

# Differential pro-inflammatory responses of TNF- $\alpha$ receptors (TNFR1 and TNFR2) on LOX-1 signalling

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**Abstract** TNF- $\alpha$  potently induces LOX-1 expression in THP-1 macrophages at concentrations between 1.25–50 ng/mL. The interplay between the two TNF receptors (TNFR1 and TNFR2) was apparent in the expression pattern of LOX-1 in response to TNF- $\alpha$ . Interestingly, R1 signal abrogation depleted both TNFR2 as well as LOX-1 transcript expression, suggesting that TNFR1 holds priority in the relative signaling mechanism between TNFR1 and TNFR2. TNF- $\alpha$  was also found to abrogate the oxidized-LDL (ox-LDL) mediated increase in intracellular pool of NO, a known downstream intermediate of LOX-1 pro-inflammatory signaling cascade. At the level of ox-LDL clearance, TNF- $\alpha$  inhibited the uptake (scavenging) of ox-LDL via LOX-1. Our study demonstrates the ability of TNF- $\alpha$  to enhance the signaling propensity of LOX-1 by increasing its expression and inhibiting its scavenging property.

**Keywords** LOX-1 · TNF receptors · Atherosclerosis · Nitric oxide · Oxidized-LDL

## Abbreviations

TNF- $\alpha$  Tumor necrosis factor alpha  
TNFR TNF receptor

LOX-1 Lectin-like oxidized low density lipoprotein receptor-1  
NO Nitric oxide  
ox-LDL Oxidized-low density lipoprotein  
PMA (WO) PMA washout

## Introduction

The relationship between ox-LDL and atherosclerosis has been supported by many studies and lectin-like ox-LDL receptor (LOX-1) has gained attention as the receptor involved in mediating the signaling cascade of ox-LDL [1–3]. LOX-1 is highly expressed in the large arteries (aortic, carotid and thoracic) [1, 4] which are the predilection sites for atherosclerosis. It is primarily expressed in macrophages [5], smooth muscle cells (SMC) and vascular endothelial cells [6] which are the three most important cell types involved in the development of atherosclerosis. Lectin-like ox-LDL receptor has also been reported to function as a cell-adhesion molecule by mediating the platelet-endothelial interaction which may initiate and promote atherosclerosis [4]. Attempts by different groups to elucidate the mechanism of action of this receptor in disease processes have produced ambiguous results. Further exploration of the different facets of LOX-1 signaling and its modulation may throw light on the immune-inflammatory facet of atherosclerosis [6].

Macrophages are the primary cells involved in the secretion of TNF- $\alpha$  in the atherogenic arena and are also involved in the differential expression and regulation of scavenger receptors required for lipid clearance in the atherogenic plaque [6, 7]. The stimulatory effects of TNF- $\alpha$  on LOX-1 receptor expression have been previously

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demonstrated in endothelial cells [8] and macrophages of *in vivo* origin [9]. However, most of these reports have used either single/low concentration or are time restricted studies. So a more comprehensive analysis of the interaction between TNF- $\alpha$  and LOX-1 is still lacking.

External TNF- $\alpha$  is capable of binding to its cognate receptors viz. TNFR1 and TNFR2, which vary in their respective functions and contribute to complex cross-talks, an aspect that still remains under-explored. For instance, co-expression of these two receptors on glial and neuronal membranes not only mediates apoptotic responses, mediated exclusively via TNFR1, but also neuroprotective responses by activation of NF $\kappa$ B, a pathway seemingly shared by both TNFR1 and TNFR2 [10]. However, in case of endothelial cells, where TNFR2 signaling promotes migratory and survival responses, TNFR1 signaling inhibits the same responses in models of arteriogenesis [11].

Due to a scarcity of evidence related to crosstalk of the two TNF receptors in macrophages, we wanted to carry out a comprehensive approach towards understanding the effects of externally added TNF- $\alpha$  on the signaling of LOX-1, involving the two TNF receptors or vice versa. Therefore, this study was designed to explore the differential effects of TNF- $\alpha$  on its two receptors and to delineate the string of R1/R2 interplay involved in LOX-1 signaling.

## Materials and methods

### Cell culture

Human monocyte leukemia cell line, THP-1 (procured from National Centre for Cell Science, Pune) was cultured in RPMI-1640 medium containing 10 % FCS, 100 units of penicillin, streptomycin and amphotericin B at 37 °C in a humidified environment in presence of 5 % CO<sub>2</sub>. For differentiation into macrophages, the monocytes were plated in RPMI containing 10 % FCS in presence of 100 nM PMA (Phorbol-12-myristate-13-acetate), without any antibiotic/anti-mycotic for 24 h under the same culture conditions as mentioned above.

