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Oxidized low-density lipoproteins induce tissue factor expression in T-lymphocytes via activation of lectin-like oxidized low-density lipoprotein receptor-1

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Aims T-lymphocytes plays an important role in the pathophysiology of acute coronary syndromes. T-cell activation *in vitro* by pro-inflammatory cytokines may lead to functional tissue factor (TF) expression, indicating a possible contribution of immunity to thrombosis. Oxidized low-density lipoproteins (oxLDLs) are found abundantly in atherosclerotic plaques. We aimed at evaluating the effects of oxLDLs on TF expression in T cells and the role of the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1).

Methods and results $CD3^+$ cells were isolated from healthy volunteers. Gene, protein, and surface expression of TF, as well as of LOX-1, were assessed at different time-points after oxLDL stimulation. To determine whether oxLDL-induced TF was LOX-1 dependent, T cells were pre-incubated with an LOX-1 inhibiting peptide (L-RBP) or with an anti-LOX-1 blocking antibody. To exclude that TF expression was mediated by reactive oxygen species (ROS) generation, oxLDL-stimulated T cells were pre-incubated with superoxide dismutase + catalase or with 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), an intracellular free radical scavenger. Finally, to determine if the observed findings *in vitro* may have a biological relevance, the presence of $CD3^+/TF^+/LOX\text{-}1^+$ cells was evaluated by immunofluorescence in human carotid atherosclerotic lesions. oxLDLs induced functionally active TF expression in T cells in a dose- and time-dependent manner, independently on ROS generation. No effect was observed in native LDL-treated T cells. LOX-1 expression was also induced by oxLDLs in a time- and dose-dependent manner. Pre-incubation with L-RBP or anti-LOX-1 antibody almost completely inhibited oxLDL-mediated TF expression. Interestingly, human carotid plaques showed significant infiltration of $CD3^+$ cells (mainly $CD8^+$ cells), some of which were positive for both TF and LOX-1.

Conclusion oxLDLs induce functional TF expression in T-lymphocytes *in vitro* via interaction of oxLDLs with LOX-1. Human carotid atherosclerotic plaques contain $CD3^+/CD8^+$cells that express both TF and LOX-1, indicating that also in patients these mechanisms may play an important role.

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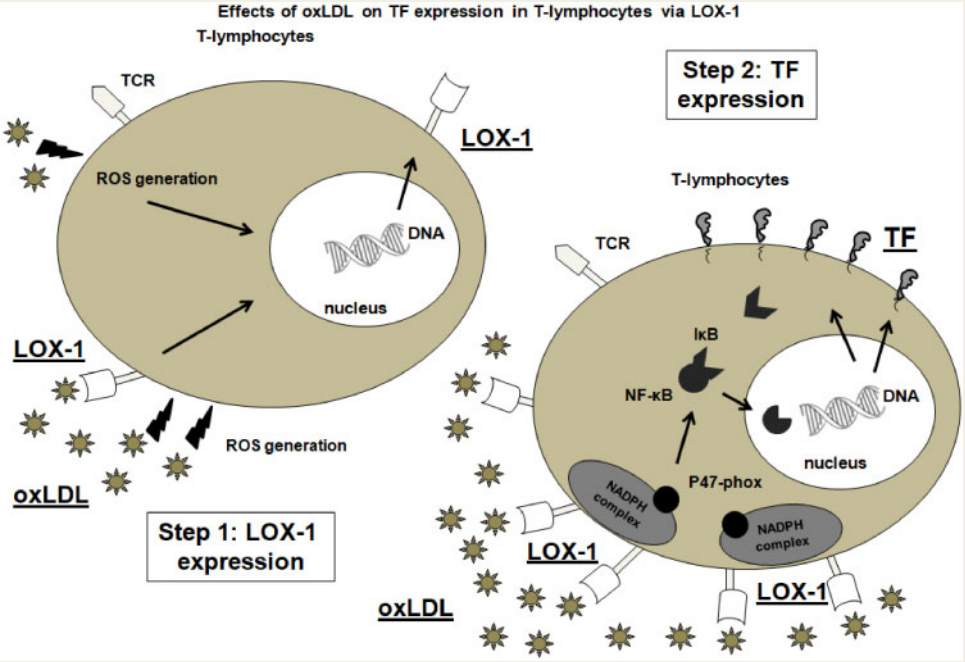
Keywords Lipoproteins • Atherosclerosis • Inflammation • T-lymphocyte • Tissue factor

Graphical Abstract



1. Introduction

Atherosclerosis is a lipoprotein-driven, inflammatory disease characterized by deposition of low-density lipoproteins (LDLs) and other blood-borne materials within the arterial wall of almost all vascular districts.[1] Furthermore, it has been convincingly demonstrated that within coronary vessels, infiltration of immune-competent cells, such as macrophages and T-lymphocytes, plays an important role not only in the progression of atherosclerosis but also in plaque destabilization, leading to the clinical scenario of acute coronary syndromes (ACS).[2]

We have previously reported that coronary plaques obtained from ACS patients show a selective, oligoclonal expansion of T cells, indicating a specific, antigen-driven recruitment of T-lymphocytes within the unstable lesions.[3] To date, however, the antigen(s) responsible for this recruitment still remain elusive, although it has been postulated that oxidized-LDLs (oxLDLs) might represent a good candidate.[4–6] Quite recently indeed, Liu *et al.*[7] have demonstrated that oxLDLs induce activation of T-lymphocytes in presence of dendritic cells, further supporting the notion that oxLDLs may act, under certain conditions, as a non-self-antigen. However, within the atherosclerotic plaque microenvironment, several stimuli may act simultaneously on the resident cells, including T-lymphocytes, thus contributing to their activation via mechanisms different from the 'classical' interaction of the antigen with the T-cell receptor.[8] For example, as reported by our group, stimulation of T-lymphocytes with selected pro-inflammatory cytokines *in vitro* induce a pro-thrombotic phenotype via expression of functional tissue factor (TF), the main initiator of the coagulation cascade.[9] Interestingly, this phenomenon seems to occur also *in vivo* in ACS patients.[8]

Internalization and degradation of oxLDLs is mediated by a lectin-like 52-kD receptor, namely lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1).[10] Since its discovery, several studies have described the role of LOX-1 in atherosclerosis and cardiovascular disease.[11]

According to the available data, we hypothesized that oxLDLs might induce TF expression in T-lymphocytes via a mechanism different from the immunological activation of T cells, i.e. antigen/T-cell receptor interaction. In particular, the role of LOX-1, also expressed in T-lymphocytes,[12] was investigated. We report here that oxLDLs, but not native LDLs, induce functionally active TF expression in T cells via interaction with LOX-1 and that human carotid plaques contain $CD3^+$/TF^+/$LOX\text{-}1^+$ cells, providing evidence for a novel mechanism of T-cell activation within atherosclerotic plaques. The potential implications of these findings in human disease are also discussed.

2. Methods

Expanded methods are provided in Supplementary material online.

2.1 Experimental protocol

LDLs (density 1.019–1.063 g/mL) and oxLDLs were prepared as previously described.[13] T-lymphocytes were isolated from daily fresh human buffy coats of 15 healthy volunteers as previously described.[9] A written informed consent was obtained from each subject. The protocol was approved by the local ethical committee and conform the principles outlined in the declaration of Helsinki.

