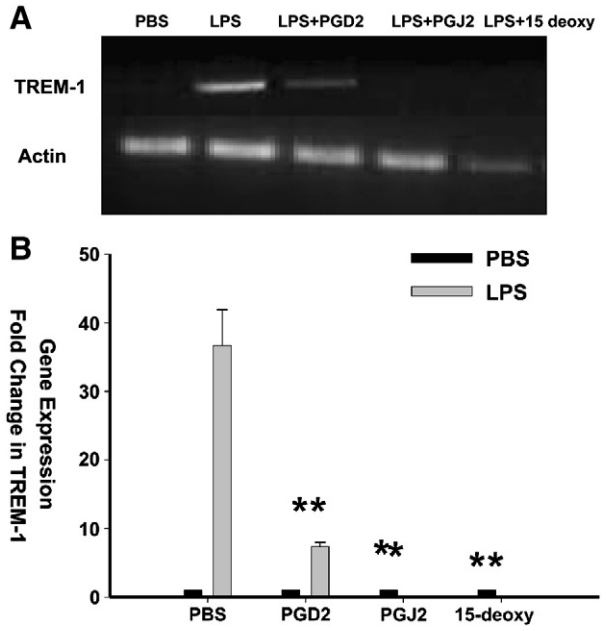


**Fig. 1 – $PGD_2$ and $PGJ_2$ inhibits the expression of TREM-1 message in RAW cells in response to LPS. RAW 264.7 cells were treated with 10 μM $PGD_2$, $PGJ_2$, and 15 deoxy $PGJ_2$ an hour before treatment with 100 ng/ml LPS for 4 hours. TREM-1 expression was determined by (A) RT, and (B) real-time PCR. TREM-1 message was attenuated by all the three prostaglandins. Data are represented as mean ± SE for three separate experiments with at least $n = 3$ for each experiment; $^{**}p < 0.01$ between comparison mediated using ANOVA.**

TREM-1 protein in $PGD_2$ and $PGJ_2$ treated groups (Fig. 2B). Immunofluorescence microscopy showed decrease in cytoplasmic as well as membrane staining of TREM-1 protein, in cells treated with $PGD_2$, $PGJ_2$, and 15-d$PGJ_2$ as compared to cells treated with LPS alone (data not shown). Additional experiments were performed to determine the effects of the prostaglandins in response to whole bacteria which showed that the expression of TREM-1 in response to *P. aeruginosa* is inhibited by $PGD_2$, and $PGJ_2$ (Fig. 2C). LPS-induced expression of cox2 is unaffected by $PGD_2$ and $PGJ_2$ (Fig. 2D).

Together, these data suggest that the expression of TREM-1 in macrophages is inhibited by these prostaglandins. Since TREM-1 amplifies the inflammatory response, inhibition of TREM-1 by prostaglandins can attenuate the inflammatory response. These findings suggest an additional mechanism by which these lipid mediators may be anti-inflammatory. Similar experiments were also performed in peritoneal macrophages and bone marrow-derived macrophages from wild type mice. We were able to demonstrate similar effects in these cell types (data not shown).

### *The inhibition of TREM-1 by $PGD_2$ is a not mediated by the DP receptors*

Prostaglandin $D_2$ ($PGD_2$) regulates inflammation via both receptor-dependent (DP1 and DP2 receptors) and receptor-independent mechanisms. Both DP1 and DP2 (CRTH2) receptors are expressed in various cell types and elicit divergent effects [17]. Thus, we next investigated whether the inhibition of TREM-1 in macrophages was mediated by a receptor-dependent mechanism. To investigate these effects, we examined the effects of selective DP1 agonist BW245C or the selective DP2 agonist 15(*R*)-methyl $PGD_2$ on LPS induced change in TREM-1 expression. RAW cells were treated with LPS (100 ng/ml) with BW245C or 15(*R*)-methyl $PGD_2$ (100 nmol). Control cells were treated with vehicle alone. Expression of TREM-1 receptor was determined by FACS analysis at 24 hours. As shown in Fig. 3A, treatment with BW245C or 15(*R*)-methyl $PGD_2$ did not significantly change the expression of TREM-1 in response to LPS at 24 hours (similar results were obtained at 4 hours; data not shown). These data suggest that the inhibitory effects of $PGD_2$ are independent of their receptors.