### Treatments

PMA-differentiated macrophages were washed and subjected to the following treatment strategies: (i) PMA(WO): cells were incubated with normal growth medium for 5 h and then subjected to immunoblotting for detection of TNF- $\alpha$  protein. The supernatants from 24 h PMA differentiation [PMA(24)] and PMA(WO) were subjected to ELISA for detection of secreted TNF- $\alpha$ . (ii) Cells were

incubated with varying concentrations of ox-LDL protein for 5 h and subjected to immunoblot analysis with anti-LOX-1 antibody. (iii) Cells were incubated with varying concentrations of human recombinant TNF- $\alpha$  (Peprotech, Inc. Rocky Hill, NJ, USA) for 24 h and processed for FACS analysis for cell surface expression of LOX-1 protein. (iv) In the pulse-chase study, THP-1 macrophages were incubated with 20  $\mu$ g/mL ox-LDL protein for 5 h. This served as the 'pulse' group representing the effects mediated directly in presence of ox-LDL. Following pulse, the cells were washed and incubated with normal growth medium (without ox-LDL) for up to 12 and 24 h respectively. These two sets comprised the experimental 'chase', representing the temporal signaling effects of ox-LDL, initiated by its pulse.

### Isolation of LDL and oxidized LDL preparation

Pooled human plasma procured from the Blood Bank Facility, AIIMS, New Delhi, India, after Institutional Ethics Committee clearance was used for LDL isolation by density gradient ultracentrifugation [12]. LDL was subjected to oxidative modification by copper oxidation method as previously described by us [13]. Briefly, 500  $\mu$ g/mL LDL protein was modified with 20  $\mu$ M CuSO<sub>4</sub>, following which the copper was removed by chelation with 1.2 mM EDTA. The extent of oxidation of the ox-LDL preparation was determined by TBARS (Thiobarbituric acid reactive substances) assay using an MDA standard [14], and relative electrophoretic mobility (REM: to decide net increase in negative charge on ox-LDL as compared to native LDL) by tube gel electrophoresis. The resultant ox-LDL preparation was found to have an MDA content of 30–40 nmoles/mg protein and REM  $\sim$  1.48.

### Dil-oxidized LDL labeling

Oxidized-LDL was tagged with fluorescent Dil (1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) according to the method described earlier for LDL labeling [15]. The preparation was filter-sterilized and protein content was determined by Bradford assay [16]. It was stored in dark vials under nitrogen and used within 1 week.

### Immunoblot analysis

Cell lysate preparation and immunoblot analysis were carried out as described earlier [15]. Briefly, 50  $\mu$ g protein was separated on 12 % SDS-PAGE and subjected to immunoblot analysis. Rabbit anti-LOX-1, mouse anti-TNF- $\alpha$  and mouse anti-GAPDH primary antibodies were

used at a final dilution of 1:2,000, 1: 1,500 or 1:1,000 in 2 % BSA at 4 °C overnight respectively. The band intensities were determined densitometrically by Alpha-Inno-tech gel documentation system using Alpha-view software and expressed in terms of percentage imaging density value (% IDV).

#### cDNA synthesis and quantitative real time PCR

cDNA was reverse transcribed from 1 µg of RNA using random hexamer primers (Fermentas). Power SYBR green (2×) master mix (Applied Biosystems Inc., USA) was used for carrying out qRT-PCR. The primers were either designed (for LOX-1) or selected (for TNFR1 and TNFR2) so as to have an annealing temperature of around 60 °C. Amplification was carried out on 7500 SDS real time PCR machine (Applied Bioscience) and analysis was done by using SDS-V2.04 software. The sequence of primers used was as follows: **FP-ATGGTGGTGCCTGGCTGCTG** and **RP-GCCGGGCTGAGATCTGTCCCT** for LOX-1, **FP-GGTGACTGTCCCAACTTTGC** and **RP-GGGTCATCAGTGTCTAGGCT** for TNFR1, **FP-CTTCGCTCTCCAGT TGG** and **RP-AGGCAAGTGAGGCACCTT** for TNFR2 [17] and **FP-GTAACCCGTTGAACCCCAT** and **RP-CCATCCAATCGGTAGTAGCG** for 18S rRNA. Program used for the amplification: Reaction volume-20 µL, 50 °C/2 min-Hold, 95 °C/10 min-Hot start, 95 °C/15 s-Denaturation, 60 °C/1 min-Annealing, 72 °C/30 s-Extension, Cycle number-40 cycles.

#### Flow cytometry

Following PMA-differentiation, the adherent cells were washed and challenged with varying concentrations of recombinant TNF-α for 24 h. FACS analysis for cell surface expression of LOX-1 receptor was performed as per the protocol described by us [13]. Expression was determined using anti-LOX-1 mouse IgG2B antibody (specific for the C-terminal extracellular domain of LOX-1; R&D Systems, Inc., USA) and IgG2B isotype antibody was used as control. Following incubation with an anti-mouse IgG-Fluorescein conjugated goat F(ab')<sub>2</sub> secondary antibody, the cells were again washed and resuspended in 2 % paraformaldehyde and immediately acquired for FACS analysis in BD-FACS-Canto instrument with FACS Diva acquisition software. The voltage parameters were set against an untreated control which contained THP-1 macrophages simply washed and fixed in 2 % paraformaldehyde. The mean fluorescence intensity (MFI) values were deducted from those of the isotype controls respectively.

#### TNF-receptor Inhibition

Inhibition of TNFR1 specific signaling pathway was carried out by pre-treating THP-1 macrophages with 25 µg/mL of D609 (Sigma St. Louis, USA) for 30 min following which varying concentrations of TNF-α were added to the medium and incubated for 24 h under standard culture conditions. As an inhibitor control, one set of cells were incubated only with the inhibitor with no TNF-α in the medium. Since D609 is known to cross react with HEPES buffer system, TNFR1 inhibition experiments were carried out in HEPES free RPMI with 2 % FCS concentration without antibiotic [18].

