TNF-induced LDL cholesterol accumulation involve elevated LDLR cell surface levels and SR-B1 downregulation in human arterial endothelial cells

Emmanuel Ugochukwu Okoro (eokoro@mmc.edu)
Meharry Medical College https://orcid.org/0000-0001-6010-6315

Research Article

Keywords: cell surface receptor, cholesterol, endothelial cell, lipoprotein, low-density lipoprotein (LDL), tumor necrosis factor (TNF)

Posted Date: March 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-267515/v2

Version of Record: A version of this preprint was published on June 9th, 2021. See the published version at https://doi.org/10.3390/ijms22126236.

Abstract

Excess lipid droplets are frequently observed in arterial endothelial cells at sites of advanced atherosclerotic plaques. Here, the role of tumor necrosis factor alpha (TNF) in modulating low density lipoprotein (LDL) content in confluent primary human aortic endothelial cells (pHAECs) was investigated. TNF promoted up to 2 folds increase in cellular cholesterol, which was resistant to ACAT inhibition. The cholesterol increase was associated with increased ^{125}I-LDL surface binding. Using the non-hydrolysable label, DiI, TNF could induce a massive increase in DiI-LDL by over 200 folds. The elevated intracellular DiI-LDL was blocked with excess unlabeled LDL and PCSK9, but not oxidized LDL (oxLDL), receptor associated protein (RAP), or apolipoprotein (apoE) depletion. Moreover, TNF-induced increase of LDL-derived lipids was elevated through lysosome inhibition. Using specific LDLR antibody, the DiI-LDL accumulation was reduced by over 99%. Effects of TNF included LDLR cell surface increase by 138%, and very large increases in ICAM-1 total and surface proteins, respectively. In contrast, that of scavenger receptor B1 (SR-B1) was reduced. Additionally, LDLR antibody bound rapidly in TNF-treated cells by about 30 folds, inducing a migrating shift in the LDLR protein. The effect of TNF on DiI-LDL accumulation was inhibited by the antioxidant tetramethythiourea (TMTU) dose-dependently, but not by inhibitors against NF-κB, stress kinases, ASK1, JNK, p38, or apoptosis caspases. Grown on transwell inserts, TNF did not enhance apical to basolateral LDL cholesterol or DiI release. It is concluded that TNF promotes LDLR functions through combined increase at the cell surface and SR-B1 downregulation.

Introduction

TNFα was originally described as a tumor-selective hemorrhagic factor [1, 2]. However, it is now recognized to have multiple cell-type dependent effects [3, 4]. Released as a 17 kD soluble protein from a transmembrane precursor by tumor necrosis factor converting enzyme (TACE) [5-7], this cytokine mainly promotes changes that facilitate leukocyte (inflammatory) functions. [3, 8]. Acting in part through the rapid-acting transcription factor, nuclear factor kappa B (NF-κB), it can induce expression of pro-inflammatory mediators such as interleukin 1 and 2 [9, 10]. Likewise, it is capable of inducing apoptosis [11-13], possibly through c-jun N-terminal kinase (JNK) activation [14-16]. Thus TNF may clear way for passage of leukocytes through target epithelial cell apoptosis. On the other hand, it can induce proliferation of lymphocytes and fibroblasts [12, 17-19]. This ability to induce proliferation have been reported to be due to upregulation of growth factor receptors [20, 21] or growth factor release, such as vascular endothelial growth factor (VEGF) [21-23] .

Background inflammation is an independent risk factor for atherosclerotic cardiovascular disease [24]. Serum concentration of TNF increases with severity of atherosclerotic lesion [25]. Thus, it has been reported to be about 200 folds higher in the atherosclerotic lesion than in the circulating blood [26]. Further, age [27], infection [28, 29], and psycho-environmental stress [30] are associated with elevated TNF.

The effect of TNF on atherosclerosis development is mixed. On one hand, it has been reported to promote advanced lesion development [31-34]. In contrast, it but suppresses early fatty streak [34] and antibodies against TNFα have been reported to induce advanced lesions [35]. The pro-atherogenic arm of TNF is in part due to its ability to enhance monocyte entry into the intima through induction of adhesion molecules on arterial endothelial cells. In atherosclerotic lesions, macrophages [36] and smooth muscle cells [36, 37] have been found to express TNF, where it acts on overlying endothelial cells to stimulate expression of leukocyte adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) [38]. More, it induces expression of monocyte chemoattractant protein I (MCP-1) [39], which stimulates gradient migration of lymphocytes and monocytes [40, 41].

