PKCδ-IRAK1 axis regulates oxidized LDL-induced IL-1β production in monocytes

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Abstract This study examined the role of interleukin (IL)-1 receptor-associated kinase (IRAK) and protein kinase C (PKC) in oxidized LDL (Ox-LDL)-induced monocyte IL-1β production. In THP1 cells, Ox-LDL induced time-dependent secretory IL-1β and IRAK activity; IRAK4, IRAK3, and CD36 protein expression; PKCδ-JNK1 phosphorylation; and AP-1 activation. IRAK1/4 siRNA and inhibitor (INH)-attenuated Ox-LDL induced secreted IL-1β and pro-IL-1β mRNA and pro-IL-1β and mature IL-1β protein expression, respectively. Diphenyleneiodonium chloride (NADPH oxidase INH) and N-acetylcysteine (free radical scavenger) attenuated Ox-LDL-induced reactive oxygen species generation, caspase-1 activity, and pro-IL-1β and mature IL-1β expression. Ox-LDL-induced secretory IL-1β production was abrogated in the presence of JNK INH II, Tanshinone (PKC pathway inhibitor) and pro-IL-1β and mature IL-1β protein expression. Ox-LDL-induced secretory IL-1β production was dependent on CD36-induced heterodimerization of toll-like receptor (TLR)4 and TLR6 (6). Binding of Ox-LDL to CD36 was found to be the initial step that was important for TLR heterodimerization and induction of sterile inflammatory response (6). IL-1β-induced sterile inflammation is also reported during acute pancreatitis (9). In addition, IL-1β has been shown to induce sterile inflammation by regulating macrophage migration (10). Moreover, evidence for IL-β-induced sterile inflammation also comes from studies in which mice were subjected to sterile injuries (11).

Traumatic injury often induces a sterile systemic inflammatory response syndrome (SIRS) in humans, and involves

Supplementary key words oxidized low density lipoprotein • protein kinase Cδ • interleukin-1 receptor-associated kinase • inflammation

Oxidized LDL (Ox-LDL) in various acute or chronic inflammatory diseases can be an independent risk factor for cardiovascular complications (1, 2). Ox-LDL itself serves as a pro-inflammatory molecule and contributes to the generation of various inflammatory cytokines (3, 4). Elevated Ox-LDL and inflammatory response were observed in extremely obese pediatric subjects (5). Recent reports suggest that Ox-LDL can induce sterile inflammation by stimulating production of various inflammatory cytokines, including interleukin (IL)-1β (6, 7). Sterile inflammation is characterized by the recruitment of neutrophils and macrophages and production of inflammatory cytokines like IL-1β and TNF-α (8). Several exogenous agents like asbestos and silica, and endogenous stimuli like RNA, DNA, and cytokines can induce sterile inflammation (8).

In monocytic cells, Ox-LDL-induced sterile inflammation was dependent on CD36-induced heterodimerization of toll-like receptor (TLR)4 and TLR6 (6). Binding of Ox-LDL to CD36 was found to be the initial step that was important for TLR heterodimerization and induction of sterile inflammatory response (6). IL-1β-induced sterile inflammation is also reported during acute pancreatitis (9). In addition, IL-1β has been shown to induce sterile inflammation by regulating macrophage migration (10). Moreover, evidence for IL-β-induced sterile inflammation also comes from studies in which mice were subjected to sterile injuries (11).

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; CSIR, Council of Scientific and Industrial Research; DPI, diphenyleneiodonium chloride; IL, interleukin; IL-1R, interleukin-1 receptor; INH, inhibitor; IRAK, interleukin-1 receptor-associated kinase; MBP, myelin basic protein; NAC, N-acetylcysteine; Ox-LDL, oxidized LDL; PKC, protein kinase C; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SE, standard error; SIRS, systemic inflammatory response syndrome; SOFA, Sequential Organ System Failure Assessment; TLR, toll-like receptor.

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activation of the innate immune response (12). The severity of immune response is often associated with the amount of circulating cytokines present in the patient (12, 13). Incidence of multiple organ failure and mortality increases with the increasing inflammatory load (12). TLR2/4 expression on peripheral blood mononuclear cell, as well as serum TNF-α, IL-β, and IL-8, was significantly higher in SIRS patients (14).

Exposure of monocytic cells to organic dust leads to production of several inflammatory cytokines, like TNF-α and IL-6, which seems to be mediated by protein kinase C (PKC)δ, along with some other PKC isoforms (15). PKCδ is a serine-threonine protein kinase that can be activated by calcium and diacylglycerol and plays an important role in inflammation (16–18). It has been shown to be involved in sepsis (19, 20) and seems to mediate sepsis-induced lung injury (19). PKCδ also mediates high glucose-induced activation of the TLR pathway and production of inflammatory cytokines in monocytic cells (21). Interestingly, asbestos-induced peribronchiolar cell proliferation and cytokine production are attenuated in the lungs of protein kinase Cδ knockout mice (22).

The IL-1 receptor (IL-1R)-associated kinase (IRAK) family of kinases represents important mediators of innate immunity and plays a crucial role in the signaling cascade induced by the TLR/IL-1R family (17, 23–25). The IRAK family consists of four members, namely IRAK1, IRAK2, IRAK3 (IRAKM), and IRAK4. IRAK1, IRAK2, and IRAK4 positively regulate the immune response, and IRAK3 usually antagonizes their effect by disrupting the IRAK1/TNFR-associated factor 6 complex (17, 23, 25). Out of all of these kinases, IRAK1 and IRAK4 are widely studied proteins and have been proposed to be true kinases, but their kinase activity is still under investigation (17, 25).

In the present study, we hypothesized that Ox-LDL can modulate the PKC and IRAK pathways in monocytic cells to induce sterile inflammation by stimulating IL-β production. The present study demonstrates the role of PKCδ in Ox-LDL-induced sterile inflammation by directly activating the IRAK1-JNK axis for IL-1β production. This hypothesis has clinical relevance because high Ox-LDL plasma in SIRS individuals primes monocytes for IL-β overproduction by activating the PKCδ-IRAK1 axis.

**METHODS**

**Materials**

Pharmacological inhibitors (INHs) including IRAK1/4 INH, JNK INH II, Rottlerin, Go6976, and Ro-31-8220 were purchased from Calbiochem (San Diego, CA). N-acetylcysteine (NAC), myelin basic protein (MBP), protease INH cocktail, and antibodies against human IRAK1, IRAK2, IRAK3, and β-actin were procured from Sigma (St. Louis, MO). IRAK1, IRAK4, phospho-IRAK, phospho-PKCδ, and PKCδ antibodies were also procured from Cell Signaling Technology (Danvers, MA). Human anti-phospho[NK and anti-total [NK were from Millipore (Billerica, MA). Human anti-CD36 (SMb); anti-TLR2, -4, and -6; Tashinone Ia; PKCδ siRNA; TLR2, -4, and -6 siRNA; and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). IRAK siRNA was purchased from Santa Cruz Biotechnology, Inc. and Dharmaco (Chicago, IL). Anti-CD36 (FA6-152) antibody was procured from Abcan (Cambridge, MA). ECL reagent was from GE Healthcare (USA). Tissue culture reagents were procured from Invitrogen (USA). All other fine chemicals used in the study were procured from Sigma.