#### Dil-oxidized LDL uptake assay

The ability of LOX-1 to internalize extracellular ox-LDL represents its scavenging activity and was monitored by an in vitro uptake assay for fluorescence labeled ox-LDL (Dil-oxLDL). Briefly, PMA differentiated THP-1 macrophages were incubated with increasing concentrations of TNF-α for 24 h. 20 µg/mL of Dil-oxLDL was added to the incubation mixture for the last 5 h. The cells were then washed and subjected to uptake assay similar to that described previously for Dil-LDL uptake [15]. Internalized Dil was extracted in isopropanol and read spectrofluorimetrically (Bio-Rad Technologies, USA) at 520 nm excitation and 574 nm emission wavelength. The amount of Dil was determined against a standard curve; tabulated against a Dil-oxLDL standard, and represented as Dil-oxLDL internalized per milligram of ox-LDL protein added.

#### DAF-FM-DA assay for nitric oxide

Intracellular nitric oxide (NO) concentration was determined by exploiting the ability of NO to oxidize DAF-FM (4-amino-5-methylamino-2',7'-difluorescein), an NO-specific fluorogenic probe into a highly fluorescent, quantifiable triazole product.

PMA-differentiated macrophages were either incubated with 20 µg/mL ox-LDL for 5 h or pretreated with 50 ng/mL of TNF-α for 24 h with addition of ox-LDL in the last 5 h. Macrophages treated with 5 ng/mL IFN-γ were used as positive control for NO generation. 5 µM diacetate salt of DAF-FM (DAF-FM-DA; Sigma St. Louis, USA) was added to all the groups 30 min before the end of their respective incubation periods and further incubation was continued in the dark at 37 °C [19, 20]. Untreated THP-1 macrophages were incubated with DAF-FM-DA and used as the fluorescence baseline. The cells were then washed twice and incubated with serum-free RPMI (without phenol red) for another 30 min in the dark. Following this, the change in fluorescence was monitored with FLUO-star

OMEGA-ELISA reader (BMG LABTECH GmbH, Allmendgruen, Ortenberg/Germany) using 485 nm excitation and 515 nm emission wavelengths.

#### Enzyme-linked immuno sorbant assay (ELISA)

Culture supernatant was collected after respective challenges, centrifuged at 3,000 rpm and stored in endotoxin-free vials at  $-20^{\circ}\text{C}$  until further use. Secreted TNF- $\alpha$  was detected in the culture supernatant with Human US TNF $\alpha$  ELISA kit (Invitrogen Bioservices India Pvt. Ltd, Bangalore) and OD was read at 450 nm on a FLUO-star OMEGA-ELISA reader.

Fixation of baseline parameters for the study

#### Control/PMA (WO) cells

PMA(WO) is the baseline in our study, i.e. the basal expression of LOX-1 protein/mRNA after the THP-1 macrophages have adhered to the Petri plate, and without additional stimulation by either PMA or by residual TNF- $\alpha$ . Hence the washout. After treatment with PMA (100 nM), the cells were completely washed off PMA, followed by which experiments were carried out on those adhered THP-1 macrophages.

Since our study has different temporal parameters in various experiments, we had to essentially take respective temporally matching controls. For example, incubation of external TNF- $\alpha$  was carried out for 24 h on the PMA (WO) cells, so the respective control PMA (WO) cells were incubated with only media without any TNF- $\alpha$ . In case of Fig. 2b, since the treatment of ox-LDL was for 5 h, the control was PMA (WO) cells maintained without Ligand for 5 h.

#### mRNA expression by real time PCR

We have represented all values as relative fold expression values. It is to note that the relative fold expression values are not absolute receptor expression values but only a depiction of the relative change in expression from the baseline. So, we normalized all the expression values from the baseline PMA (WO) cells which was considered as the limit for baseline and apparently it was considered as null or origin to calculate the fold expression of the entity when other inducer(s) (besides PMA) acted on PMA (WO) cells.

#### Statistical analysis

Comparison of means was done by one-way analysis of variance followed by Dunnett's multiple comparison; \* =  $p < 0.05$ , \*\* =  $p < 0.01$ .

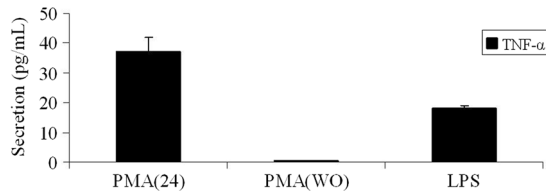
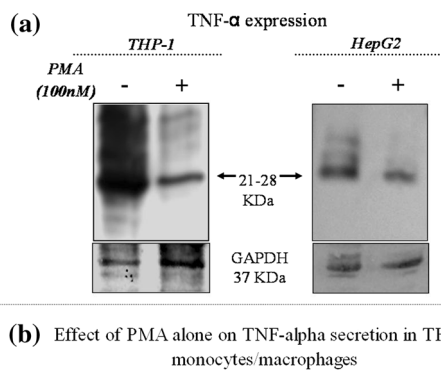
- (i) *Representation of data for experiments on FACS analysis* The data have been represented as mean fluorescent intensity  $\pm$  SD of four experimental repeats ( $n = 4$ ).
- (ii) *Representation of data for Realtime qRT-PCR experiments* All data have been represented as relative fold expression ( $2^{-\Delta\Delta\text{CT}}$ ) of LOX-1 mRNA expressed as 'X' fold over PMA baseline calculated against 18SrRNA internal control carried out in triplicates.
- (iii) *Representation of data on Dil-oxLDL uptake* All data has been represented as mean  $\pm$  SD of three experimental repeats ( $n = 3$ ).
- (iv) *Representation of data on NO level detection* All data represents mean  $\pm$  SD of four experimental repeats ( $n = 4$ ).