In preliminary experiments, a dose–response curve to oxLDLs was performed (25, 50, and 100 μg/mL). oxLDLs at 25 μg/mL induced a slight but non-significant increase in TF expression, while a significant increase in TF expression was observed at 50 and 100 μg/mL, although the latter was associated with some cytotoxicity. Based on these preliminary experiments, T cells (1×10^6/mL) were incubated with either oxLDLs (50 and 100 μg/mL) or native, non-oxidized LDLs as internal control. To determine whether oxLDLs induce expression of TF in T cells, TF mRNA analysis was performed at different time-points (4, 8, 12, 18, 24, 48, and 72 h) following stimulation. TF protein levels by western blot, as well as flow cytometry analysis and TF functional assay in intact cells were performed at 72 h. To determine whether oxLDL-induced TF expression in T-lymphocytes was mediated by expression and activation of LOX-1, further experiments were performed. First, LOX-1 expression was evaluated following stimulation with oxLDLs, as above described, both at gene and protein level. In a second set of experiments, T cells were pre-incubated for 30 min with an LDL receptor blocking peptide (L-RBP, Cayman Chemical, Ann Arbor, MI) (5 and 10 μg/mL final concentration) or a neutralizing monoclonal antibody against human LOX-1 (TS92, 10 μg/mL; characteristics of the antibody were described in previous reports).[14,15] Then, T-lymphocytes were incubated with oxLDLs (50 μg/mL). TF gene, protein, and activity levels were evaluated as above described.

To determine which intracellular signalling pathways are activated by LOX-1/oxLDL interaction, the contributions of p47 (one of the major constituent of NADPH oxidase [NOX]), as well as of NF-κB (by western blot and translocation assay as previously described[16]), were investigated. The effects of NOX inhibition by diphenyleneiodonium (DPI, obtained from Sigma-Aldrich, St. Louis, MO, D2926) were also evaluated. T cells were pre-treated with DPI at final concentration of 500 μM as already described[17] for 1 h and subsequently stimulated with oxLDL (50 μg/mL) for 24 and 48 h for mRNA analysis.

Furthermore, to determine whether the observed effects on TF expression in oxLDLs stimulated T cells were specific of LOX-1/oxLDL interaction or caused by a generic lipid peroxidation/reactive oxygen species (ROS) generation phenomenon, additional experiments were performed: (i) T cells were stimulated with H_2O_2 (50 and 100 μM)[18] and TF expression was evaluated at gene and protein levels. LOX-1 gene expression was also investigated; (ii) T cells were pre-incubated with superoxide dismutase (SOD, 500 U/mL)[16,19] and catalase (300 U/mL)[20,21] for 30 min and then stimulated with oxLDLs 50 μg/mL; (iii) T cells were pre-incubated for 1 h with 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, 10 μM), a membrane-permeable aminoxyl-type free radical scavenger, with unique antioxidant properties.[22,23] Cells were then stimulated with oxLDLs and expression of TF was evaluated as above described. The amount of lipid peroxidation was determined by measuring lipid peroxidation products both after oxLDLs and H_2O_2 stimulation.

For each peripheral blood mononuclear cell (PBMC) preparation, three independent experiments for all of the conditions above described were performed.

2.2 Real-time PCR

Total RNA was isolated using Trizol Reagent, according to the manufacturer's protocol. Amplification of LOX-1 from cDNA was performed using the following primers (Sigma-Aldrich): fw: 5′-TGGGAAAAG AGCCAAGAGAA-3′, rev: 5′-TAAGTGGGGCATCAAAGGA-3′. TF and housekeeping gene TBP (TATA Box protein) primers were previously described.[9]

2.3 Collection of cell lysates and protein expression

Protein analysis was performed on T-cell lysates at 72 h following stimulation with oxLDLs and at 4, 8, 12, 18, and 24 h after stimulation with H_2O_2. For molecular pathways analysis, cell lysates were collected 8, 16, and 24 h following stimulation with oxLDLs (50 μg/mL) with or without L-RBP (10 μg/mL) or a blocking monoclonal antibody against human LOX-1 (TS92, 10 μg/mL). Blots were incubated overnight at 4°C with anti-TF antibody (Affinity Biologicals) diluted 1:1000 in TBS with 0.1% Tween-20 and 2.5% non-fat dry milk. For molecular pathways analysis, anti-NF-κB (Biorbyt, Cambridge, UK), anti-IκB (Thermo Fisher, Waltham, MA), and anti-p47 phosphorylated antibody (Thermo Fisher) were used.

2.3 Flow cytometry analysis

Ten million T cells, isolated as above described, were plated in X-Vivo medium and treated with oxLDL (50 μg/mL) for 72 h. Surface TF expression as well as CD8/CD4 subpopulations characterization was performed.

2.4 TF activity in intact T cells

TF procoagulant activity was measured as previously described.[9]

2.5 Quantification of lipid peroxidation products

The extent of lipid peroxidation in cell lysates was determined by measuring malonyldialdehyde (MDA) levels, a secondary lipid peroxidation product. The thiobarbituric acid method (TBARS assay kit, Cayman Chemical) was used and results were expressed as micromolar of MDA generated.[13]

2.6 Confocal microscopy of T cells

To evaluate surface expression and distribution of LOX-1 on T-cell membrane, we performed confocal microscopy experiments. Briefly, T-lymphocytes (3×10^6) were incubated with oxLDLs (50 μg/mL) for 72 h. Cells were then incubated with primary antibody (Anti-LOX 1, purchased from Abcam, Cambridge, UK) for 1 h at RT, extensively washed in phosphate buffer saline (PBS) and incubated with secondary antibody (Alexafluor 546 goat anti mouse) for 1 h at RT. Cells were extensively washed in PBS, spotted on microscope slides, and analysed.

2.7 Immunohistochemistry of human carotid atherosclerotic lesions

To determine whether T-lymphocytes co-localize with TF *in vivo*, immunohistochemistry staining was performed using 4 μm thick formalin-fixed, paraffin-embedded (FFPE) tissue sections of atherosclerotic plaques. Serial adjacent sections were treated as previously described.[9,24]

2.8 Immunofluorescence of human carotid atherosclerotic lesions

Immunofluorescence was performed using 4 μm thick FFPE tissue sections of carotid plaques. Slides were treated as previously described.[25] To evaluate the CD3 subset population co-expressing TF/LOX-1, immunofluorescence (IF) was carried out by mean of Opal Multiplex IF Assay[26] (Perkin Elmer, Waltham, MA, USA) (gift of Prof. G. Matarese, University 'Federico II', Naples, Italy) as suggested by the manufacturer using 4 μm thick FFPE tissue sections from atheroma.