**Study population**

In the present study, 74 healthy volunteers and 41 SIRS patients were recruited and evaluated for circulating Ox-LDL and plasma IL-1β. Ethical approval was taken from the institutional ethics committee (human research) of CSIR-Central Drug Research Institute, King George’s Medical University, and Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, and written consent was obtained from the patients’ surrogates. Kin, caretakers, or guardians consented on the behalf of participants whose capacity to consent was reduced and the institutional committee approved this consent procedure. Ethical guidelines were in agreement with the Declaration of Helsinki. Critically ill patients were admitted to the intensive care unit of King George’s Medical University, Lucknow, and SIRS was diagnosed by the presence of two or more of the following criteria: temperature >38°C or <36°C; heart rate >90 beats/min; respiratory rate >20 breaths/min or PaCO₂ <32 mm Hg; and an alteration in the white blood cell count >12,000 cells/μL. Inclusion criteria for the patients enrolled in the present study were patients of trauma, postoperative surgical patients, and patients with respiratory illness (COPD, asthma). The exclusion criteria for SIRS patients were patients older than 80 years, cardiac failure (class III or IV), liver insufficiency, and the presence of HIV, HBV, HCV, infection, or cancer. Disease severity index [Acute Physiology and Chronic Health Evaluation (APACHE) II scores and Sequential Organ System Failure Assessment (SOFA) scores] (26), along with other clinical pathology tests, were monitored at the time of admission in the intensive care unit. Among the SIRS patients, 68% were men and 32% were women, with a mean ± standard error (SE) age of 44 ± 5 years (Table 1). The mean ± SE age of healthy subjects was 42 ± 4 years, out of which 75% were male participants and 25% were female participants (Table 1). Blood samples were collected in tubes containing 3.8% trisodium citrate (9:1 ratio) from healthy subjects and SIRS patients with the help of a central venous catheter, and plasma was separated after centrifugation at 13,000 g for 7 min (27). Plasma was used immediately or stored at −70°C for assessment of circulating Ox-LDL level and plasma IL-1β. Repeated freezing and thawing of samples were avoided to prevent degradation of plasma Ox-LDL and IL-1β levels.

**Circulating Ox-LDL measurement**

Circulating Ox-LDL was measured using an Ox-LDL competitive ELISA kit (Mercodia AB, Uppsala, Sweden). As per manufacturer’s protocol, plasma samples were initially diluted with sample buffer. Calibrator (25 μL), control and diluted samples, along with 100 μL of assay buffer were added into appropriate wells precoated

| TABLE 1. Patient demographic characteristics |
|---------------------------------------------|
| **Patient Detail** | **Control (n = 74)** | **SIRS (n = 41)** |
| Age (years) | 42 ± 4 | 44 ± 5 |
| Male/female (n) | 56/18 | 28/13 |
| Temperature (°C) | 37 ± 0.1 | 39 ± 1 |
| Heart rate (beats/min) | 96.7 ± 2 | 124 ± 1 |
| Respiratory rate (breaths/min) | 21 ± 2 | 27 ± 4 |
| Mean arterial pressure (mm Hg) | 81 ± 5 | 91 ± 4 |
| SOFA score | — | 9.9 ± 0.7 |
| APACHE II score | — | 22 ± 1.3 |

Results are expressed as the mean ± SE.
with anti-Ox-LDL monoclonal antibody. Plates were incubated on a plate shaker (700–900 rpm) for 2 h at room temperature (23–25°C). After rinsing with wash buffer, 100 μl of enzyme conjugate was added to each well and incubated for 1 h at room temperature. After subsequent washing, 3,3′,5,5′-tetramethylbenzidine substrate was added and the developed color was measured using an ELISA reader (BioTek Instruments, Inc., USA) at a wavelength of 450 nm (28). Standard curve was prepared for each assay run using calibrators and control supplied along with the assay kit. Cu²⁺-modified LDL (50–500 ng/ml) was used as standard solution (4) to quantify the circulating plasma Ox-LDL in micrograms per milliliter for the treatment in primary monocytes isolated from healthy volunteers.

**Human monocyte isolation, THP1 cell culture, and treatments**

Primary human monocytes were isolated, as described earlier with slight modification (17, 29), from healthy donors after their informed consent. Whole blood was centrifuged at 250 g for 20 min and the upper layer (rich in platelets) platelet-rich plasma (PRP) was removed. The remaining blood was centrifuged at 650 g for 20 min and the buffy coat was collected. It was mixed with saline and subjected to dextran sedimentation. The upper layer (rich in leukocytes) was collected and centrifuged at 500 g for 5 min at room temperature. Pellets were resuspended in HBSS containing glucose. Density gradient centrifugation utilizing Percoll 1080 and 1065 was done at 700 g for 15 min and the interface layer was collected and washed with glucose HBSS. The pellet was resuspended in RPMI-1640, loaded on hyper-osmotic gradient, and the interface layer of monocytes was adhered in RPMI-1640 containing 10% FBS for 1 h and subsequently used for experiments (17, 29). Viability of cells was found to be >95%, as assessed by CD14+ cells by flow cytometry. In addition to this human monocytic cell line, THP1 was cultured in RPMI-1640 containing glucose, 10% FBS for 1 h and subsequently used for experiments (17, 29). Viability of cells was found to be >95%, as assessed by trypan blue staining, and purity of cells was found to be >95%, as assessed by CD14+ cells by flow cytometry. In addition to this human monocytic cell line, THP1 was cultured in RPMI-1640 containing 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. THP1 monocytic cells were treated for 15 min, 30 min, 1 h, 6 h, 12 h, 24 h, 48 h, and 72 h with Ox-LDL (40 μg/ml) (6, 30). As per requirement, cells were also pretreated for 1 h with different pathway INHs, their vehicle control, and antibodies at reported concentrations before Ox-LDL treatment. INHs were always compared with their vehicle control for ruling out nonspecific effects. INHs used in the present study were IRAK1/4 INH (0.3 μM), JNK INH II (10 μM), general PKC INH (Ro-31-8220, 1 μM), classical PKC INH (Go6976, 20 mM), PKC⁰ INH (Rotterlin, 2 μM), AP-1 INH (Tanshinone Ia, 1 μM), DPI (10 μM), and NAC (10 mM). DMSO (<0.1%) was used as vehicle control. Treatments with Fa6-152 and isotype control antibodies were used at 5 μg/ml.

Primary monocyes were preincubated with CD36 antibody (5 μg/ml) or with respective isotype and vehicle control for 1 h. Subsequently, monocyes were treated with 40% (v/v) plasma (4) from healthy subjects with low (6.7 ± 0.3 μg/ml) and high (26.5 ± 0.5 μg/ml) Ox-LDL and plasma from SIRS patients with low (12 ± 0.07 μg/ml) and high (32 ± 2 μg/ml) Ox-LDL (4). After respective treatments, supernatant was collected for IL-1β measurement and cell lysates were prepared for Western blotting.