## Results

We used PMA for differentiation of THP-1 monocytes into macrophages since PMA is known to induce a baseline expression of LOX-1 [5]. However, since PMA is a potent mitogen and can itself have an effect on TNF- $\alpha$ , we first delineated the PMA-elicited effects (100 nM/24 h, used in this study) on the expression (western blotting) and secretion (ELISA) of TNF- $\alpha$ , independent of LOX-1 expression. The PMA-induced effects on THP-1 cells were compared against HepG2 cells, which do not express LOX-1 (Fig. 1a). In both cell lines, 24 h PMA-treatment showed an inhibition of TNF- $\alpha$  expression as compared to untreated cells, demonstrating that the inhibition of TNF- $\alpha$  was independent of any existing LOX-1 expression. At the level of secretion, we compared the 24 h PMA-treated group against a PMA(WO) group, where following 24 h PMA treatment, the THP-1 macrophages were washed and incubated in media without any PMA for 5 h. We found a marked decrease in the secretion of TNF- $\alpha$  following PMA(WO) as compared to 24 h PMA treatment (Fig. 1b). Lower expression of TNF- $\alpha$  after PMA treatment might be due to an effect on TNF- $\alpha$  secretion, enhanced by PMA. LPS was used as a positive control for TNF- $\alpha$  secretion in the medium. This control experiment demonstrated that the baseline in our study i.e., PMA(WO) was devoid of the effects of PMA on TNF- $\alpha$ . These cells also maintained a basal LOX-1 expression expected on PMA differentiated macrophages (Fig. 2a and b, concentration 0).

The effect of TNF- $\alpha$  on the cell surface expression of LOX-1 protein in THP-1 macrophages was studied by FACS analysis following staining with anti-LOX-1 antibody specific for its extracellular, C-terminal lectin-like domain (CTLD). The PMA(WO) cells were challenged





**Fig. 1** Baseline effects of PMA on TNF- $\alpha$  expression and secretion. **a** Depicts immunoblot analysis of TNF- $\alpha$  protein in THP-1 macrophages and HepG2 cells with and without PMA (100 nM) treatment. The inhibition of TNF- $\alpha$  expression was an independent property of PMA regardless of the presence of LOX-1 (THP-1 macrophages) or absence (HepG2 cells). **b** Shows the secretory profile of TNF- $\alpha$  in the culture supernatant of cells treated with 100 nM PMA for 24 h i.e. PMA (24) and macrophages washed off PMA and incubated for 5 h i.e. PMA (WO). Treatment of 100 ng/mL of LPS for 4 h following PMA differentiation served as a positive control for TNF- $\alpha$  secretion

with varying concentrations of TNF- $\alpha$  for 24 h. Although there was a significant increase in LOX-1 expression at all concentrations of TNF- $\alpha$ , peak receptor expression was observed at 1.25 and 50 ng/mL (Fig. 2a). This raised the probability of differential signaling responses by the two surface TNF receptors (R1 and R2) at two different concentrations of TNF- $\alpha$ .