### *The effects of $PGJ_2$ are mediated independent of PPARγ*

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the ligand-activated nuclear steroid hormone receptor family that can function as a transcription factor [32]. Several small-molecule agonists of this family of receptors, including several prostaglandins and antidiabetic agents like rosiglitazone and pioglitazone possess pronounced anti-inflammatory effects [32,33] by binding to PPARγ. We thus questioned whether the inhibitory effect of $PGJ_2$ on TREM-1 is mediated by PPARγ. The effects of rosiglitazone and pioglitazone were investigated on LPS induced TREM-1 expression. RAW cells were treated with LPS (100 ng/ml) with or without 20 μM rosiglitazone or pioglitozone. Control cells were treated with vehicle alone. The expression of TREM-1 in response to LPS was not inhibited at this concentration of PPARγ agonists at 24 hours (Fig. 3B). These data suggest that the inhibitory effects of $PGJ_2$ on the expression of TREM-1 are not mediated by PPARγ.

### *TREM-1 expression is inhibited by $PGJ_2$ through modulation of transcription factors NF-κB and Nrf2*

We have previously shown that the expression of TREM-1 is regulated at a transcriptional level by NF-κB and PU.1 [9]. Upon activation by LPS, NF-κB binds to the TREM-1 promoter which leads to the expression of TREM-1 [9]. 15-d$PGJ_2$ and $PGJ_2$ have also been shown to mediate some of their effects by inhibiting transcription factors and signal regulated kinases (Erk). Thus, we hypothesized that the inhibitory effects of $PGJ_2$ on TREM-1 expression may be mediated by inhibition of NF-κB. Thus, we examined if in RAW cells the effects of $PGJ_2$ on TREM-1 expression are mediated by modulation of NF-κB activation in response to LPS. RAW cells were treated LPS (100 ng/ml) and $PGJ_2$ (10 μmol) for 4 hours. Western blot analysis for p65 was performed on nuclear proteins. In cells that were treated with $PGJ_2$, the nuclear translocation of p65 was reduced compared to the LPS treated cells (Figs. 4A and B). To further confirm these findings we treated TREM-1 luciferase reporter cells with LPS in the presence and absence of $PGJ_2$ and NF-κB inhibitor BMS-345541 (1 μg/ml) (Bristol Myer Squib). We showed that the expression of TREM-1 is significantly reduced in the presence of $PGJ_2$ and BMS (Fig. 4C). LPS-induced degradation of IκB-α is also inhibited by $PGJ_2$ (Fig. 4D). These data confirm that the modulation of TREM-1 by $PGJ_2$ is at least partially mediated by inhibition of NF-κB.

Some of the anti-inflammatory effects of $PGJ_2$ and 15-d$PGJ_2$ are also mediated by activation of Nrf2. Nrf2 is a transcription factor that has been indentified to play an important role in the induction of