**Isolation, purification, and characterization of Ox-LDL**

LDL (d = 1.019–1.063 g/ml) was isolated from the plasma of healthy volunteers by sequential ultracentrifugation (31). Ox-LDL was prepared by dialyzing the LDL in PBS overnight at 4°C. LDL protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Native LDL (0.2 mg/ml) diluted in PBS was oxidized by exposure to 5 μM CuSO₄ in PBS at 37°C for 24 h. The oxidation was terminated by addition of Na₂ EDTA (0.2 mM) and butylated hydroxytoluene (50 μM). The LDL oxidation was determined by measuring the relative electrophoretic mobility and thiobarbituric acid-reactive substances (6, 30, 32).

Endotoxin concentration in the Ox-LDL preparations was <0.1 EU/ml/mg protein, as measured by ToxinSensor chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ) (33). Samples, including human plasma, were routinely tested and excluded for endotoxin contamination.

**Assay for secretory IL-1β production**

The production of IL-1β after treatment with Ox-LDL, different pathway INHs and antibodies was measured in the media by conventional ELISA (BD OptEIA™ set human IL-1β; BD Biosciences, San Diego, CA) as described earlier (17). In brief, supernatants were collected from control and treated mononcytic cells and incubated for 2 h at room temperature in overnight capture antibody-coated ELISA plates. After incubation, wells were washed with PBS containing 0.05% Tween-20 and incubated with detection antibody followed by washing and enzyme reagent incubation. The color was developed by adding 3,3′,5,5′-tetramethylbenzidine substrate reagent set (BD Biosciences) and subsequently read at 450 nm and 570 nm on an ELISA plate reader (BioTek Instruments, Inc.). Standard IL-1β provided in the kit was used for drawing the standard and calculation of absolute IL-1β levels.

**Western and phospho blotting**

Cells were harvested after the desired treatments and lysed in lysis buffer containing 0.1M NaCl, 0.01M Tris HCl (pH 7.4), 0.001M EDTA (pH 7.4), aprotinin (1 μg/ml), phenylmethysulfonyl fluoride (100 μg/ml), pepstatin (20 μg/ml), sodium orthovanadate (2 mM), sodium fluoride (2 mM), and 1% Triton X-100. The cell extracts were clarified at 15,000 g for 5 min and protein contents were measured by using Bradford reagent. Equal amounts of lysate were boiled in Laemmli buffer and separated on a denaturing 7–10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking (5% BSA in TBST), the membranes were incubated with primary antibody against various candidate proteins like IRAKs (1:1000), phospho-IRAK1 (1:1000), phospho-JNK (1:1000), JNK (1:1000), phospho-PKCS (1:500), PKCS (1:1000), IL-1β (1:1000), CD36 (1:1000), and actin (1:2000), as per the manufacturer’s protocol. This was followed by incubation with specific HRP-conjugated secondary antibody. The specific bands were detected by enhanced chemiluminescence as described earlier (17). The relative intensities of the bands were measured by LAS 4000 and image quant software. Results were expressed as fold change in relative image quant units.

**Immunoprecipitation and in vitro kinase assay**

Cells from different experimental groups were lysed in 0.1% Nonidet P-40 lysis buffer [50 mM Tris-Cl (pH 8.0), 157 mM sodium chloride, 2 mM EDTA, 5% glycerol, and 0.1% Nonidet P-40] supplemented with 1:100 protease INH cocktail. The lysates were centrifuged at 15,500 g, supernatants were collected, and protein concentration was measured. Pre-clearing of cell lysates was performed by incubating 400 μg of cell extracts from different experimental groups with 20 μl protein A Sepharose beads (50% slurry) for 45 min at 4°C. After centrifugation at 14,000 g for 10 min, the supernatant was mixed with 2.0 μg/ml rabbit anti-IRAK1 antibody and incubated at 4°C overnight. Subsequently, 20 μl of protein A Sepharose beads (50% slurry) was mixed and further rotated for 2 h at 4°C. The protein A Sepharose beads were spun down and washed four times with lysis buffer and two times with 0.1M LiCl. The immunoprecipitates were processed for immunoblotting as desired.
IRAK1 kinase assay was performed as described earlier (17). Briefly, the immune complexes were washed with kinase assay buffer (20 mM MOPS (pH 7.2), 50 mM MgCl₂, 2 mM EGTA, and 1 mM dithiothreitol). The reaction was carried out in the presence of 5 μg of MBP substrate, 0.5 mM ATP, and 10 μg of [γ⁻³²P]-ATP for 30 min at 30°C (17). Reactions were stopped by the addition of 15 μl of 6× SDS-PAGE sample buffers and subsequently boiled. Supernatants were subjected to SDS-PAGE and transferred to PVDF membranes. Phosphorylation of the substrate was measured by autoradiography.

**AP-1 activity assay**

AP-1 activity was measured at different time points of Ox-LDL treatment by using a commercially available ELISA kit (TransAM™ AP-1-c-Jun, Active Motif Co., Ltd., Carlsbad, CA). Nuclear extracts were prepared as per the instructions in the kits. Briefly, after treatment, mononuclear cells were collected and washed with ice-cold phosphatase INH buffer (125 mM NaF, 250 mM para-nitrophenyl phosphate, and 25 mM NaVO₃) and resuspended in 1 ml of ice-cold hypotonic glycerophosphate, 250 mM para-nitrophenyl phosphate, and ice-cold phosphatase INH buffer (125 mM NaF, 250 mM para-nitrophenyl phosphate, and 0.1 mM EDTA). The cells were allowed to swell for 15 min on ice. Fifty microliters of 10% Nonidet P-40 was added and the tube was shaken for 10 s. Cell homogenate was centrifuged for 10 s at 4°C and the supernatant (cytoplasmic fraction) was removed. The nuclear pellet was suspended in 50 μl of complete lysis buffer for 30 min on a rocking platform. The lysate was centrifuged at 15,000 × g for 10 min and the nuclear extract was used for AP-1 (c-Jun) assay after protein quantification. AP-1 was measured by loading 10 μg of nuclear extract onto a well of a 96-well microtiter plate coated with oligonucleotide 5′-TGAGTCA-3′ for 1 h. After washing three times, monoclonal antibody against c-Jun was added to the appropriate wells and incubated further for 1 h at room temperature. Anti-IgG HRP conjugate, in a volume of 100 μl, was then added and further incubated for 1 h at 25°C. Absorbance at 450 nm was measured after the addition of tetramethylbenzene solution. Absolute levels of the transcription factor were quantified by setting up standard curves with the help of reagents provided in the kit. **siRNA transfection**

Transfections were performed by using an Amaxa Nucleofector machine (Amaxa, Cologne, Germany), as described earlier (17), and in the optimized protocol for THP1 and primary monocytes as provided by the manufacturer. Briefly, 1 × 10⁶ cells in 100 μl transfection reagent provided in the kit (Cell Line Nucleofector kit V) were transfected with 3.0 μg of control, IRAK1, IRAK2, IRAK3, IRAK4, TLR2, TLR4, TLR6, CD36, or PKCδ siRNA. Nucleofector machine program V001 was used for THP1 and Y001 for primary monocytes. After transfection, cells were removed in 0.5 ml RPMI and plated in 1 ml of prewarmed medium in 6-well plates. THP1 macrophages were transfected with control, PKCδ, TLR2, TLR4, TLR6, or CD36 siRNA using Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions. Briefly, THP1 cells were differentiated with PMA (100 nM) for 24 h. Lipofectamine and siRNA (3 μg) were incubated together at room temperature for 20 min and the complex formed was added to the cells. After 18 h of transfection, Ox-LDL treatment was given for 15 min to measure PKCδ and IRAK1 phosphorylation in THP1 cells, primary monocytes, and THP1 macrophages. CD36 expression was also measured in THP1 macrophages. Secretory IL-1β was measured after 48 h of Ox-LDL treatment. Expression of recombinant green fluorescent protein (provided in the kit) and FITC-labeled control siRNA were used as markers for monitoring the transfection efficiency. Gene silencing was measured by Western blotting.