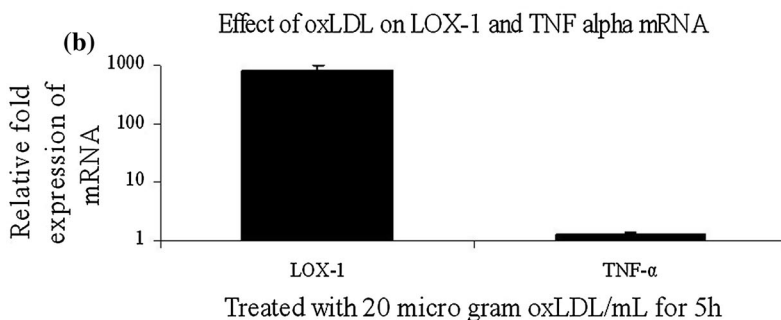
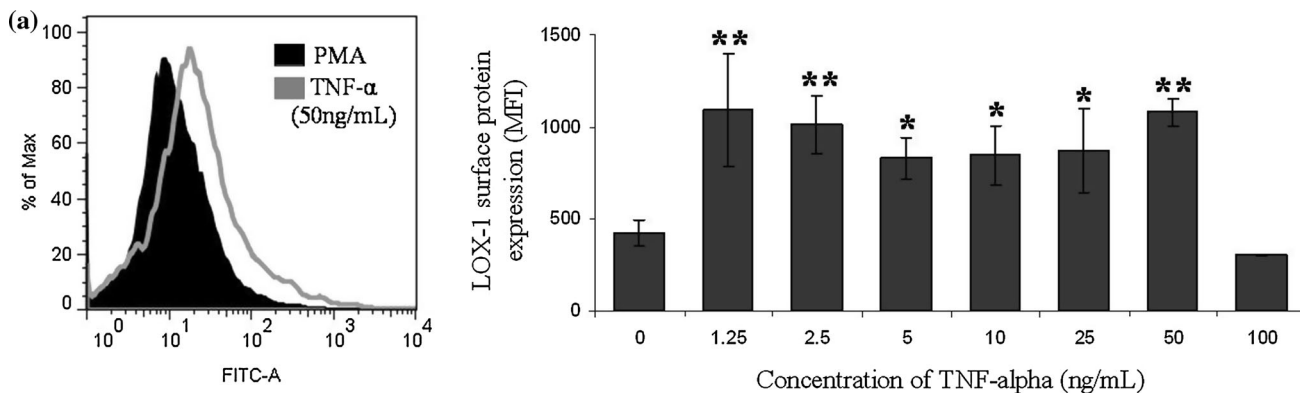
Oxidized-LDL, a pro-inflammatory ligand, not only stimulates LOX-1 expression but also stimulates TNF- $\alpha$  expression (Fig. 2b). TNF- $\alpha$ , which itself is pro-inflammatory, can also stimulate LOX-1 expression, hence contributing to a vicious cycle probably involving TNF receptor signaling/crosstalk. In a previous report we have already demonstrated an optimal LOX-1 protein and mRNA expression in the presence of 20  $\mu$ g/ml of ox-LDL for 5 h [13]. Figure 2b represents the relative expression profiles of LOX-1 and TNF- $\alpha$  transcript in response to ox-LDL (20  $\mu$ g/ml) challenge on a logarithmic scale where the value 1 represents the basal expression after PMA differentiation. Since activation of LOX-1 by its ligand promotes expression of pro-inflammatory TNF- $\alpha$ ; the expression of its cognate receptors viz. TNFR1 and TNFR2 was also expected to be influenced by ox-LDL-mediated LOX-1 signaling. As shown in Fig. 3a, following 5 h pulse of ox-LDL, there was an increase in TNFR2 expression over

PMA baseline which was sustained till 12 h of chase. However, there was a significant fall in receptor expression below its own pulse value at 24 h of chase. In the case of TNFR1, receptor expression was below PMA baseline value at all observation points. However there was a significant increase in TNFR1 expression at 12 h chase followed by a significant decrease in expression at 24 h respectively as compared to its own expression at 5 h pulse (Fig. 3a).

Following the view that TNFR1 is more sensitive to soluble extracellular TNF- $\alpha$  as compared to TNFR2, which responds to TNF expressed as an integral membrane protein [11], R1 could probably stimulate LOX-1 expression even at low concentrations (1.25 ng/mL) via its own signaling (Fig. 2a). To determine the priority of TNFR1 in LOX-1 expression we inhibited TNFR1 signaling. We used D609, a specific inhibitor of R1 to evaluate R1-mediated responses over R2. The TNFR1/PC-PLC/PKC $\alpha$  is the most common pathway to relay TNFR1 mediated signal which is selectively inhibited by this compound as also reported previously [18, 21]. As shown in Fig. 3b, presence of D609, inhibitor of downstream signaling cascade of TNFR1, completely abolished the expression of LOX-1 mRNA. This inhibition of LOX-1 expression by D609 also indicated towards the probable dependence of TNFR2 expression on TNFR1 signaling.

Figure 4 depicts the relationship between TNFR1 and TNFR2 in presence and absence of D609. The dotted line represents the expression of TNFR1 and TNFR2 in presence of the inhibitor. The concentration 0 ng/mL shows the expression of R1/R2 in presence of 25  $\mu$ g/mL of D609 and in the absence of any TNF- $\alpha$  (0 ng/mL). It is to be noted that the relative change in the expression of R1 and R2 have been compared with their own respective baseline values and are not depictive of mRNA levels relative to each other.