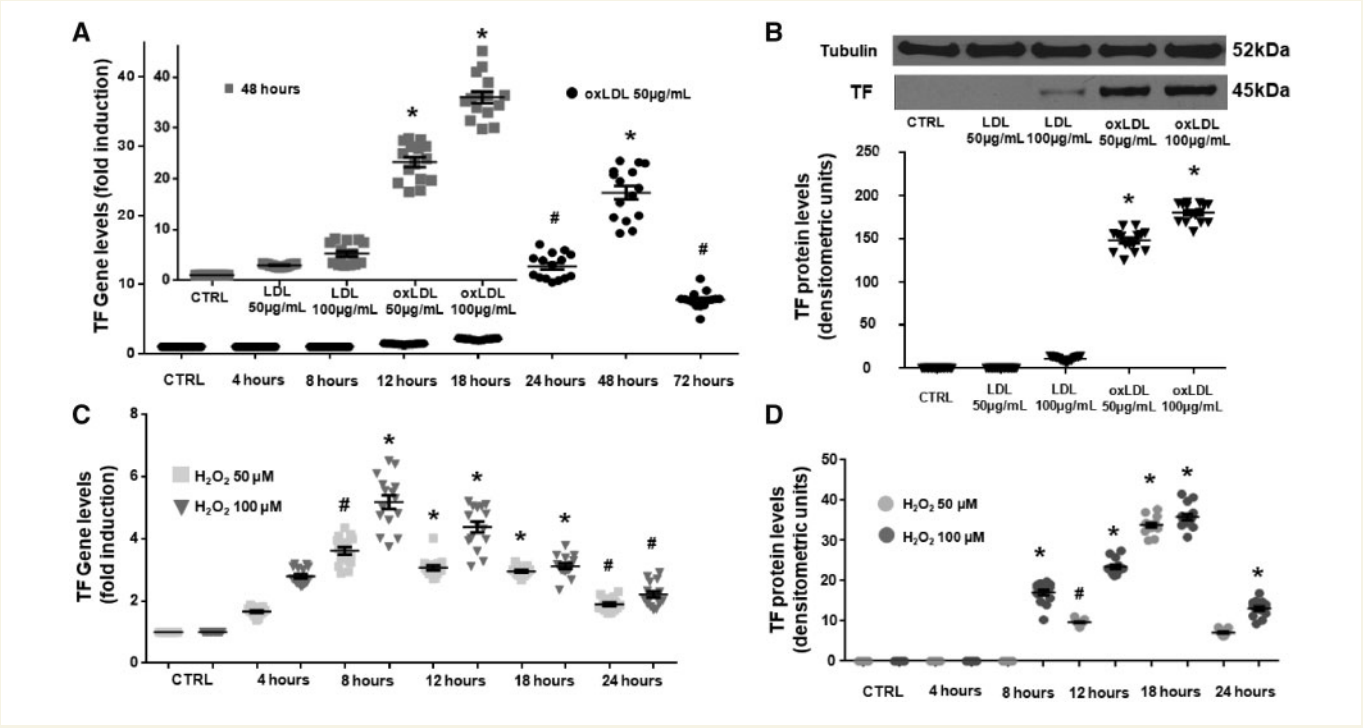


Figure 1 Effects of oxLDLs and H_2O_2 on TF expression by T-lymphocytes *in vitro*. $CD3^+$ lymphocytes were isolated from freshly prepared buffy coats and stimulated with either native LDLs, oxLDLs, or H_2O_2. (*A*) oxLDLs induced a time- and dose-dependent increase in TF gene levels, as TF gene expression increased significantly at 24 h, peaked at 48 h, and decreased significantly at 72 h following incubation with oxLDLs. Each column represents the mean ± SE of 15 experiments in triplicate ($^{\#}P < 0.01$, $^{*}P < 0.001$ vs. Control; one-way ANOVA with Tukey's *post hoc* test). Graph in the inset shows the dose-dependent effects of oxLDLs (50 and 100 μg/mL) at 48 h. (*B*) Changes in TF protein levels (measured by western blot analysis on cell lysates) paralleled those observed for TF gene. TF protein significantly increased at 72 h in oxLDL-treated cells in a dose-dependent manner, while only a slight effect was observed in LDL-stimulated cells. Each column represents the mean ± SE of 15 experiments in triplicate ($^{*}P < 0.001$ vs. Control; one-way ANOVA with Tukey's *post hoc* test). (*C*) Incubation of T-lymphocytes with H_2O_2 induced a slight expression of TF gene in a dose- and time-dependent manner. However, the time-course of TF expression was much earlier as compared to oxLDL-treated cells: TF gene levels reached the highest value at 8 h with a five-fold increase as compared to unstimulated cells. Each column represents the mean ± SE of 15 experiments in triplicate ($^{\#}P < 0.01$, $^{*}P < 0.001$ vs. Control; one-way ANOVA with Tukey's *post hoc* test). (*D*) Changes in TF protein levels (measured by western blot analysis on cell lysates) paralleled those observed for TF gene. TF protein significantly increased at 8 h in H_2O_2-treated cells in a dose-dependent manner. Each column represents the mean ± SE of 15 experiments in triplicate ($^{\#}P < 0.01$, $^{*}P < 0.001$ vs. Control; one-way ANOVA with Tukey's *post hoc* test).

2.9 Statistical analysis

Results are expressed as mean ± SE. All data from *in vitro* experiments were evaluated by D'Agostino–Pearson test for normal distribution. One-way ANOVA followed by the Tukey test was used for multiple comparisons. All analyses were performed using the GraphPad Prism 6.00 for Windows software (GraphPad Software, San Diego California, USA).

3. Results

3.1 oxLDLs induce TF gene and protein expression in T-lymphocytes

Incubation of T-lymphocytes with oxLDLs, but not with native LDLs, induced TF gene expression in a dose- and time-dependent manner (*Figure 1A* and inset), without affecting cell viability up to 50 μg/mL (data not shown). TF mRNA levels remained stable at baseline values up to 18 h, increased up to about 25-fold the control values at 48 h of incubation with oxLDLs 50 μg/mL, and significantly decreased at 72 h (*Figure 1A*). Taking into account the time-course of TF mRNA, protein levels were investigated by western blot up to 72 h following oxLDL stimulation. No detectable bands were observed at earlier time point (18 and 24 h, while a faint band was present at 48 h, data not shown). TF protein levels significantly increased at 72 h following exposure to oxLDLs (*Figure 1B*). No significant effect was observed in T cells exposed to native LDLs.

It has been previously demonstrated that oxLDLs result in a significant peroxidation of cellular lipid membranes.[27,28] Thus, to determine if the observed effects of oxLDLs on TF expression in T cells were non-specifically related to an increase in lipid peroxidation and/or ROS generation, T-lymphocytes were stimulated with H_2O_2 (50 and 100 μM). H_2O_2 at both concentrations determined an increase in TF expression both at gene and protein levels (*Figure 1C and D*). LOX-1 was also induced in a dose- and time-dependent manner (Supplementary material online, *Figure S1*). However, H_2O_2 stimulation of T cells resulted in a much lower TF expression, as compared to oxLDLs. Most importantly, induction of TF by H_2O_2 was much earlier with respect to oxLDLs, as TF gene was already significantly induced at 4 h of stimulation. This time-lag in TF expression between oxLDLs and H_2O_2 suggests that the effects

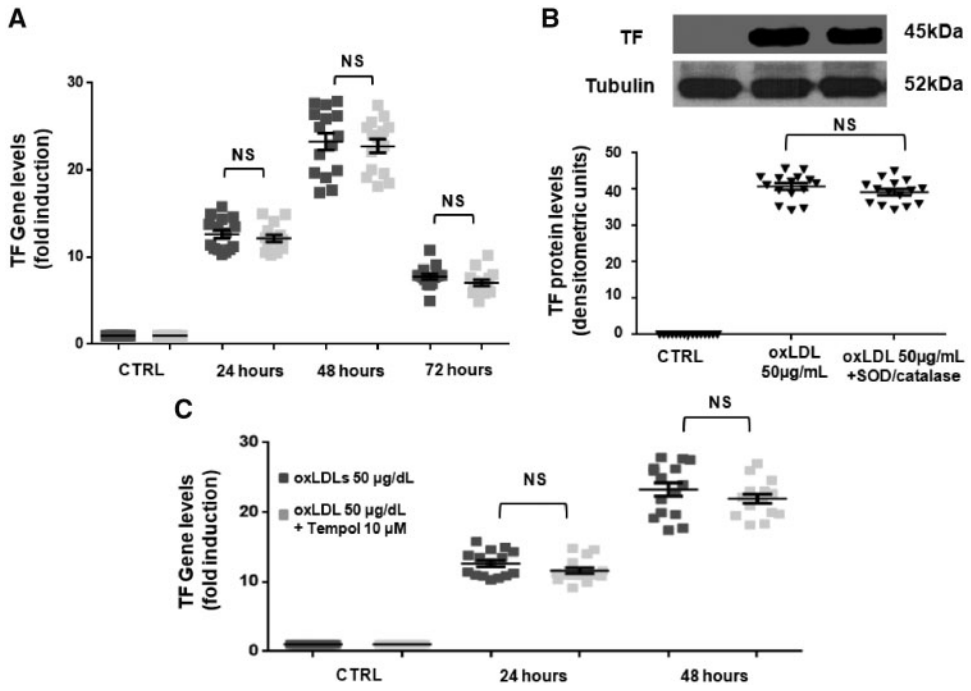


Figure 2 Effects of antioxidants (superoxide dismutase, catalase, and Tempol) on TF expression induced by oxLDLs. T-lymphocytes were pre-incubated with superoxide dismutase (SOD, 500 U/mL) and catalase (300 U/mL) or Tempol (10 µM) before stimulation with oxLDLs. SOD and catalase or Tempol did not significantly affect TF expression at gene (*A* and *C*) and protein levels (*B*). Each column represents the mean ± SE of 15 experiments in triplicate. Statistical analysis made by one-way ANOVA with Tukey's *post hoc* test. NS, not significant.