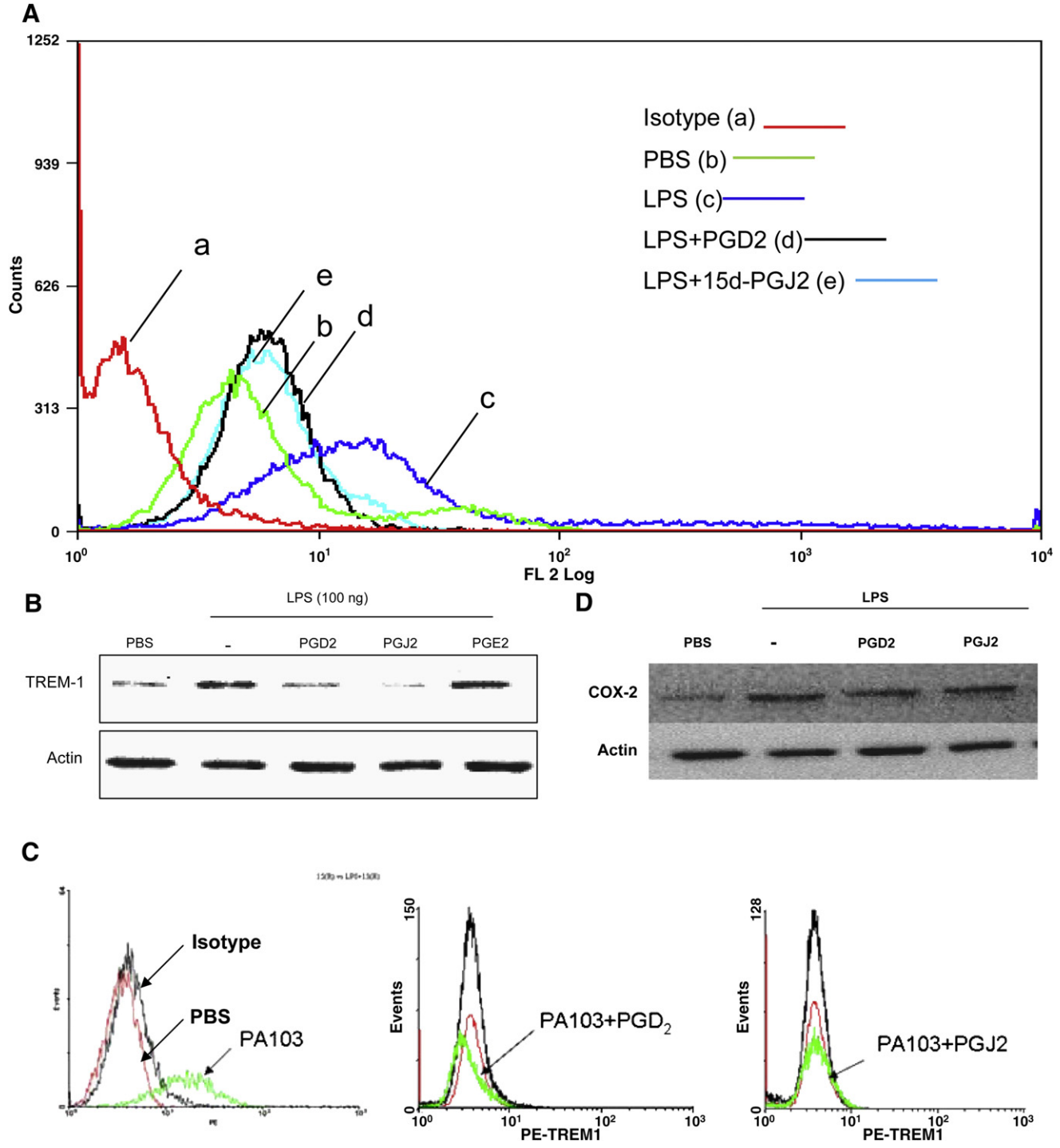


**Fig. 2 – Expression of TREM-1 protein in response to LPS and *P. aeruginosa* is inhibited by $PGD_2$ and $PGJ_2$ in RAW cells. RAW cells were treated with $PGD_2$, $PGJ_2$, and $PGE_2$ before (1 hour) treatment with LPS (100 ng/ml). Expression of TREM-1 protein was inhibited by all the prostaglandins at 24 hours as determined by (A) FACS analysis. (B) Western blot analysis of TREM-1. (C) RAW cells that were treated with PA103 (MOI of 1) and $PGD_2$ and $PGJ_2$ also showed an inhibition of TREM-1 protein at 24 hours and no changes in isotype- (red) and vehicle- (black) treated cells. (D) Western blot of COX-2 in prostaglandins-treated macrophages.**

antioxidant enzymes [34–36]. It is reported that 15-d$PGJ_2$ binds to keap-1 and activates Nrf2 in macrophages [34]. In addition, the expression of TREM-1 is increased in Nrf2 knockout mice [37]. We thus hypothesized that the effects of $PGJ_2$ may also be mediated by Nrf2. RAW cells were treated with LPS in the presence and absence of $PGJ_2$ and determined the expression of Nrf2 by Western blot analysis. Nuclear translocation of Nrf2 following treatment with LPS and $PGJ_2$ was increased at 4 hours as compared to cells that were treated with