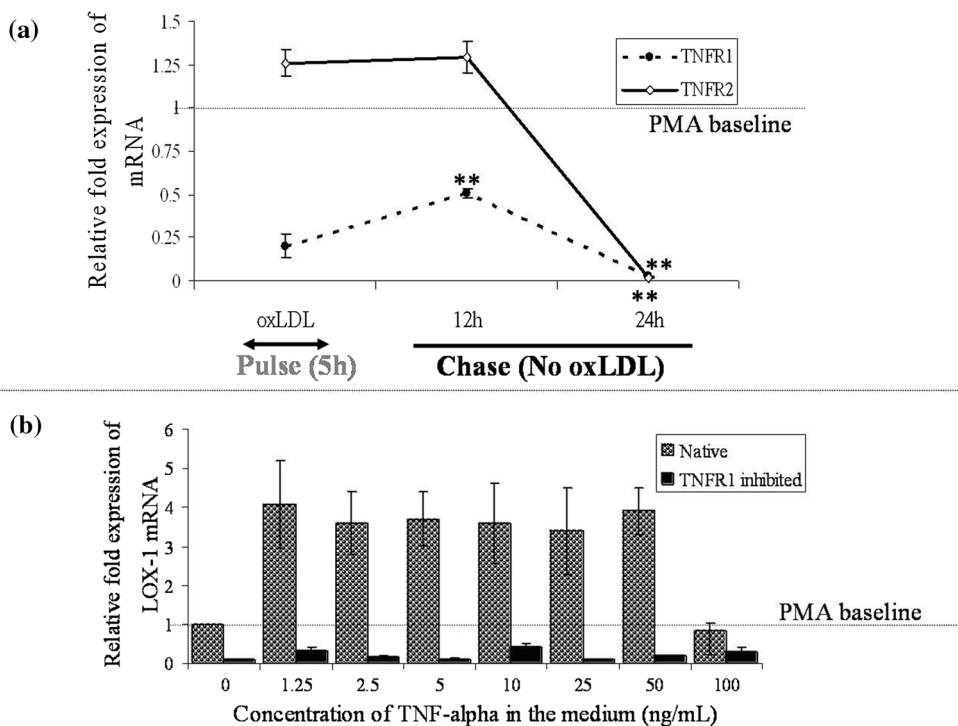
Inhibition of both TNFR1 (panel-a) and TNFR2 (panel-b) transcripts was noted in presence of the inhibitor D609. This provides evidence that the downstream signaling cascade of TNFR1 elicited by external TNF- $\alpha$  regulates the transcription of both the receptors. Furthermore, LOX-1 transcript was upregulated at most of the concentrations of TNF- $\alpha$  used, but in presence of D609, even LOX-1 expression was abrogated (Fig. 3b). Since, upon inhibition of TNFR1 signaling, transcription of both TNFR2 as well as LOX-1 was inhibited, the possibility of the direct involvement of TNFR2 signaling in LOX-1 expression is also eliminated. These results suggest that in our study model, TNF- $\alpha$ -mediated signal for LOX-1 expression was primarily mediated through TNFR1. Further, the results also demonstrate a probability that TNF- $\alpha$  can mediate its signal through TNFR2 only when TNFR1 signaling is functional.



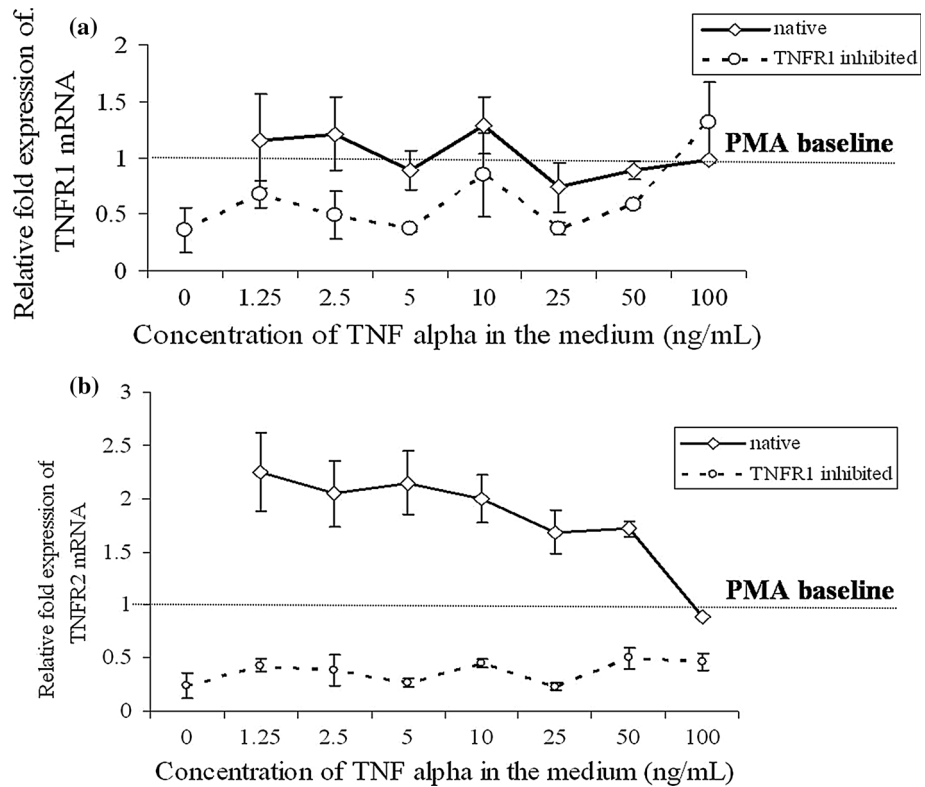
**Fig. 2** Comparative effect of TNF- $\alpha$  and ox-LDL on LOX-1 expression: **a** Effect of TNF- $\alpha$  on LOX-1 expression determined by FACS analysis. Varying concentrations of TNF- $\alpha$  were incubated with THP-1 macrophages for 24 h and the cell surface expression of LOX-1 was determined by FACS analysis. The histogram is a

representative image of baseline (PMA) expression of LOX-1 overlaid with the expression found at 50 ng/mL TNF- $\alpha$ . **b** Shows the comparative effect of ox-LDL (20  $\mu$ g/mL protein) treatment on the expression of LOX-1 and TNF- $\alpha$  mRNA by qRT-PCR and has been depicted on a logarithmic scale

**Fig. 3** Modulation of the expressions of LOX-1, TNFR1 and TNFR2: **a** Depicts the mRNA expression of TNFR1 and TNFR2 in a pulse-chase set up. **b** Depicts inhibition of LOX-1 mRNA transcription in TNFR1 inhibited cells demonstrating a prime role of TNFR1 in TNF- $\alpha$  stimulated expression of LOX-1