**Caspase-1 fluorometric assay**

Caspase-1 activity was assayed by using a caspase-1 fluorometric assay kit (R&D Systems, Inc., Minneapolis, MN). After various treatments, cells were collected by centrifugation at 250 g for 10 min. The kit buffer was used for cell lysis. The supernatant obtained after centrifugation at 10,000 g was used for caspase-1 assay. Total protein (200 μg) was mixed with an equal volume of 2× reaction buffer in a microplate. Reactions were initiated by the addition 5 μl of caspase-1 fluorogenic substrate (WEHD-afc). The reaction was carried out at 37°C for 2 h. Plates were read at excitation 400 nm and emission 505 nm in an LS 55 fluorescence plate reader (Perkin Elmer, Waltham, MA). The results were expressed as fold increase in caspase-1 activity of induced cells over that of noninduced cells (34).

**Expression of IL-1β by real-time PCR**

Total RNA from THP1 cells was extracted by using TRI reagent. For quantitative (q)RT-PCR analysis of IL-1β, cDNA was synthesized from 1 μg of RNA by using a commercially available DNA synthesis kit (Fermentas RevertAid first strand DNA synthesis kit, Lithuania). Real-time PCR was done in a 25 μl reaction by using Maxima® CYBR Green/ROX qPCR Master Mix (2×) (Fermentas Life Sciences, Lithuania), IL-1β (forward primer-5′CTCTCTCACCTCCTCCTAC, reverse primer-ACACTGCTACCTTGGCC), actin (forward primer-AACCTGGACCTGGAAGGTG, reverse primer-CGTGTGGT-GACTTGGGAGG) specific primers, and LightCycler® 480 real-time PCR system (Roche Applied Science, Mannheim, Germany). Three step cycling protocol (initial denaturation at 95°C for 10 min, 35 cycles of 15 s denaturation at 95°C, 30 s annealing at 60°C, and 30 s extension at 72°C) was used to amplify the genes (35, 36). Relative fold difference between an experimental and calibrator sample was calculated by using comparative Ct (2⁻ΔΔCt) method. Actin was used as internal standard to calculate the relative expression (37).

**Determination of intracellular reactive oxygen species**

Intracellular reactive oxygen species (ROS) generation was measured by using cell permeable indicator 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma). Briefly, THP1 cells were treated with 5 μM H2DCF-DA for 30 min and PBS washed cells were pre-treated with DPI (10 μM), NAC (10 mM), and IRAK1/4 (0.3 μM) INH for 1 h before stimulation with Ox-LDL (40 μg/ml, 1 h) (38). ROS-dependent fluorescence was measured by a microplate reader at excitation 480 nm and emission 530 nm.

**Statistical analysis**

Results are expressed as the mean ± SE. The data obtained from control and SIRS patient samples were analyzed by Kolmogorov-Smirnov test for normal distribution. The Pearson product-moment correlation coefficient (r) was used to establish the association of the two variables. Unpaired Student’s t-test was used to calculate the significant difference between two groups. The significance of difference between the means of three or more groups was determined by one-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test. P ≤ 0.05 was considered statistically significant. Blots represent one of three or more similar experiments. All statistical analyses were performed with the GraphPad Prism 5.0 program (GraphPad Inc., San Diego, CA).

**RESULTS**

Ox-LDL induces IL-1β production and activation of IRAK pathway

THP1 monocytic cells were treated with Ox-LDL (40 μg/ml) for the indicated time points and secretory PKCδ mediates Ox-LDL-induced IL-1β production 1229
IL-1β was measured in the supernatant (Fig. 1A). A time-dependent increase in IL-1β production was observed after Ox-LDL treatment (Fig. 1A). The treatment with Ox-LDL for 6 h significantly increased secreted IL-1β (~4-fold), and this was further increased with time reaching maximum at 48 h (~25-fold). At 72 h, the secreted IL-1β was not significantly different from that observed at 48 h (Fig. 1A). However, LDL (40 μg/ml) treatment for 72 h had no effect on IL-1β production (Fig. 1A). Because the IRAK family of proteins mediates innate immune response generated by the TLR/IL-1R receptor (39), activation of different IRAK proteins was studied. We monitored time-dependent expression of all IRAK isoforms up to 72 h in THP1 monocytes, after Ox-LDL treatment (Fig. 1B, C). A moderate but significant increase in expression of IRAK1 was observed after 15 and 30 min of Ox-LDL stimulation without any further increase at later time points (Fig. 1B). Furthermore, we also observed increased IRAK3 expression in a time-dependent manner up to 72 h of Ox-LDL stimulation, but no change was found in expression of IRAK2 (Fig. 1B, C). No difference in expression of IRAK2 was observed after Ox-LDL stimulation. Expression of IRAK3 was increased in a time-dependent manner up to 72 h of Ox-LDL stimulation (Fig. 1B, C). Expression of IRAK4 was also significantly increased at 30 min of Ox-LDL stimulation, and this was maximum at 24 h. A decrease in IRAK4 expression was observed at 48 and 72 h of Ox-LDL stimulation, but this was still significantly more than the control levels (Fig. 1B, C). Because it is reported that IRAK1 is downstream to IRAK4 and relays the signal forward (25), we performed IRAK1 kinase assay to ascertain the activation of the IRAK4-IRAK1 signaling pathway. A significant

![Fig. 1. Ox-LDL induces time-dependent IL-1β production and IRAK activation. THP1 monocytes were stimulated with Ox-LDL (40 μg/ml) for different times and the following parameters were measured. A: IL-1β production in culture media by ELISA (in triplicate, n = 8). B: IRAK1, -2, -3, and -4 expressions by Western blotting (n = 8). C: Densitometric quantification of the expressed IRAK isoforms in relative image quant units, fold of control (n = 8). D: IRAK1 kinase activity in an in vitro kinase assay in which cells were lysed and immunoprecipitated IRAK1 was subjected to kinase assay in the presence of 32P-PATP and MBP as substrate (n = 3). Values represent the mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.](image-url)
PKCδ mediates Ox-LDL-induced IL-1β production

**Fig. 2.** IRAK1 and IRAK4 mediate IL-1β production in THP1 cells. Ox-LDL-induced (40 μg/ml) IL-1β production at 48 h in THP1 cells was measured in triplicate after pretreatment with IRAK1/4 INH (0.3 μM, n = 6) (A), IRAK1 siRNA (3 μg, n = 4) (B), IRAK2 siRNA (3 μg,
To determine the role of each IRAK isoform in Ox-LDL-induced IL-1β production, isoform-specific siRNAs were used. A significant reduction in IRAK1, -2, -3, and -4 expression was observed on treatment with their specific siRNA (Fig. 2B–E). IRAK1- and IRAK4-specific siRNA significantly inhibited Ox-LDL-induced secretory IL-1β, while no change was observed with IRAK2 and IRAK3 siRNA (Fig. 2B–E).