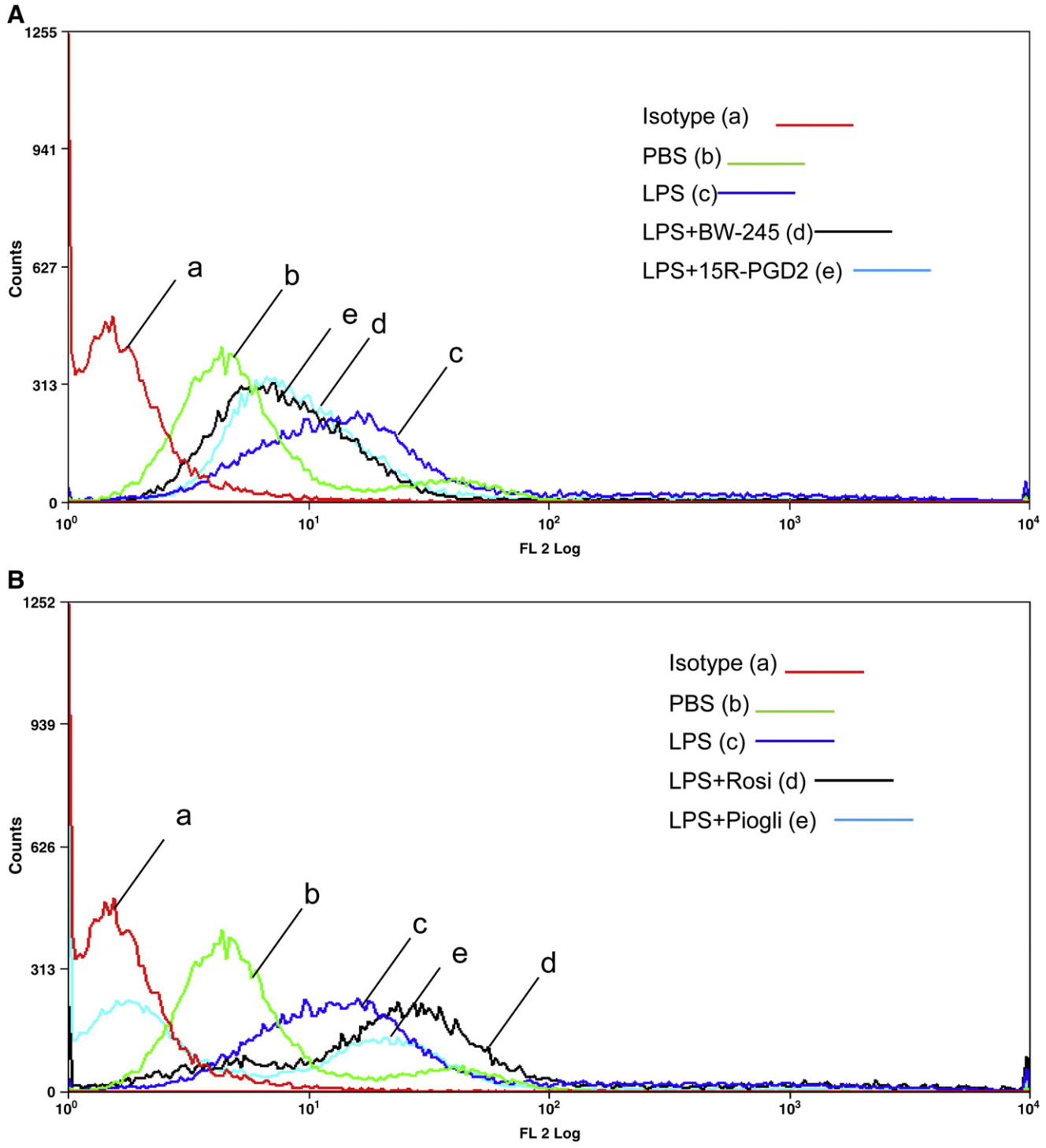


**Fig. 3 – Inhibition of TREM-1 in response to LPS is independent of DP2 receptors and PPARγ. Cells were treated with (A) DP1 agonist BW245C and DP2 agonist 15(*R*)-methyl $PGD_2$ (100 nM) 1 hour before treatment with LPS (100 ng/ml for 24 hours). The expression of TREM-1 was not altered by treatment of the agonist, indicating that the effects mediated by $PGD_2$ are independent of the receptor activation. (B) Pretreatment (1 hour) with PPARγ agonist rosiglitazone and pioglitazone (20 μM) did not alter the expression of TREM-1 protein upon treatment with LPS for 24 hours, suggesting that the effects mediated by $PGJ_2$ are independent of PPARγ.**