of oxLDLs were not related to an increase in lipid peroxidation but rather mediated via activation of some other intracellular pathways. Furthermore, pre-incubation with SOD and catalase did not significantly affect oxLDL-induced TF expression, both at gene and protein levels (*Figure 2A and B*), indicating that ROS generation does not significantly contribute to TF induction by oxLDLs. To strengthen these findings, we have also evaluated the effect of Tempol, an antioxidant that permeates biological membranes, on oxLDL-treated T cells. Pre-incubation with Tempol, similarly to SOD and Catalase, did not significantly affect oxLDL-induced TF expression (*Figure 2C*).

3.2 TF induced in T-lymphocytes by oxLDLs is translocated to the plasma membrane and is functionally active

Fluorescence-activated cell sorting (FACS) analysis showed that oxLDL-induced TF was translocated to the cell membrane of T cells as compared to LDL-stimulated or control cells (Supplementary material online, *Figure S2*). Of the overall $CD3^+$ T-lymphocyte population, 61.1 ± 2.9% were $CD4^+$ cells, while 33.6 ± 2.4% were $CD8^+$ cells. Both unstimulated subtypes did not significantly express TF on their membrane, while following stimulation with oxLDLs, 7.3 ± 2.1% of $CD4^+$ and 4.9 ± 1.5% of $CD8^+$ were also TF^+ (*Figure 3A*). Most importantly, surface-expressed TF was also functional as shown in the activity assay experiments reporting a significant increase in FXa generation from intact T-lymphocytes (*Figure 3B*).

3.3 oxLDLs induce TF in T-lymphocytes via expression of LOX-1 and activation of NF-κB pathway: role of NADPH oxidase

The time-lag of TF expression in oxLDL-stimulated T cells as compared to H_2O_2-stimulated cells suggested that the effects of oxLDLs were mediated by activation of a different intracellular pathway. Thus, we investigated whether oxLDL-induced TF expression was mediated by expression of LOX-1, the scavenger receptor of oxLDLs. Incubation of T cells with oxLDLs, but not with LDLs, induced expression of LOX-1 in a dose- and time-dependent manner (*Figure 4A* and inset). Gene levels remained stable at baseline values up to 12 h. At 18 h, a two-fold increase in LOX-1 gene levels was observed when T cells were incubated with oxLDLs (50 µg/mL), peaking up to six-fold the control values at 48 h (*Figure 4A*). Interestingly, confocal microscopy analysis revealed that unstimulated T cells express almost undetectable levels of LOX-1 on their surface (*Figure 4B*); in contrast, exposure to oxLDLs (50 µg/mL) for 72 h highly increased LOX-1 expression on T-cell surface (*Figure 4B*).

To verify whether TF expression in oxLDL-treated T-lymphocytes was related to LOX-1 activation, we pre-treated T cells with a low-density lipoprotein receptor blocking peptide (L-RBP) and anti-LOX-1 blocking antibody. Pre-incubation with L-RBP, as well as with the anti-LOX-1 antibody resulted in a significant suppression of oxLDL-induced TF expression both at gene and protein levels (*Figure 5A, B, D, and E*). TF procoagulant activity was also significantly reduced by both, L-RBP and TS92 (*Figure 5C*). Of note is the finding that L-RBP did not affect LOX-1 gene expression, as shown in *Figure 5D*.

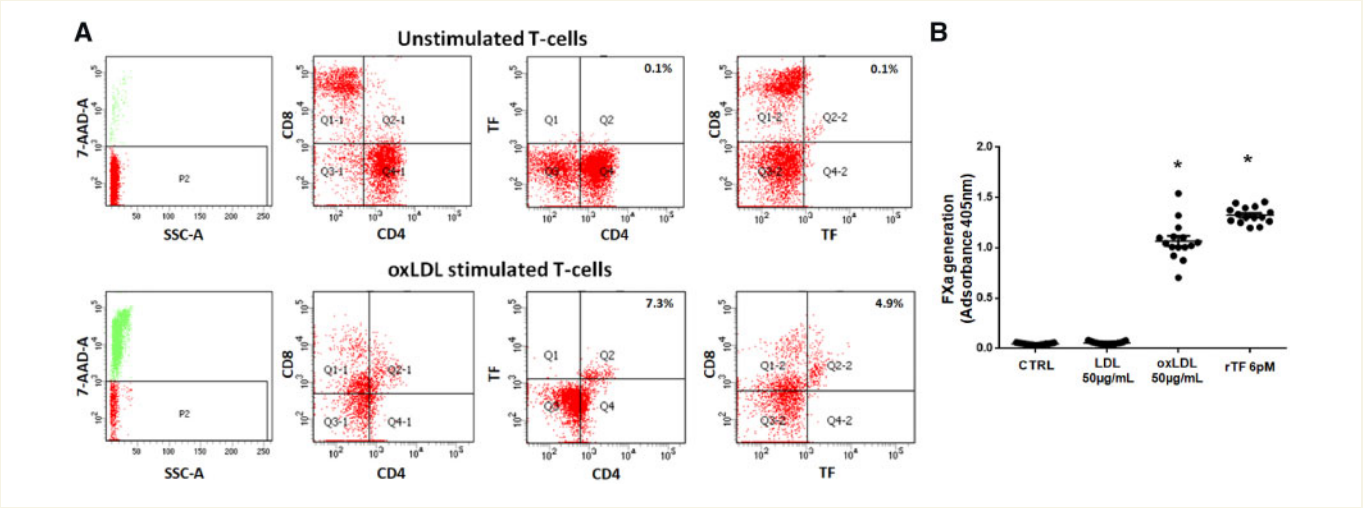


Figure 3 TF surface expression in oxLDL-treated T-lymphocytes. (*A*) FACS analysis was performed on live cells (red colour in 7-AAD-A box) and showed that oxLDL-induced TF was translocated to the cell membrane of T cells. Because of oxLDL toxicity some cells died (green colour), thus reducing the total cell population. Of the overall $CD3^+$ T-lymphocyte population, 61.1 ± 2.9% were $CD4^+$ cells, while 33.6 ± 2.4% were $CD8^+$ cells. Both unstimulated subtypes did not significantly express TF on their membrane, while following stimulation with oxLDLs, 7.3 ± 2.1% of $CD4^+$ and 4.9 ± 1.5% of $CD8^+$ were also TF^+ (*Figure 3A*). (*B*) TF procoagulant activity in intact T cells was measured by a modified chromogenic TF assay (see text for details). oxLDL stimulation resulted in a significant increase in FXa generation, as compared to unstimulated cells or cells treated with native LDLs, indicating that TF was translocated to the cell membrane in an active form. Activity of 6 pM recombinant re-lipidated TF (rTF) is reported as an internal control. Each column represents the mean ± SE of 15 experiments in triplicate (*$P < 0.001$ vs. Control; one-way ANOVA with Tukey's *post hoc* test).