IRAK1/4 regulates Ox-LDL-induced IL-1β transcription

Because IL-1β production is regulated at multiple levels, including gene transcription, translation, and processing, expression of IL-1β at mRNA level was measured by real-time RT-PCR and at protein level by Western blotting.
For assessing the processing of pro-IL-1β into mature IL-1β, caspase-1 activity was also evaluated by a fluorometric assay. A significant induction in IL-1β mRNA (~6.5-fold) was observed after Ox-LDL treatment (Fig. 3A). A significant reduction (~3-fold) in IL-1β mRNA was observed in cells that were pretreated with IRAK1/4 INH and subsequently stimulated with Ox-LDL (Fig. 3A). Ox-LDL stimulation also induced ROS generation (~1.9-fold) in THP1 cells, and this was reduced by DPI (~1.4-fold) and NAC (~1.8-fold) (Fig. 3B). Induction in pro-IL-1β (~2.5-fold) and IL-1β (~3.6-fold) protein expression was observed after Ox-LDL stimulation, and this was significantly attenuated in the presence of DPI (~2- and 1.8-fold, respectively), NAC (1.7- and 1.5-fold, respectively), and IRAK1/4 INH (~1.9- and 1.8-fold, respectively) (Fig. 3C). Caspase-1 activity was increased (~1.5-fold) upon Ox-LDL stimulation (Fig. 3D). Pretreatment of DPI and NAC significantly reduced (~1.5- and 1.6-fold, respectively) Ox-LDL-induced caspase-1 activation (Fig. 3D). However, no change in caspase-1 activity was observed in IRAK1/4 INH pretreated cells that were stimulated with Ox-LDL (Fig. 3D).

Involvement of the JNK1-AP-1 axis in Ox-LDL-induced IL-1β production

Because downstream signaling of IRAK involves the JNK pathway (40), we performed phospho-JNK blotting in THP1 lysates obtained after Ox-LDL stimulation for different time points. An initial activation of JNK1 (~2 to 4-fold) at 15 and 30 min of Ox-LDL treatment was observed, which subsided at later time points (Fig. 4A). Interestingly, specific activation of JNK1 was observed, but there was no significant increase in JNK2 phosphorylation after Ox-LDL treatment (Fig. 4A). Because further downstream signaling of JNK involves AP-1-induced gene transcription, we therefore evaluated nuclear AP-1 DNA binding activity by using a TransAM™ AP-1 c-Jun ELISA kit (Fig. 4B). Ox-LDL

![Graphs](image_url)

**Fig. 4.** Ox-LDL-induced IL-1β production is JNK1-AP-1 dependent. A: JNK activation was measured after Ox-LDL (40 µg/ml) treatment for various times by probing the blots with activated phospho-JNK antibody that recognizes both phospho-JNK1 and phospho-JNK2. Blots were also probed with total JNK and β-actin antibody (n = 8). B: AP-1 activity was measured in nuclear extracts of THP1 monocytes stimulated with Ox-LDL for different times by using a TransAM™ AP-1-c-Jun kit (in triplicate, n = 4). C: Secretory IL-1β was measured at 48 h of Ox-LDL stimulation after pretreatment with JNK INH II (10 µM) and Tanshinone Ila (1 µM) (in triplicate, n = 5). Blots represent one of eight similar experiments. Values represent mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 versus control; ###P < 0.001 versus Ox-LDL alone.
induces time-dependent activation of AP-1 (~2 to 3-fold) from 15 min to 24 h. Maximum induction was observed at 30 min (~3-fold) of Ox-LDL stimulation, which decreases subsequently (Fig. 4B). Pretreatment with JNK and AP-1 INHs significantly reduced secreted IL-1β, indicating the positive role of the JNK-AP-1 axis in Ox-LDL-induced IL-1β production (Fig. 4C).

**PKCδ mediates Ox-LDL-induced IRAK1 activation and IL-1β production**

Previous reports suggest a crucial role of PKC in IL-1β production from monocytes (18). To evaluate the role of various PKC isoforms in secretory IL-1β production, experiments were carried out in the presence of different classes of PKC INHs (Fig. 5A). Ox-LDL-induced IL-1β production was measured in the presence of general (Ro-31-8220) and classical (Go6976) PKC INHs. The Ro-31-8220 and Go6976 significantly reduced Ox-LDL-induced secretory IL-1β production (Fig. 5A). More importantly, PKCδ-specific INH Rottlerin also significantly reduced Ox-LDL-induced IL-1β production (Fig. 5A). Previous studies have also suggested a role of PKCδ in IL-1β production from monocytes (18). On expected lines, we did see a time-dependent activation of PKCδ after Ox-LDL treatment (Fig. 5B). PKCδ activation was observed starting from 15 min up to 72 h and activation was maximum (~5-fold) at 12 h, confirming that Ox-LDL treatment activates PKCδ (Fig. 5B).

PKCδ-specific siRNA significantly inhibited Ox-LDL-induced IL-1β production (Fig. 5C). To test whether PKC and specific isoform δ feeds into the IRAK pathway, IRAK1 kinase assay was performed in THP1 lysates obtained after Ox-LDL, Ro-31-8220, Go6976, and Rottlerin treatment.

![Image of IRAK1 assay](image.png)

Fig. 5. IRAK1 mediates PKCδ-induced IL-1β production. A: Secretory IL-1β was measured in culture supernatant of THP1 monocytes at 48 h of Ox-LDL (40 μg/ml) stimulation after pretreatment with Go6976 (20 nM), Ro-31-8220 (1 μM), and Rottlerin (2 μM) (in triplicate, n = 4). B: Phosphorylation of PKCδ in THP1 cells was measured at various time points by Western blotting. Cell extracts were resolved on SDS-PAGE and, after transfer to PVDF membrane, were probed with a phospho antibody that recognizes activated PKCδ. At the same time, expression of total PKCδ and β-actin were monitored by probing the blots with specific antibodies (n = 3). C: Bar graph representing IL-1β level in the supernatant obtained from Ox-LDL-stimulated THP1 monocytes treated with control siRNA or PKCδ siRNA (3 μg, in triplicate, n = 4). D: IRAK1 kinase activity measured by an in vitro kinase assay at 30 min of Ox-LDL stimulation after pretreatment with Go6976, Ro31-8220, and Rottlerin. Cells were lysed and immunoprecipitated. IRAK1 was subjected to kinase assay in the presence of [γ-32P]ATP and MBP as substrate (n = 3). E: Bar graph represents fold change in the expression of phospho-IRAK1 in Ox-LDL-stimulated THP1 monocytes with control siRNA or PKCδ siRNA treatment (n = 3). Blots represent one of three similar experiments. Values represent mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 versus control; #P < 0.05, ###P < 0.001 versus Ox-LDL alone.
PKCδ-IRAK1 axis activates the JNK1-AP-1 pathway during Ox-LDL-induced IL-1β production