**Fig. 4** Relative expression of TNFR1 and TNFR2 mRNAs: **a** Represents the expression of TNFR1 transcript in presence and absence of its down signaling inhibitor D609. **b** Represents the expression of TNFR2 transcript in presence and absence of D609, the down signaling inhibitor of TNFR1. All the panels represent the relative fold expression of respective transcripts in response to TNF- $\alpha$  and in presence and absence of D609, the inhibitor for TNFR1



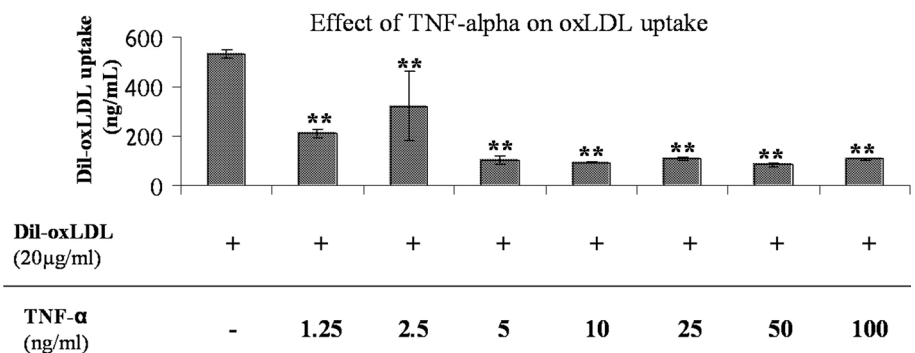
We have previously demonstrated the endocytic propensity of LOX-1 for its ligand ox-LDL [13]. The interaction between LOX-1 and its cognate Ligand has been interpreted in terms of the uptake of fluorescence labeled ox-LDL. Figure 5 depicts the ox-LDL uptake profile of THP-1 macrophages in presence of TNF- $\alpha$ . Interestingly we found that TNF- $\alpha$  potently inhibited the uptake of Dil-oxLDL at all tested concentrations as compared to control. The results of this experiment suggested that TNF- $\alpha$  inhibited the scavenging ability of LOX-1 and probably predisposed it for pro-inflammatory signaling.

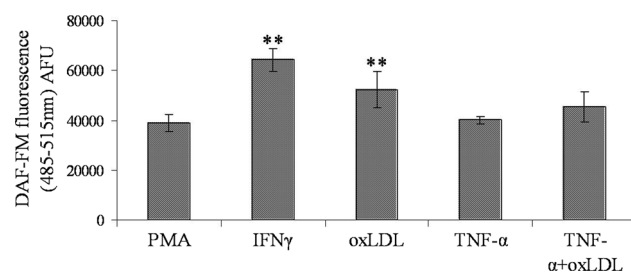
To explore the effects of TNF- $\alpha$  on the downstream signaling of LOX-1, we determined intracellular NO concentration, one of the intermediates of the LOX-1 signaling cascade that contributes to a pro-inflammatory response

[4]. Figure 6 shows the intracellular concentration of NO in THP-1 macrophages in response to ox-LDL and TNF- $\alpha$  treatment. IFN- $\gamma$  was used as positive control for induction of intracellular NO. Oxidized-LDL and TNF- $\alpha$  were used at 20  $\mu$ g/mL and 50 ng/mL respectively, the concentration at which maximal expression of LOX-1 was observed with these ligands. Downstream signaling cascade of activated LOX-1 utilizes intracellular NO as a substrate for the synthesis of peroxynitrite (ONOO<sup>-</sup>), an activator of NF $\kappa$ B which in turn activates pro-inflammatory genes [4, 22]. Therefore, the increase in intracellular levels of NO observed in the presence of ox-LDL (Fig. 6) can be attributed to LOX-1 activation.

As TNF- $\alpha$  increases LOX-1 expression (Fig. 2a), it also increases the interaction of extracellular ox-LDL (when

**Fig. 5** Effect of TNF- $\alpha$  on Dil-ox-LDL uptake by THP-1 cells: The graph shows the uptake of Dil-oxLDL in presence of varying concentrations of TNF- $\alpha$  (0–100 ng/mL). The presence of TNF- $\alpha$ , the uptake via LOX-1 was inhibited at all concentrations





**Fig. 6** Effect of ox-LDL and TNF- $\alpha$  on the generation of intracellular NO radical: Intracellular NO was measured by the DAF-FM-DA assay. The data shows a fall in NO concentration in presence of TNF- $\alpha$

present) with its cell surface LOX-1 receptors. However, there was no increase in intracellular NO levels in the TNF- $\alpha$  treated group as compared to PMA control (Fig. 6). Also, TNF- $\alpha$  inhibited the ox-LDL mediated increase in intracellular NO levels (Fig. 6), suggesting the plausibility of an increased utilization of intracellular NO under the influence of TNF- $\alpha$ , translating into enhanced LOX-1 signaling.