LPS alone (Figs. 5A and B). To further confirm these findings, we treated the TREM-1 luciferase reporter cells in the presence of Nrf-2 activator (L-sulforaphane, 10 μmol). Cells that were treated with L-sulforaphane showed significant inhibition of expression of TREM-1 compared to control cells, and suppression of Nrf2 by SiRNA also showed increased TREM-1 expression at the basal level or after LPS

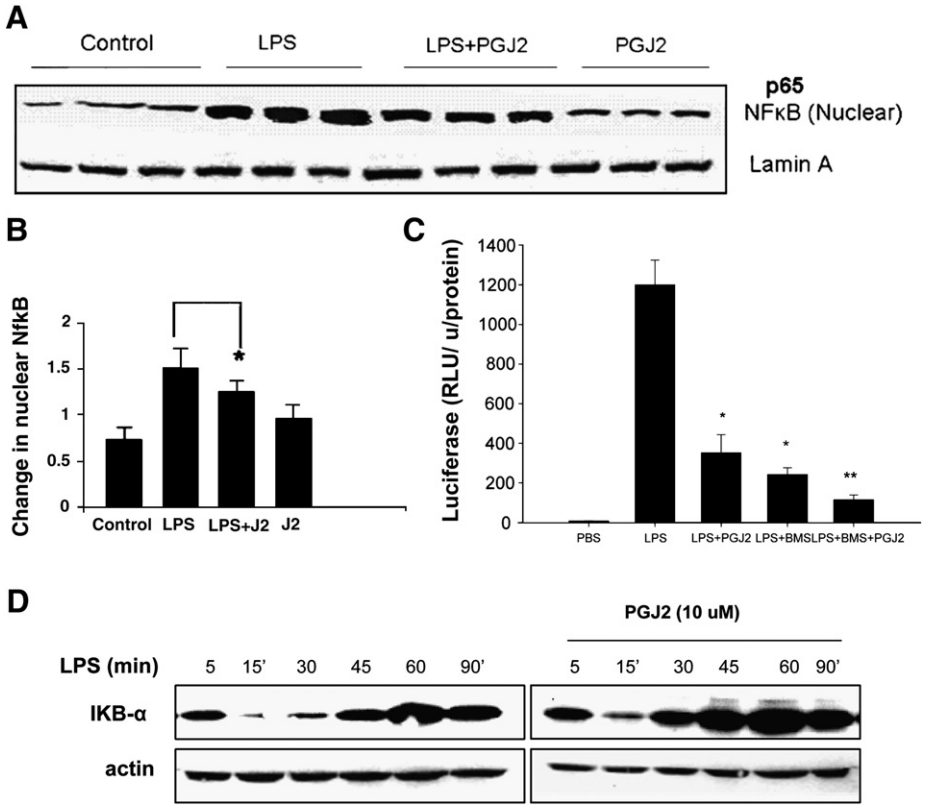


**Fig. 4 – $PGJ_2$ inhibits the nuclear translocation of p65 in response to LPS. RAW cells were pretreated with $PGJ_2$ (10 μmol) 30 min before LPS (100 ng/ml) treatment. After 4 hours, nuclear translocation of p65 was determined by (A) Western blot analysis. (B) Densitometry confirmed that there was significant inhibition of nuclear p65 in cells that were treated with $PGJ_2$ and LPS compared to those that were treated with LPS. (C) TREM-1 reporter cells that were treated with LPS and $PGJ_2$ also showed a significant decrease in the expression of TREM-1 similar to cells that were treated with BMS-345541 (1 μg/ml) (NF-κB inhibitor). (D) Immunoblotting analysis of BMDM cells treated with LPS and $PGJ_2$ with IκBα and actin antibodies ($n=4$, $^{*}p<0.05$, $^{**}p<0.01$).**

induction (Figs. 5C and D). Together, these data suggest that the inhibition of TREM-1 in macrophages by $PGJ_2$ may be mediated by activation of Nrf-2 and inhibition of NF-κB.