Although we did observe activation of the PKCδ-IRAK1 and JNK-AP-1 axis during Ox-LDL-induced IL-1β production, experiments were performed to test whether the PKCδ-IRAK pathway feeds into the JNK-AP-1 axis during Ox-LDL-induced IL-1β production. Ox-LDL-induced JNK-AP-1 axis activation was evaluated in the presence of Rottlerin and IRAK1/4 INH (Fig. 6A). Significant inhibition in JNK phosphorylation was observed in the presence of these INHs and PKCδ siRNA, thus indicating that the PKCδ-IRAK1 axis feeds into the JNK pathway. Because AP-1 inhibition by Tanshinone IIa also inhibits Ox-LDL-induced IL-1β production, we determined the AP-1 level at 30 min of Ox-LDL stimulation in Rottlerin, IRAK1/4 INH, JNK INH II, and PKCδ siRNA pretreated THP1 cells (Fig. 6C). Significant inhibition in Ox-LDL-induced AP-1 activity by these INHs and PKCδ siRNA indicates that PKCδ-induced IL-1β production involves the PKCδ-IRAK1-JNK-AP-1 axis. In primary human monocytes also, Ox-LDL induced time-dependent PKCδ phosphorylation (Fig. 6D).

PKCδ mediates Ox-LDL-induced IL-1β production
Moreover, treatment with TLR6, TLR4, TLR2, and CD36 siRNA significantly prevented Ox-LDL-induced PKC\(\beta\) phosphorylation (\(\sim\)1.5-, 1.5-, 1.7-, and 1.3-fold, respectively; Fig. 7A), IRAK1 activation (\(\sim\)1.6-, 1.5-, 1.3-, and 1.3-fold, respectively; Fig. 7B), and IL-1\(\beta\) production (\(\sim\)1.4-, 1.2-, 1.3-, and 1.3-fold, respectively) in THP1 cells (Fig. 7C).

Further, we explored the role of CD36 and TLRs in Ox-LDL-induced PKC\(\beta\) and IRAK1 activation and IL-1\(\beta\) production in THP1 monocyte-derived macrophages. Ox-LDL enhanced PKC\(\beta\) phosphorylation (\(\sim\)3.3-fold, supplementary Fig. IIIA), IRAK1 phosphorylation (\(\sim\)2.8-fold, supplementary Fig. IIIB), and IL-1\(\beta\) production (\(\sim\)14-fold, supplementary Fig. IIIC) in THP1 macrophages (supplementary Fig. III).

Treatment with TLR6, TLR4, TLR2, and CD36, and PKC\(\beta\) siRNA significantly reduced respective protein expression (\(\sim\)1.4-, 1.5-, 1.3-, and 1.5-fold, respectively; supplementary Fig. IIA–D), from as early as 5 min of Ox-LDL treatment, and a significant increase was observed from 15 min of treatment. The increase in PKC\(\beta\) phosphorylation was sustained until the last point of analysis. Ox-LDL significantly enhanced IL-1\(\beta\) production in primary human monocytes, while LDL had no significant effect (Fig. 6E). Rottlerin (Fig. 6E) and PKC\(\beta\) siRNA (Fig. 6F) pretreatment significantly attenuated Ox-LDL-induced IL-1\(\beta\) production in these cells as well (Fig. 6E and Fig. 6F, respectively).

**Role of CD36 and TLR in Ox-LDL-induced IL-1\(\beta\) production**

To explore the involvement of CD36 and TLRs in Ox-LDL-induced IL-1\(\beta\) production, THP1 cells were pretreated with TLR6, TLR4, TLR2, and CD36 siRNA, and subsequently stimulated with Ox-LDL.

TLR6-, TLR4-, TLR2-, and CD36-specific siRNA significantly reduced respective protein expression (\(\sim\)1.4-, 1.5-, 1.3-, and 1.5-fold, respectively; supplementary Fig. IIA–D).

**Fig. 7.** TLR and CD36 mediate PKC\(\beta\) and IRAK1 activation and IL-1\(\beta\) production in THP1 cells. THP1 cells were treated with control, TLR6, TLR4, TLR2, or CD36 siRNA for 18 h. Total and phosphorylated PKC\(\beta\) (A) and IRAK1 (B) were measured after 15 min of Ox-LDL stimulation by immunoblotting (n = 3). C: IL-1\(\beta\) level was measured in the supernatant after Ox-LDL treatment for 48 h (in triplicate, n = 3). Blots represent one of three similar experiments. Values represent mean ± SE. \(^*\)P < 0.05, \(^{**}\)P < 0.01, \(^{***}\)P < 0.001 versus control siRNA.
IRAK1 activation (≈1.6-, 1.6-, 2-, 2-, and 1.3-fold, respectively; supplementary Fig. VB), and IL-1β production (≈1.4-, 1.3-, 1.3-, 1.6-, and 1.3-fold, respectively; supplementary Fig. VC) in THP1 macrophages. Interestingly, PKCδ siRNA significantly reduced Ox-LDL-induced CD36 upregulation, indicating a positive feedback of the kinase on the receptor (supplementary Fig. VD).

**Elevated Ox-LDL and IL-1β in SIRS patients**

Ox-LDL and IL-1β were measured in the plasma of healthy subjects (n = 74) and SIRS patients (n = 41). Patient demographic characteristics including heart rate, mean arterial pressure, and disease severity scores (SOFA and APACHE II) are listed in Table 1. A significant increase in the circulating Ox-LDL (P < 0.001; Fig. 8A) and IL-1β (P < 0.001; Fig. 8B) levels was observed in SIRS patients when compared with healthy controls. Because both Ox-LDL and IL-1β were augmented in SIRS patients, in order to examine any correlation between them, we performed Pearson correlation coefficient analysis in both healthy subjects and SIRS patients. A positive correlation between Ox-LDL and IL-1β was observed both in healthy subjects (r = 0.7, P < 0.0001; Fig. 8C) and SIRS patients (r = 0.58, P < 0.0001; Fig. 8D). The positive correlation between

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

![Graph E](image5.png)

![Graph F](image6.png)

**Fig. 8.** Plasma Ox-LDL and IL-1β augmented in SIRS patients. Plasma analysis was done in healthy and SIRS individuals. Bar diagrams representing Ox-LDL (A) and IL-1β (B). To analyze the association between Ox-LDL and IL-1β, the Pearson product-moment correlation coefficient (r) was used. Line graphs represent correlation between Ox-LDL and IL-1β in healthy subjects (n = 74) (C) and SIRS patients (n = 41) (D). E: Bar graph representing Ox-LDL-LDL ratio in healthy and SIRS individuals. F: Line graph representing correlation between Ox-LDL-LDL ratio and IL-1β in SIRS patients. Values represent mean ± SE. *P < 0.05, ***P < 0.001 versus healthy subjects.
SIRS plasma with enhanced Ox-LDL primes monocytes for PKCδ-IRAK1 hyper-phosphorylation and IL-1β overproduction

Because IL-1β and Ox-LDL increase showed a positive correlation in both healthy subjects and SIRS patients, and PKCδ and IRAK are known to modulate IL-1β production from human monocytes (17, 18), the dose-dependent effect of Ox-LDL on phospho-PKCδ, phospho-IRAK1, and IL-1β was monitored by treating primary monocytes with control and SIRS plasma containing low and high levels of Ox-LDL.