## Discussion

TNF- $\alpha$ , a pro-inflammatory cytokine of the TNF superfamily is a central player in the atherosclerotic process and is known to act via its receptors TNFR1 (p55) and TNFR2 (p75). TNF- $\alpha$  elicits its signals via the NF $\kappa$ B/p38/JNK/ERK pathways [23]. Though previous reports have demonstrated the ability of TNF- $\alpha$  to stimulate LOX-1 expression [24], most of these studies were carried out on plaque transections, arterial biopsies, endothelial cells and vascular SMC [6, 25]. Although evidence for the effect of TNF- $\alpha$  on LOX-1 exists on macrophages as well, these are not elaborate enough in relation to the LOX-1 signaling cascade. Therefore we have carried out a comprehensive study to understand the interaction of TNF- $\alpha$  with its receptors (TNFR1 and TNFR2) and their subsequent cross talk with LOX-1 receptor (expression and downstream signaling) in THP-1 macrophages. We incubated PMA differentiated macrophages with various concentrations of TNF- $\alpha$  for 24 h. TNF- $\alpha$  was found to stimulate LOX-1 expression at two different concentrations (Fig. 2a). We predicted that the observed peaks in LOX-1 expression at 1.25 and 50 ng/mL of TNF- $\alpha$  may in fact depict a probable differential interaction of TNF- $\alpha$  with TNFR1 and TNFR2 respectively. On the other hand, it was noted that ox-LDL itself stimulates LOX-1 as well as TNF- $\alpha$  as shown in Fig. 2b. Therefore, we hypothesized that ox-LDL-mediated activation of the downstream signaling cascade of LOX-1 may be responsible for the increased expression of the TNF-receptors. In this direction, as shown in Fig. 3a,

we found TNFR2 expression to increase over PMA baseline in response to ox-LDL ‘pulse’, with sustained expression up to 12 h in the absence of ox-LDL (chase). However the receptor expression levels significantly decreased at 24 h in the absence of ox-LDL (chase) as compared to its own ‘pulse’ value. On the other hand, TNFR1 expression significantly increased as compared to its own ‘pulse’ value followed by a significant decrease at 24 h in the absence of ox-LDL (chase). Interestingly, inhibition of downstream signaling of TNFR1 by using a specific signaling inhibitor ‘D609’ (PC-PLC specific) almost completely abrogated the TNF- $\alpha$  mediated increase in LOX-1 expression (Fig. 3b). Further, the expression of TNFR1 transcript in response to different concentrations of TNF- $\alpha$  (except 100 ng/mL) was also inhibited by D609 (Fig. 4a). However, this inhibition was overcome at 100 ng/mL of TNF- $\alpha$ .

Surprisingly, under similar conditions TNFR2 transcript was also found to be inhibited (Fig. 4b). This suggested that, inhibition of TNFR1-mediated downstream signaling probably abrogated the cross talk between the two TNF receptors, thus raising the possibility of a preferential bias in receptor signaling. Such a bias in TNF receptor signaling has also been reported previously. For example, in rats with Rheumatoid arthritis (a chronic inflammatory disease) macrophages infiltrating the synovium have been shown to exhibit a greater expression of TNFR2 as compared to TNFR1 [26]. Furthermore, Defer N, et al., have demonstrated that TNFR1-dependent responses in cardiac myocytes are under the yoke of TNFR2 [27].

Dil-oxLDL uptake by THP-1 cells was found to be inhibited by TNF- $\alpha$  (Fig. 5) thus increasing the extracellular accumulation of ox-LDL and creating an athero-promotive environment favoring more LOX-1 signaling and expression (Fig. 2b). This could in turn send pro-inflammatory signals for more TNF- $\alpha$  synthesis. Intracellular NO is an important substrate for the down stream signaling cascade of LOX-1 [4]. As shown in Fig. 6, TNF- $\alpha$  inhibited the ox-LDL mediated increase in the intracellular NO level. A possible explanation for this decrease in intracellular NO pool could be due its increased utilization for generation of peroxynitrite intermediate through LOX-1 signaling.

Our study demonstrates a possible cross-talk between LOX-1 and the two TNF receptors (R1 and R2), with TNF- $\alpha$  acting as a mediating factor. On one hand, TNFR1 (Fig. 3a) appears to take priority in responding to TNF- $\alpha$  and generates more LOX-1. On the other hand TNF- $\alpha$  increases extracellular accumulation of ox-LDL (Fig. 5). Hence, exploiting the interaction between ox-LDL and LOX-1 could be a possible mechanism by which TNF- $\alpha$  contributes to the atherogenic arena.



## Conclusion and perspectives

Overall our study demonstrates the interdependency of LOX-1 and TNF- $\alpha$ . On one hand ox-LDL-mediated LOX-1 activation induces TNF- $\alpha$ , while on the other hand, extracellular TNF- $\alpha$  potently induces LOX-1 cell surface expression. However, TNF- $\alpha$  significantly inhibits the ability of LOX-1 to scavenge extracellular ox-LDL, hence predisposing it towards signaling. Further, while ox-LDL significantly increased intracellular NO levels, this was inhibited in the presence of TNF- $\alpha$ . This was probably due to the increased utilization of intracellular NO to form peroxynitrite, an activator of NF $\kappa$ B mediated pro-inflammatory responses. Furthermore, we found that inhibition of TNFR1 signaling (in presence of D609), abrogated both TNFR2 as well as LOX-1 expression, suggesting that the latter two effects are under the regulation of TNFR1 signaling.

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