Binding of oxLDLs to LOX-1 resulted in activation of NADPH oxidase (NOX) on the cell surface, as suggested by the increase in intracellular p47 levels, which in turn resulted in increased cytosolic levels of NF-κB and reduced levels of IκB (*Figure 6A*). The NOX is a multicomponent enzyme with up to six subunits, including p47-phox[29]; NOX activation on the cell surface usually results in intracellular ROS formation, ultimately leading to the redox-sensitive NF-κB signalling pathway.[30,31] Interestingly, pre-incubation with the L-RBP significantly reduced p47 phosphorylation and NF-κB activation, while it increased IκB levels (*Figure 6A*). NF-κB translocation is also reduced by anti-LOX-1 antibody (*Figure 6B*). Furthermore, DPI, a potent inhibitor of NADH/NADPH oxidase,[17] resulted in a significant inhibition of TF gene expression (*Figure 6C*).

To evaluate if the observed effects oxLDLs on TF expression were related at least in part to the lipid peroxidation induced by oxLDLs, we have measured lipid peroxidation products, expressed as concentration of MDA levels following stimulation with oxLDLs or H_2O_2. MDA levels were significantly higher in H_2O_2-treated cells at both concentrations, as compared to oxLDL-treated cells (Supplementary material online, *Figure S3A*). However, while incubation of T cells with H_2O_2 induced expression of LOX-1 mRNA in a dose- and time-dependent manner to a higher extent as compared to oxLDLs (Supplementary material online, *Figure S3B*), H_2O_2 induced only a slight increase in TF expression, again as compared to oxLDL-stimulated cells (Supplementary material online, *Figure S3C*).

3.4 T-lymphocytes in human carotid atherosclerotic plaques express both LOX-1 and TF on their surface

Immunohistochemistry staining of atherosclerotic lesions collected from patients undergoing thromboendarterectomy (TEA) resulted cell enriched, as evidenced by haematoxylin and eosin staining (*Figure 7*, Panel 1A). Some of these cells were T-lymphocytes, as they were positive for CD3 antigen (*Figure 7*, Panel 1B), some of which were also positive for TF antigen (*Figure 7*, Panel 1C).

3.5 Immunofluorescence on human carotid atherosclerotic lesion

We asked if T-lymphocytes present in atherosclerotic lesions expressing TF were also able to express LOX-1 protein. We performed immunofluorescent staining of human carotid atherosclerotic lesions with rabbit monoclonal anti-CD3 and mouse monoclonal anti-LOX-1 antibodies, or on consecutive serial section, rabbit polyclonal anti-TF. Results are shown in Supplementary material online, *Figure S4*. T-lymphocytes $CD3^+$ expressed LOX-1 and TF. Successively we asked which class of T-lymphocytes was represented in human carotid atherosclerotic lesions. We performed immunofluorescence and confocal microscopy on FFPE 4 µm thick sections from human carotid atherosclerotic lesions using the Opal kit (see Section 2) with antibodies anti CD4, TF, LOX-1, and on a consecutive serial section with antibodies anti CD8, TF, and LOX-1. As shown in *Figure 7*, Panel 2, in carotid atherosclerotic lesions, lymphocytes $CD8^+$ that were also TF+ and LOX-1+ were present. Antibody anti CD4 gave no staining.

4. Discussion

In the present study, we report for the first time that oxLDLs but not native LDLs induce a pro-thrombotic phenotype in T-lymphocytes by inducing TF expression via activation of LOX-1 receptor. Of note is the observation that TF was translocated to the cell membrane and was functionally active. This observation is in line with and extends our

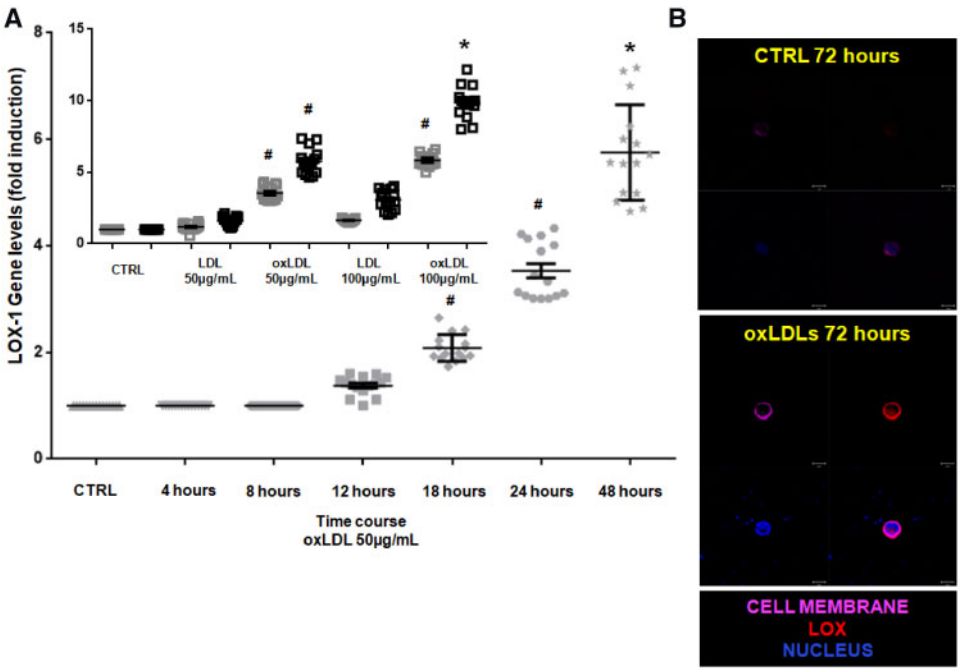


Figure 4 Effects of oxLDLs on LOX-1 expression by T-lymphocytes *in vitro*. $CD3^+$ lymphocytes were isolated from freshly prepared buffy coats and stimulated with either native LDLs or oxLDLs. (*A*) oxLDLs induced a time- and dose-dependent increase in LOX-1 gene levels, as its expression increased significantly at 18, 24, and 48 h following incubation with oxLDLs. Each column represents the mean ± SE of 15 experiments in triplicate ($^{\#}P < 0.01$, $^{*}P < 0.001$ vs. Control; one-way ANOVA with Tukey's *post hoc* test). Graph in the inset shows the dose-dependent effects of oxLDLs (50 and 100 μg/mL) at 24 and 48 h. (*B*) For confocal microscopy experiments, T-lymphocytes (3×10^6) were incubated with oxLDLs (50 μg/mL) for 72 h. Cells were then incubated with 0.5% Cell Marker Deep Red, fixed in 4% para-formaldehyde and stained with a primary antibody anti-human LOX-1 and incubated with a secondary antibody. Experiments were carried out on an inverted and motorized microscope. Fluorescence emission was revealed by main dichroic beam splitter and variable secondary dichroic beam splitter. Triple staining fluorescence images were acquired separately in the blue, red, and infra-red channel. Unstimulated cells expressed very low (almost undetectable) levels of LOX-1 on cell surface (red colour). Exposure to oxLDLs (50 μg/mL) for 72 h highly increases LOX-1 expression on T-cell surface.

previous findings that selected pro-inflammatory cytokines may induce active TF expression in isolated T cells (cells membrane and bearing microparticles[9]) *in vitro* and in patients with ACS.[8]

Another interesting finding of the present study is that the effects on T cells were not related to an increase in oxidative stress via lipid peroxidation/ROS generation but instead were mediated by the induction/activation of LOX-1. Indeed, experiments conducted in presence of L-RBP, an inhibitor of LDL binding to its receptor, or a blocking monoclonal antibody against human LOX-1, almost completely suppressed oxLDL-induced TF expression.