Monocytes from healthy volunteers were treated with 40% plasma (v/v) from healthy or SIRS patients containing low \([6.7 ± 0.3 \mu g/ml \text{ (control)} \text{ and } 12 ± 0.07 \mu g/ml \text{ (SIRS)}\), respectively] and high \([26.5 ± 0.5 \mu g/ml \text{ (control)} \text{ and } 32 ± 2 \mu g/ml \text{ (SIRS)}\), respectively] amounts of Ox-LDL with or without Ox-LDL receptor CD36 FA6 antibody and its isotype control.

Plasma from healthy subjects containing low or high Ox-LDL dose-dependently induced PKCδ phosphorylation (~1.3- and ~2-fold, respectively) (Fig. 10A), IRAK1 activation (~1.5- and ~2-fold, respectively) (Fig. 10B), and IL-1β production (~3- and ~4.2-fold, respectively) (Fig. 10C) in primary human monocytes. CD36 FA6 antibody pretreatment significantly reduced high Ox-LDL plasma-induced PKCδ phosphorylation (~2-fold; Fig. 10A), IRAK1 activation (~1.9-fold; Fig. 10B), and IL-1β production (~1.5-fold; Fig. 10C).

Similarly, plasma from SIRS patients containing low (12 ± 0.07 \mu g/ml) or high (32 ± 2 \mu g/ml) Ox-LDL dose dependently induced PKCδ phosphorylation (~2.2- and ~4.1-fold, respectively) (Fig. 11A), IRAK1 activation (~1.7- and ~2.8-fold, respectively) (Fig. 11B), and IL-1β production (~5- and ~8.5-fold, respectively) (Fig. 11C) in primary human monocytes. FA6 antibody pretreatment significantly reduced high Ox-LDL plasma-induced PKCδ phosphorylation (~2-fold; Fig. 11A), IRAK1 activation (~1.3-fold; Fig. 11B), and IL-1β production (~1.3-fold; Fig. 11C).

Both in control and SIRS subjects, the FA6 isotype control antibody had no significant effect on plasma-induced PKCδ phosphorylation, IRAK1 activation, and IL-1β production (Figs. 10, 11).

To elucidate the role of CD36 and TLRs in plasma Ox-LDL-induced IL-1β production, human primary monocytes
PKC\(\text{\textregistered}/\text{H9254}\) mediates Ox-LDL-induced IL-1\(\text{\beta}/\text{H9252}\) production during Ox-LDL-induced IL-1\(\text{\beta}/\text{H9252}\) production. Because Ox-LDL treatment induces inflammation and subsequent cholesterol accumulation causes cell death (41, 42), we have used an optimal concentration that induces significant IL-1\(\text{\beta}/\text{H9252}\) production along with minimal cell death; this has been routinely used by other investigators as well (6, 30).

A time-dependent increase in Ox-LDL-induced IL-1\(\text{\beta}/\text{H9252}\) production and IRAK1 kinase activity indicated that there is a positive correlation between the two. IRAK1 activation preceded a significant increase in IL-1\(\text{\beta}/\text{H9252}\) production, thus indicating its role in the production of the inflammatory cytokine. A significant increase in IRAK4 expression was also observed, thus confirming the induction of IRAK1 and IRAK4 during Ox-LDL-induced IL-1\(\text{\beta}/\text{H9252}\) production. A time-dependent increase in IRAK3 can explain the saturating levels of IL-1\(\text{\beta}/\text{H9252}\) observed at later time points. IRAK3 can negatively regulate a positive inflammatory response (43).

**DISCUSSION**

In the present study, we have evaluated the role of the PKC and IRAK kinase families and associated signaling events during Ox-LDL-induced IL-1\(\text{\beta}/\text{H9252}\) production. Because Ox-LDL treatment induces inflammation and subsequent cholesterol accumulation causes cell death (41, 42), we have used an optimal concentration that induces significant IL-1\(\text{\beta}/\text{H9252}\) production along with minimal cell death; this has been routinely used by other investigators as well (6, 30).

A time-dependent increase in Ox-LDL-induced IL-1\(\text{\beta}/\text{H9252}\) production and IRAK1 kinase activity indicated that there is a positive correlation between the two. IRAK1 activation preceded a significant increase in IL-1\(\text{\beta}/\text{H9252}\) production, thus indicating its role in the production of the inflammatory cytokine. A significant increase in IRAK4 expression was also observed, thus confirming the induction of IRAK1 and IRAK4 during Ox-LDL-induced IL-1\(\text{\beta}/\text{H9252}\) production. A time-dependent increase in IRAK3 can explain the saturating levels of IL-1\(\text{\beta}/\text{H9252}\) observed at later time points. IRAK3 can negatively regulate a positive inflammatory response (43).
IL-1β production can be regulated at several levels, including transcription, translation, processing, and secretion (44). In the present study, Ox-LDL induces IRAK1-dependent IL-1β transcription because significant inhibition in pro-IL-1β transcription was observed with IRAK1/4 INH. Accumulation of cholesterol crystals in the monocytes is known to increase the caspase-1 activity via activating the NLRP3 inflammasome (45). Recently, it was shown in macrophages that Ox-LDL induces IL-1β production by stimulating IL-1β transcription and also processing by activating the NLRP3 inflammasome-caspase-1 pathway (7). However, in the present study, caspase-1 activation seems to be independent of the IRAK pathway. This can be explained by the fact that production of IL-1β involves two steps: 1) TLR-induced transcription of IL-1β to form pro-IL-1β; and 2) inflammasome-induced activation of caspase-1, which then processes the pro-IL-1β to form the mature IL-1β. In the present study, Ox-LDL induced ROS generation and caspase-1 activity. Because Ox-LDL-induced pro-IL-1β and mature IL-1β protein expression were significantly attenuated by free radical scavenger NAC and NADPH oxidase INH DPI, it is quite possible that ROS play a role in IL-1β processing. Earlier studies have also shown that free radicals mediated caspase-1 activation and IL-1β production in THP1 monocytes and macrophages after Ox-LDL stimulation (7, 46).