## Discussion

Long regarded as proinflammatory molecules, prostaglandins (PGs) also have anti-inflammatory effects. $PGJ_2$ and/or 15D-$PGJ_2$ suppress LPS have been shown to induce expression of a variety of inflammatory molecules in different cells and in animal models of inflammation [17,18,20,21]. The mechanisms by which prostaglandins mediate their anti-inflammatory effects have not been fully elucidated. Recent reports suggest that $PGD_2$ and $PGJ_2$ may modulate the expression of cell surface receptors such as TLR2 in microglial cells [31]. TREMs are a class of superimmunoglobulin receptors which have an important role in innate and adaptive immune response. In particular, the role of TREM-1 in sepsis is intriguing [1–5]. Blockade of TREM-1 has shown to improve survival in animal models of sepsis [10–12]. The ligand for TREM-1 has not been identified, and the regulation and expression of these receptors is not fully characterized [9]. In the present studies, we investigated the role of prostaglandin $D_2$ and its products $PGJ_2$ and 15-d$PGJ_2$ on the expression of TREM-1 in response to LPS in macrophage cells. Our study, for the first time, shows that $PGD_2$ and $PGJ_2$ inhibit the expression of TREM-1 in macrophages. These effects are mediated at higher doses and are independent of the DP2 receptors and PPARγ. Thus, we further investigated the mechanisms by which $PGJ_2$ mediates these effects. We show that the inhibition of TREM-1 is mediated by activation of Nrf2 and inhibition of NF-κB. These data provide novel insights into the role of $PGJ_2$ as anti-inflammatory molecules.

The prostanoids are a family of lipid mediators that are produced when arachidonic acid is released from the plasma membrane by phospholipases and by the action of cyclooxygenase on a 20-carbon unsaturated fatty acid, arachidonic acid [13,14]. Prostanoids are generated widely in response to diverse stimuli and play important roles in normal physiology and disease including inflammation, smooth muscle tone, hemostasis, thrombosis, parturition, and gastrointestinal secretion [14]. $PGD_2$ is formed in several tissues by two distinct $PGD_2$ synthases (PGDS) selectively expressed in different cell types. The cyclopentanone prostaglandins of the J series are synthesized by the nonenzymatic dehydration of prostaglandin $D_2$. $PGD_2$ mediates its effects by specific G protein-coupled seven transmembrane receptors, whereas there are no specific transmembrane receptors identified for $PGJ_2$ [20,21,38,39]. Instead, $PGJ_2$ exerts its effects through interaction with intracellular targets. To date, numerous studies have reported various effects of $PGJ_2$ in different cell types including pro- and anti-inflammatory effects.