We have also analysed the expression of TF in T-cell resident within human atherosclerotic lesions obtained from patients undergoing TEA. Immunohistochemistry analysis showed that these lesions were enriched in $CD3^+/CD8^+$cells, most of which were also TF^+ and $LOX\text{-}1^+$. In addition, immunofluorescence analysis of the same plaques revealed the presence of T cells expressing both TF and LOX-1, indicating the potential clinical relevance of the findings observed *in vitro*.

Taken together, the data of the present study demonstrate, for the first time, that T-lymphocytes exposed to oxLDLs acquire a pro-thrombotic phenotype by expressing functional TF on their membrane, as already reported for monocytes,[32] *independently* on the potential role of oxLDLs as a non-self-antigen, namely via activation of LOX-1, and that within atherosclerotic lesions, these cells might directly contribute to the onsite thrombotic process *in vivo*, linking atherosclerosis, immunity and thrombosis in a single multi-step process.

It is widely recognized that atherosclerosis is an inflammatory disease, where immunocompetent cells producing pro-inflammatory cytokines are abundant in atherosclerotic lesions.[33] In addition, important components of the atherosclerotic plaque include dead cells, debris, and modified forms of lipids, particularly oxLDLs.[34] It has been convincingly demonstrated that T cells with pro-inflammatory properties (i.e. Th1 subsets) play a key role not only in initiating the development of the atherosclerotic plaque, but also in promoting its complication (rupture, fissuration, etc.), leading to the clinical occurrence of ACS. For example, several studies have demonstrated that complicated plaques show a higher content of inflammatory cells, often located near the site of rupture, thus suggesting an active role in plaque rupture (for review, see ref. 34). In a study from our group employing coronary plaque specimens obtained during directional coronary atherectomy from living patients with ACS we have investigated the T-cell repertoire, demonstrating a specific, immune-driven activation of T cells within unstable plaques.[3] In particular, we have shown that unstable plaques contain a much greater T-cell infiltration than stable plaques and that the T-cell receptor (TCR) repertoire within the unstable plaques was highly skewed in all patients with ACS, as compared to patients with stable angina, indicating a specific antigen-driven T-cell recruitment within the culprit lesions of patients

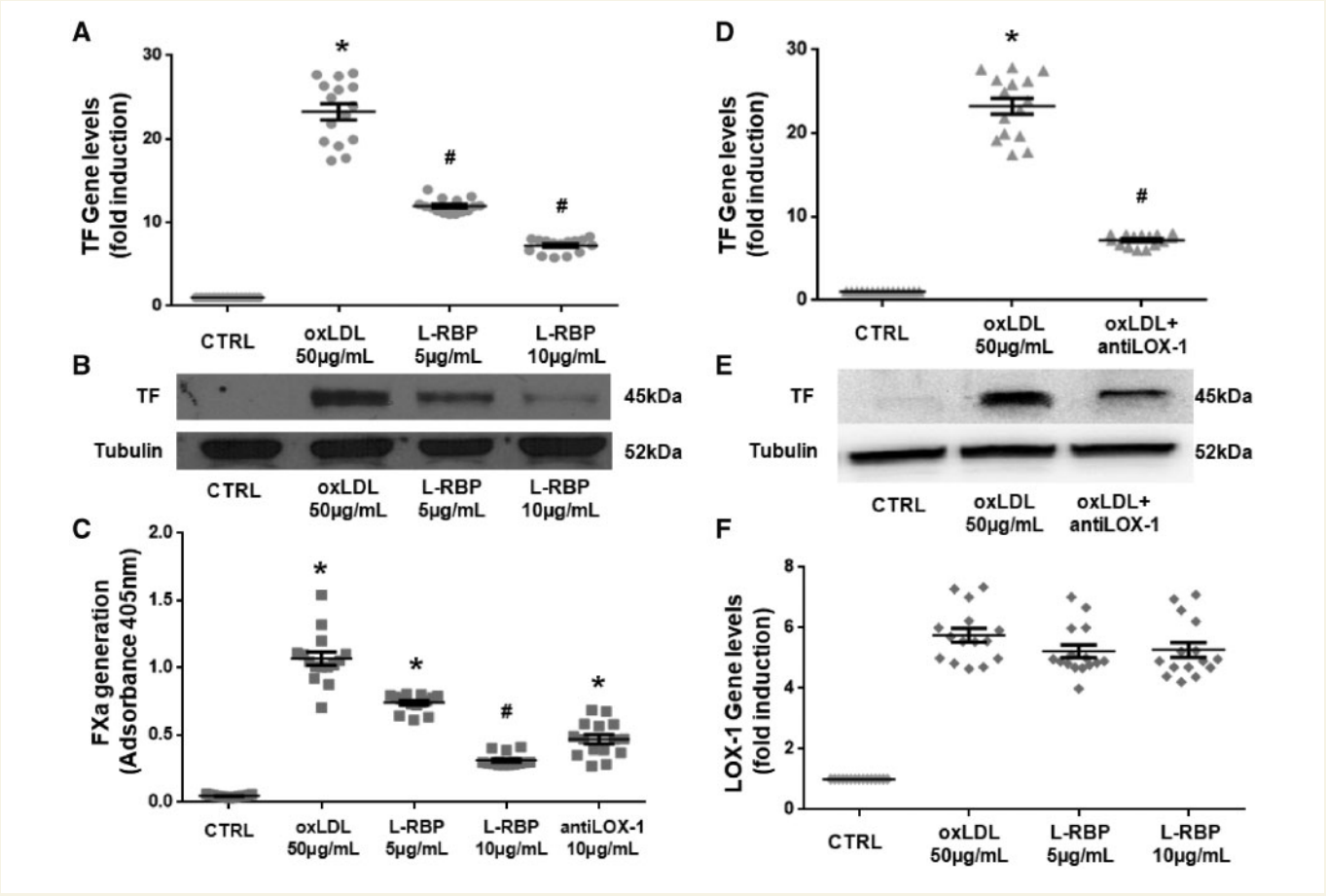


Figure 5 Effects of LOX-1 inhibition on TF and LOX-1 expression by T-lymphocytes stimulated with oxLDLs. T cells were pre-incubated with a low-density lipoprotein receptor blocking peptide (L-RBP) or anti-LOX-1 blocking antibody and then stimulated with oxLDLs. (*A* and *B*) L-RBP resulted in a dose-dependent suppression of TF expression at gene and protein levels (*$P < 0.001$, #$P < 0.01$ vs. Control; one-way ANOVA with Tukey's *post hoc* test). (*C*) TF procoagulant activity is significantly affected by L-RBP and anti-LOX-1 blocking antibody pre-treatment (*$P < 0.001$, #$P < 0.01$ vs. Control; one-way ANOVA with Tukey's *post hoc* test). (*D* and *E*) Anti-LOX-1 blocking antibody resulted in a significant suppression of TF expression at gene and protein levels (*$P < 0.001$, #$P < 0.01$ vs. Control; one-way ANOVA with Tukey's *post hoc* test).(*F*) In contrast, L-RBP had no effect on LOX-1 gene expression (*D*). Each column represents the mean ± SE of 15 experiments in triplicate.

with ACS.[3] However, the search for of the putative antigen(s) responsible for the local activation of the T cells still remains elusive.