Upstream positioning of IRAK in the JNK pathway has been done earlier (25), and in the present study also, the IRAK1/4 INH significantly attenuated Ox-LDL-induced JNK1 phosphorylation. Previous reports suggest a role of the JNK-AP-1 axis in IL-1β production (17). Ox-LDL seems to induce JNK1-specific IL-1β production because JNK2 phosphorylation was unaffected by Ox-LDL treatment. A simultaneous increase in Ox-LDL-induced AP-1 activity indicates that JNK1-mediated effects are transduced through AP-1. Because Ox-LDL-induced IL-1β production was significantly attenuated in the presence of the JNK-specific and AP-1 INHs, it can be speculated that the JNK1-AP-1
PKC\textsubscript{\(\alpha\)} mediates Ox-LDL-induced IL-1\(\beta\) production in primary human monocytes. Primary human monocytes were treated with control, TLR6, TLR4, TLR2, or CD36 siRNA for 18 h. Total and phosphorylated PKC\textsubscript{\(\alpha\)} (A) and IRAK1 (B) were measured after 15 min of plasma containing high Ox-LDL (from SIRS patients) stimulation by immunoblotting (\(n = 3\)). C: Secreted IL-1\(\beta\) was measured in the supernatant after high Ox-LDL-containing plasma (from SIRS patients) treatment for 48 h (in triplicate, \(n = 3\)). Blots represent one of three similar experiments. Values represent mean ± SE. \#\(P < 0.05\), ##\(P < 0.01\), ###\(P < 0.001\) versus control siRNA.

Fig. 12. TLR and CD36 mediate PKC\textsubscript{\(\alpha\)} and IRAK1 activation and IL-1\(\beta\) production in primary human monocytes. Earlier reports also demonstrate the role of AP-1 in IL-1\(\beta\) transcription (47). The IRAK1/4 and JNK INHs, alone or in combination, produced similar inhibitions in AP-1 activity, indicating that they are in the same pathway for IL-1\(\beta\) production.

A recent report also shows that PKC\(\delta\) mediates high glucose-induced sterile inflammatory response by upregulating nuclear factor kappa B and inflammatory cytokine expression in monocytic cells (21). Our results indicate that both classical PKC (PKC\(\alpha\) and \(\beta\)) and PKC\(\delta\) play an important role in IL-1\(\beta\) secretion, as both general Ro-31-8220 and classical PKC INH Go6976 significantly attenuated Ox-LDL-induced IL-1\(\beta\) production. However, because Ro-31-8220, Rottlerin, and PKC\(\delta\) siRNA inhibited Ox-LDL-induced IRAK1 kinase activity, it can be concluded that PKC\(\delta\) is the main PKC mediating IRAK1-induced IL-1\(\beta\) production. PKC\(\delta\) operates upstream of the IRAK-JNK-AP-1 axis because PKC\(\delta\) siRNA prevented Ox-LDL-induced IRAK1-JNK-AP-1 activation and IL-1\(\beta\) production.

There are several ways by which PKC\(\delta\) can be activated during IL-1\(\beta\) production, including direct activation by Src family kinases (48, 49), which are known to associate with CD36 during Ox-LDL-induced macrophage foam cell formation (50). Several studies implicate PKC\(\delta\) in TLR-induced cytokine production (18, 51). PKC\(\delta\) can bind to Toll-interleukin 1 receptor (TIR) domain-containing adapter protein/MyD88 adapter-like (Mal), an adaptor protein for TLR2 and TLR4, and promote TLR signaling (52). A recent report also suggests a role of PKC\(\delta\) in macrophage foam cell formation by regulating expression of SRA and CD36 (53). Our present findings have immense implications for disorders like atherosclerosis, where PKC\(\delta\) can act as a double-edged sword by preventing both Ox-LDL-induced inflammation and macrophage foam cell formation.
Ox-LDL induced time-dependent CD36 upregulation and activation of the IRAK pathway, thus indicating the possible role of CD36 and TLRs in Ox-LDL-induced IL-1β production.

Therefore, we checked the role of these receptors in Ox-LDL-induced PKCβ and IRAK1 activation and IL-1β production. For studying the effect of Ox-LDL on cell lines expressing higher amounts of CD36, we used PMA-differentiated THP1 cells, which express high amounts of this receptor (46) and were also used in studies such as (7). Gene silencing with TLR2-, TLR4-, TLR6-, or CD36-specific siRNA in THP1 monocytes and macrophages significantly attenuated Ox-LDL-induced PKCβ and IRAK1 activation and IL-1β production, suggesting that TLR2, TLR4, TLR6, and CD36 can mediate Ox-LDL-induced effects seen in the present study. Interestingly, PKCβ positively regulated Ox-LDL-induced CD36 upregulation. Therefore, inhibition in CD36 upregulation due to less PKCβ may also contribute to the reduced signaling events transduced by these receptors. However, there are several ways by which TLRs can be activated. Ox-LDL can induce CD36-dependent TLR4 and TLR6 heterodimerization during IL-1β production, as shown in THP1 monocytes (6). TLR2, TLR4, or TLR6 can also heterodimerize and interact with CD36 in a ligand-specific manner (54). Ox-LDL can also prime monocytes and peripheral blood mononuclear cell for cytokine overproduction by upregulating TLR2 and TLR4 (4, 55, 56).

Because a positive correlation existed between circulating Ox-LDL and IL-1β in both SIRS and healthy subjects, it can be speculated that increased circulating Ox-LDLs can be a factor for enhanced IL-1β production in humans. A positive correlation between Ox-LDL and IL-1β with the disease severity scores (APACHE II and SOFA) also indicated an association of Ox-LDL concentration with the extent of IL-1β production and disease severity.

The effect of low and high Ox-LDL-containing plasma of control and SIRS individuals on PKCβ and IRAK1 activation and IL-1β production was CD36-dependent, because blocking this receptor by CD36 FA6 antibody significantly attenuated these signaling events. Because CD36 FA6 antibody completely blocks binding of Ox-LDL to CD36 receptor (57), it can be speculated that plasma-induced IL-1β production due to Ox-LDL will be minimal in the antibody-treated samples. A previous report also suggests that Ox-LDL-induced IL-1β production is attenuated in monocytes derived from CD36-deficient patients (58).

Although experiments done with specific siRNA demonstrate the role of TLR2, TLR4, TLR6, and CD36 in SIRS high Ox-LDL plasma-induced PKCβ and IRAK1 activation and IL-1β production, these results can be interpreted in several ways. Plasma can be a source of several entities, including IL-1β that may induce PKCβ and IRAK1 activation and cytokine overproduction. At the same time, some studies suggest a role of minimally modified LDL in IL-1β production by upregulating and activating TLR2 and TLR4 (3, 59, 60), and its presence in the plasma cannot be ruled out.

In conclusion, we demonstrate for the first time the role of CD36, TLR2, TLR4, TLR6, and the PKCβ-IRAK1-JNK-AP-1 axis during Ox-LDL-induced IL-1β production (Fig. 13). Our findings have implications for sterile inflammatory disorders because significant increases in Ox-LDL and IL-1β were observed in SIRS patients, which positively correlated with each other. Low and high Ox-LDL-containing plasma of healthy and SIRS patients primed monocytes for IL-1β overproduction by activating PKCβ and IRAK1 in a CD36-, TLR2-, TLR4-, and TLR6-dependent manner. PKCβ is thus proposed to be an attractive target for preventing IL-1β production and sterile inflammation observed during chronic inflammatory disorders.

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