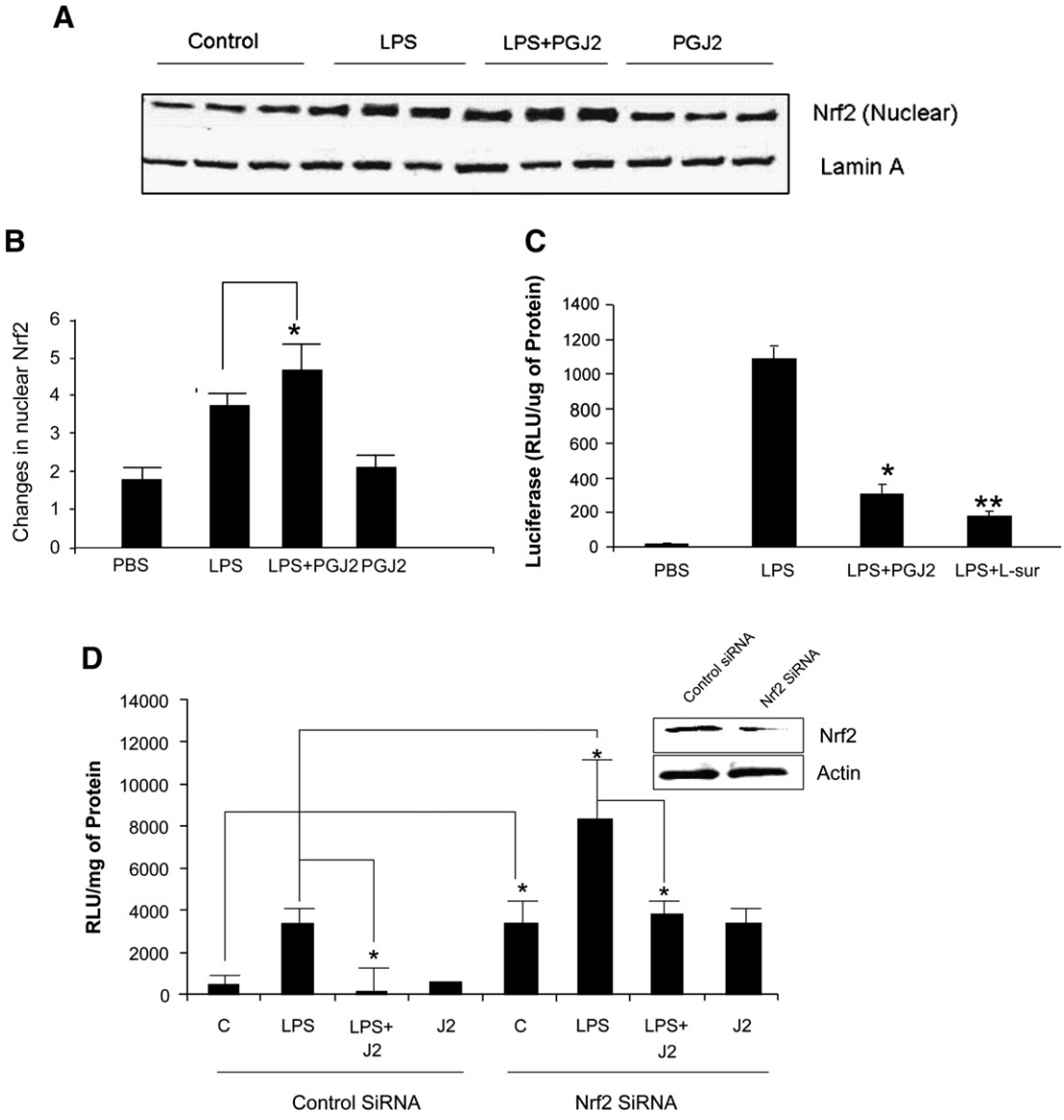


**Fig. 5 – $PGJ_2$ activates the translocation of Nrf2 in response to LPS. RAW cells were pretreated with $PGJ_2$ (10 μmol) 30 min before LPS (100 ng/ml) treatment. After 4 hours, Nrf2 expression was determined from the nuclear proteins by (A) Western blot analysis. (B) Densitometry confirmed that there was a significant increase in the nuclear Nrf2 in cells that were treated with LPS and $PGJ_2$. (C) TREM-1 luciferase reporter cells that were treated with LPS and $PGJ_2$ showed a significant decrease in luciferase activity similar to that with treatment of L-sulforaphane (10 μmol) which is an activator of Nrf2. (D) Repression of Nrf2 by SiRNA significantly increase the TREM-1 luciferase activity at basal level as well as after LPS stimulation. Data are represented as mean ± SE for three separate experiments with at least $n = 3$ for each experiment; $^*p < 0.05$ between comparison mediated using ANOVA.**

The role of prostaglandins in the innate immune responses is less well defined. To our knowledge, this is the first study to demonstrate that $PGD_2$, $PGJ_2$, and 15-d$PGJ_2$ inhibit LPS induced expression of TREM-1. TREM-1 is a superimmunoglobulin receptor present on the macrophage and neutrophils that is involved in the triggering of the inflammatory response [1–5]. The natural ligand for TREM-1 is unknown; however, Toll-like receptor agonists stimulate TREM-1 expression and function. TREM-1 activation amplifies the Toll-like receptor-initiated responses to invading pathogens allowing the secretion of proinflammatory chemokines and cytokines [6]. TREM-1 expression is upregulated in patients with microbial sepsis and mice with LPS-induced septic shock. TREM-1 activation amplifies the Toll-like receptor initiated responses to invading pathogens allowing the secretion of proinflammatory chemokines and cytokines [5,6]. In animal models of LPS-induced sepsis, blockade of TREM-1 improves survival. Since TREM-1 amplifies the inflammation induced by microbial infection and blockade of TREM-1 improves survival in animal models of sepsis, it could be a potential therapeutic target. Our study provides a novel approach to blockade of TREM-1 by $PGD_2$ and $PGJ_2$. Similar type of study by Murakami et al. [40] showed LPS induced expression of TREM-1 is enhanced by $PGE_2$.