According to the leading hypothesis, modified forms of LDLs, including oxLDLs may play an important role in the occurrence of plaque complication by being recognized as non-self-antigens by T-lymphocytes. Previous studies have indeed suggested that oxLDLs can function as T-cell antigens in the context of atherogenesis;[35,36] interestingly, Liu *et al.*[7] have recently demonstrated that enzymatically modified forms of LDLs, but not native LDLs, promote dendritic cell maturation and T-cell activation, suggesting an involvement of the TCR. Whether oxLDLs may induce TF in dendritic cells via activation of LOX-1 is not known at the moment; it should be pointed out, however, that in the present study we provide evidence of the interplay between T-lymphocytes and oxLDLs in a way different from the 'classical' TCR-mediated activation of lymphocytes. In fact, we have shown that oxLDLs may activate T cells via induction of the scavenger receptor, LOX-1. Binding of LOX-1 to oxLDLs then induced expression of functional TF on the T-cell membrane. Confocal microscopy experiments confirmed that LOX-1 is almost undetectable in unstimulated T cells, while it is highly expressed on T-cell membrane following exposure to oxLDLs.

Another potential mechanism by which oxLDLs may activate T-lymphocytes is represented by an increase in oxidative stress. For example, it is well established that oxLDLs increase intracellular levels of ROS and lipid peroxidation end-products.[28] To verify if TF expression was related to an oxLDL-induced oxidative stress, we have conducted two different sets of experiments: first, we have quantified lipid peroxidation in T cells stimulated with oxLDLs or hydrogen peroxide. Secondly, we have measured TF and LOX-1 expression in H_2O_2-treated cells and have compared it with that obtained after oxLDL stimulation. Although H_2O_2 induced significantly higher levels of lipid peroxidation than oxLDLs, the effects on TF expression were of a significantly lesser order of magnitude. Interestingly, H_2O_2 induced high levels of LOX-1 gene as early as 4 h in a time- and dose-dependent manner. We may speculate that peroxidation by H_2O_2 induces early expression of LOX-1, that is used by oxLDLs for binding and amplification of LOX-1 expression in line with other observation in different cells type.[37,38] More important,

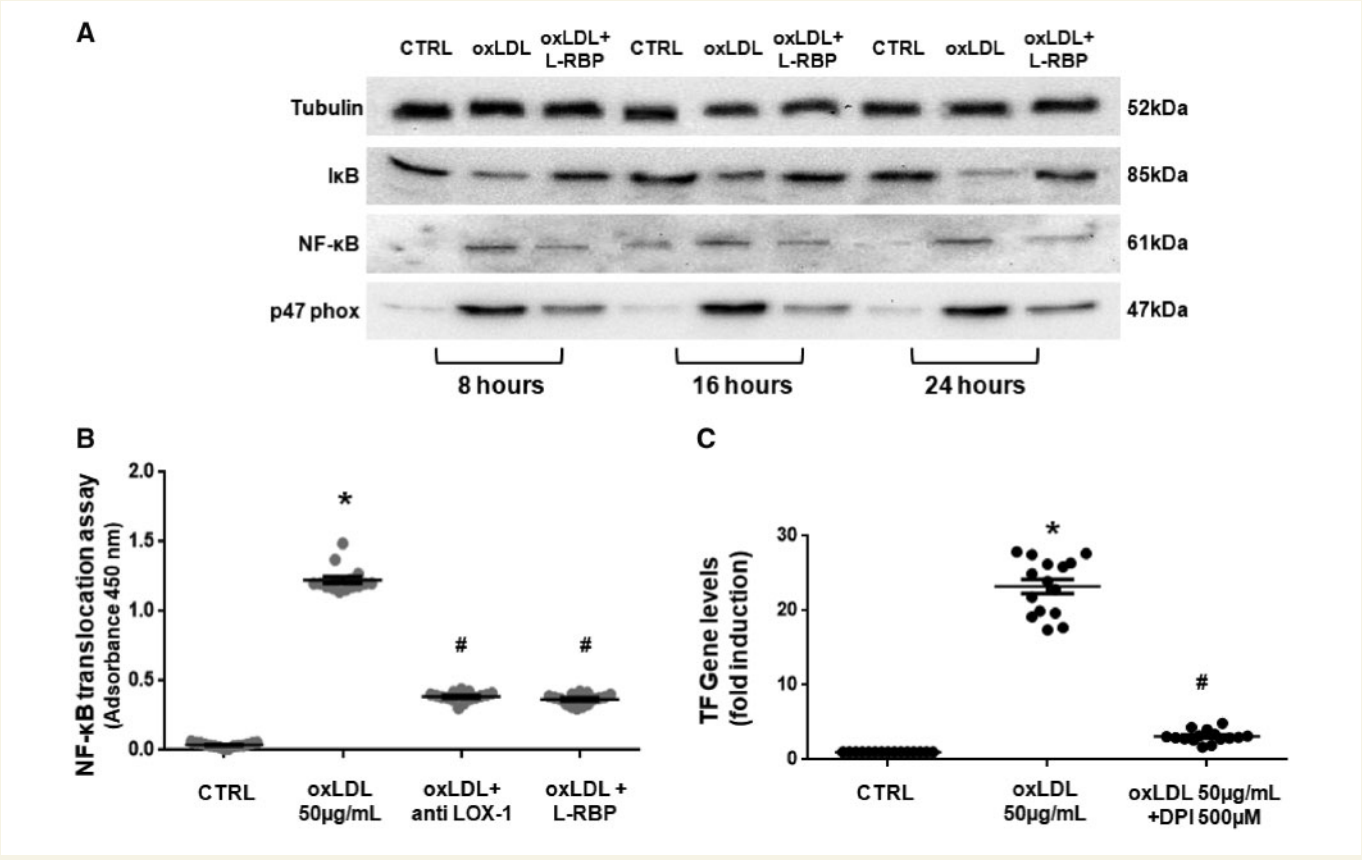


Figure 6 Intracellular molecular pathways activated by oxLDL stimulation of T-lymphocytes. T-lymphocytes were stimulated with oxLDLs for 8, 16, and 24 h. Cell lysates were used to measure cytosolic NADPH and NF-κB activation. (*A*) Western blots analyses. oxLDLs/LOX-1 interaction induced NAPDH oxidase activation, as shown by the increased levels of p47 phox (one of the intracellular subunit of the NADPH system) that in turn resulted in increased cytosolic levels of NF-κB and reduced levels of IκB. Conversely, pre-incubation with L-RBP, reduced significantly p47-phox and NF-κB and increased IκB. (*B*) NF-κB translocation to the nucleus is significantly reduced by L-RBP and anti-LOX-1 blocking antibody pre-incubation (*$P < 0.01$ vs. CTRL, #$P < 0.01$ vs. oxLDL; one-way ANOVA with Tukey's *post hoc* test). (*C*) T cells pre-incubated with DPI (500 μM) resulted in a significant inhibition of TF gene expression (*$P < 0.01$ vs. CTRL, #$P < 0.01$ vs. oxLDL; one-way ANOVA with Tukey's *post hoc* test).

in oxLDL-treated cells, pre-incubation with SOD and catalase did not affect significantly TF expression. In addition, pre-treatment with Tempol, an intracellular antioxidant, did not significantly affect oxLDL-induced TF expression. These findings, together with the observation that in T cells stimulated with oxLDLs in presence of L-RBP, an inhibitor of LDL binding to its receptor, or a blocking anti-LOX-1 antibody, TF expression was almost completely abolished clearly indicate that the effects of oxLDLs on T cells are largely mediated via interaction with LOX-1. Finally, one additional observation further supports this conclusion: binding of oxLDLs to LOX-1 activates NADPH oxidase on the cell membrane that results in rapid formation of intracellular ROS,[29] which in turn activate the redox-sensitive NF-κB signalling pathway, generating different intracellular responses.[30,31]