In this study, we also investigated the potential mechanisms by which $PGJ_2$ modulates the expression of TREM-1. 15-d$PGJ_2$ is recognized as the endogenous ligand for the intranuclear receptor peroxisome proliferator-activated receptor γ which is a member of

the steroid receptor superfamily with a variety of cellular functions [20,21]. This property is responsible for many of the 15-d$PGJ_2$ anti-inflammatory functions. We thus investigated if the inhibition of TREM-1 was PPARγ mediated. Treatment of cells with PPARγ agonists such as thiazolidinediones did not block the expression of TREM-1 in macrophages, indicating that these effects are PPARγ independent.

$PGJ_2$ also mediates its effects through modulation of transcription factors such as NF-κB and Nrf2 [20,38]. We therefore investigated if the inhibition of TREM-1 by $PGJ_2$ in macrophages is mediated by transcriptional modulation. Using a reporter cell line of TREM-1, we have shown that the expression of TREM-1 gene is modulated by $PGJ_2$ through inhibition of NF-κB and activation of Nrf-2. Activation of the NF-κB signaling cascade results in degradation of the inhibitory protein IκBα, allowing translocation of p65 into nucleus. We showed that the nuclear translocation of p65 is inhibited by $PGJ_2$. Together, these data suggest that the inhibition of TREM-1 is mediated by modulation of NF-κB activation. We also confirmed that IκBα degradation is prevented by $PGJ_2$ which is the mechanism by which $PGJ_2$ inhibits the activation of NF-κB. We have previously shown that p65 binds to the TREM-1 promoter in response to LPS in macrophages and regulates the gene. Thus, the inhibitory effects of $PGJ_2$ on TREM-1 expression are at least in part mediated by NF-κB activation.

TREM-1 gene is also regulated by Nrf-2 [37]. Nrf2 (NF-E2-related factor-2) is a member of the leucine zipper family of transcription factors that has been identified as a pivotal factor that regulates the expression of antioxidant enzymes through induction of antioxidant responsive elements. Thimmulappa et al. [37] showed that the expression of TREM-1 is highly upregulated in Nrf-2 knockout mice after nonlethal intraperitoneal injection of LPS. We therefore hypothesized that some of the effects of $PGJ_2$ on TREM-1 expression may be mediated through modulation of Nrf-2 by $PGJ_2$. By using the TREM-1 luciferase reporter cells, we have shown that activation of Nrf-2 inhibits the expression of TREM-1. We further showed that $PGJ_2$ leads to increased activation of Nrf-2. In addition, by knocking down Nrf2, we have shown that the inhibitory effect of $PGJ_2$ on TREM-1 expression is reduced. Together, these data indicate that $PGJ_2$ regulates TREM-1 expression by modulation of Nrf-2 and NF-κB.

In conclusion, our study provides novel insights into the role of $PGD_2$ and $PGJ_2$ in innate response and is the first evidence that these lipid mediators inhibit the expression of TREM-1 on macrophages which amplifies the TLR-mediated inflammation. The present study also shows that the inhibition of TREM-1 by $PGJ_2$ is mediated by activation of Nrf2 and inhibition of NF-κB. Blockade of TREM-1 improves survival in animal models of sepsis; however, specific therapeutic methodologies to inhibit TREM-1 have not been developed. Understanding the role of prostaglandins in diseases has led to development of COX blockers and prostaglandin analogues as treatment for a variety of medical conditions. Our data suggest that prostaglandins particularly $D_2$ and $J_2$ should be investigated as potential targets to block TREM-1 in animal models of inflammation.

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