The NADPH oxidase (NOX) is a multicomponent enzyme made up of six subunits: a Rho guanosine triphosphatase (GTPase) and five 'phox' units, one of which is p47.[39] NOX is known to be one of the major sources of intracellular ROS. Once activated, the G proteins Rac1 or Rac2 and the phosphorylated cytoplasmic p47phox subunit initiate migration of the cytosolic elements to the plasma membrane, to form a functional complex that generates ROS.[40] The p47phox acts as a regulatory subunit of NOX to initiate assembly of active oxidase, which is important for NOX activation.[39] It has been reported that inhibition of p47phox abrogated NOX function and diminished ROS production in ovarian and prostate cancer cells.[41] Depending on the level of ROS, different redox-sensitive transcription factors are activated and co-ordinate distinct biological responses. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB and AP-1. NF-κB was the first transcription factor shown to be redox-regulated.[42]

In the present study, oxLDLs/LOX-1 interaction induced NAPDH oxidase activation as shown by the increased levels of p47 that in turn resulted in increased cytosolic levels of NF-κB and reduced levels of IκB. Conversely, pre-incubation with L-RBP significantly blunted the increase in p47 and NF-κB, while it increased IκB. In line with these results is the observation that the use of DPI, a potent NAPDH oxidase inhibitor, significantly reduced oxLDL-induced TF expression.

In conclusion, the present study shows for the first time that T cells may acquire a pro-thrombotic phenotype via expression of functional TF in response to oxLDLs and that this phenomenon is largely mediated

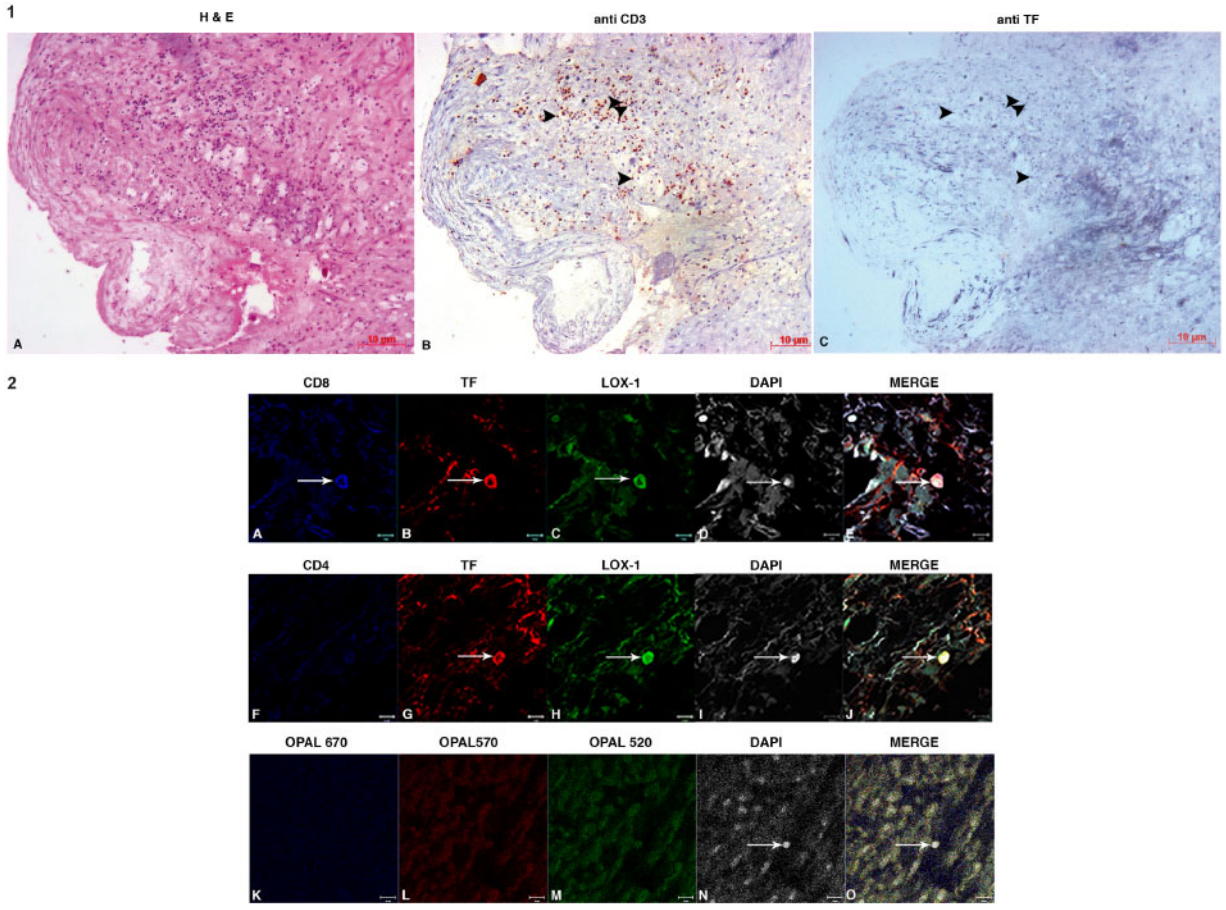


Figure 7 Panel 1: Immunohistochemistry and immunofluorescence of human carotid atherosclerotic plaques. Atherosclerotic plaques collected from patients undergoing TEA resulted cellularly enriched as evidenced by haematoxylin and eosin staining (*A*). Interestingly, some of these cells were positive for CD3 antigen (*B*, arrows), and also expressing TF (*C*, arrows). Panel 2: T-lymphocytes characterization in atherosclerotic lesion. In atheroma, cytotoxic T-lymphocytes ($CD8^+$), express TF and LOX-1. Tissue sections from representative carotid atheroma were stained, as described in Section 2, with antibodies anti-CD8 (*A*), anti-CD4 (*F*), anti-TF (*B* and *G*), and LOX-1 (*C* and *H*). Section from atheroma shows cell stained with anti-CD8 antibody (Blu, *A* white arrow), the same cell is also stained by anti-TF antibody (Red, *B* white arrow), and anti-LOX-1 antibody (Green, *C* white arrow). In sequential section from the same atheroma, antibody anti-CD4 gave no staining (Blu, *F*) while there are cells stained by anti-TF antibody (Red, *G* white arrow) and anti-LOX-1 antibody (Green, *H* white arrow). As negative control a section was stained only with Opal Polymer horseradish peroxidase (HRP) anti-Mouse–Rabbit secondary antibody solution and then incubated with the Opal Fluorophore 670 (*K*), 570 (*L*), and 520 (*M*). Nuclei were counterstained with DAPI (white *D*, *I*, *N*, white arrow), Merge (white arrow) is shown in *E*, *J*, *O* (bar = 10 μm).

by activation of LOX-1. Taken together with previous studies[3,9,43,44] data of the present study support the relevant contribution of T cells in atherosclerosis and thrombosis. Considering the key role of LOX-1 in this mechanism, future studies exploring the effects of its modulation in the clinical scenario of acute cardiovascular events are warranted.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Translational perspectives

Despite significant advancements have been made in antithrombotic therapy, current strategies still fail to prevent acute thrombotic coronary events in a substantial number of patients, indicating that the complex mechanisms underlying the pathophysiology of ACS are not fully elucidated. The present study demonstrates, for the first time, that T-lymphocytes may express functional TF in response to oxLDLs via mechanisms other than the 'classical' activation of the T-cell receptor, namely via interaction with LOX-1. Further studies aimed at interfering with LOX-1 in T-lymphocytes might offer innovative and additional therapeutical approaches for ACS patients.