Beyond its effects on leukocyte-to-endothelial adhesion proteins, the precise mechanism(s) by which TNF affects atherosclerosis development is poorly understood. For example, tumor necrosis factor receptor 1 (TNFR1) has been reported to be anti-atherogenic [42, 43], pro-atherogenic [31, 44, 45], or neutral [46]. Likewise, TNFR2 has been reported to be pro-atherogenic [47] or neutral [43]. These conflicting results suggest that the mechanisms by which TNF affects atherosclerosis development need further clarification, especially at the level of the intima.

Unlike macrophages [48, 49] and smooth muscle cells [48, 50], normal confluent endothelial cells are resistant to cholesterol accumulation [51]. However, fibro-fatty atherosclerotic lesions contain endothelial foam cells with intact monolayer [52-54]. In this report, the effect of TNF on the pro-atherogenic lipoprotein, LDL [55, 56] was investigated. Importantly, it has been suggested that the pro-atherogenic function of TNF is related to it effects on non-inflammatory cells of the arterial wall [44].

Here, evidence is pressented that native LDL uptake alteration in pHAECs may contribute to the atherogenic nature of TNF and LDL. It will be shown that TNF increases pHAEC cholesterol and lipid accumulation by enhancing LDL receptor (LDLR) functions.

Materials And Methods

Materials

Reagents	Company	Catalog number	Reagents	Company	Catalog number
[^{3}H]Cholesterol	Perkin Elmer	NET139001	JNK inhibitor	Sigma-Aldrich	420118
ABCA1 antibody	Santa Cruz Biotechnology	sc-58219	LDLR antibody	R&D Systems	AF2148
Antibiotic-antimycotic	Thermo Fisher Scientific	15240112	mHBSS	Sigma-Aldrich	H8264
Anti-goat-HRP	R&D Systems	NL001	NF-kB Inhibitor	Sigma-Aldrich	481412
ApoE antibody	Santa Cruz Biotechnology	sc-13521	NF-kB Inhibitor	Tocris	4590
ApoE-conjugated agarose	Santa Cruz Biotechnology	sc-13521 AC	Normal goat IgG	R&D Systems	AB-108-C
ASK 1 inhibitor	Tocris	MSC 2032964A	Normal mouse Ig agarose	Santa Cruz Biotechnology	sc-2343
BCA assay kit	Thermo Fisher Scientific	23227	p38 inhibitor	Sigma-Aldrich	506163
Bond elut column	Agilent Technologies	12256060	PCSK9	Caymen Chemical	20631
Caspase inhibitor	Tocris	2163	pHAECs	ATCC	PCS-100-011
Caveolin-1 antibody	Santa Cruz Biotechnology	sc-53564	Phosphatase inhibitor	Sigma-Aldrich	P0044
Centrifugal filter	Pall Corporation	MAP003C36	Protease inhibitor	Sigma-Aldrich	P8340
Cholesterol esterase	MP Biomedicals	105439	RAP	Enzo Life Sciences	BML-SE552-0100
Cholesterol oxidase	Sigma-Aldrich	C8649	Sandoz 58-035	Sigma-Aldrich	S9318
Desalting columns	Thermo Fisher Scientific	89890	Scopoletin	TCI America	S0367
Dialysis membrane	Spectrum Labs	131204	Sodium heparin	Thermo Fisher Scientific	J16920-EXT
Dil	Sigma-Aldrich	468495	Sodium taurocholate	Beantown Chemicals	141920
EZ-link-Biotin	Thermo Fisher Scientific	A39258	SR-B1	Novus Biologicals	NB400-104
		306	Strepavidin	GE Healthcare	28-9872-

					30
FBS	R&D Systems	S11550	TMTU	Alfa Aesar	L13392
Glass bottom dishes	Cellvis	P96-0-N	TNF	ProSci Inc	96-734
HRP	Alfa Aesar	J60026	VBM	ATCC	PCS-100-030
ICAM1 antibody	Santa Cruz Biotechnology	sc-107	VEGF	ATCC	PCS-100-041
Iodination beads	Thermo Fisher Scientific	28665			

Cell culture and incubations. pHAECs, ATCC, lots 70001318, 63233442, and 64323512, passages 3-7, were used during the course of the experiments, with similar results. The majority of experiments were performed using lot 70001318. Characteristics were routinely verified by criteria of cobblestone morphology at confluence and expression of von Willenbrand factor. The cells were maintained in VBM with growth factor kit (VEGF) in the presence of 15% FBS, and antibiotic, antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B. Under serum-free conditions, the cells were cultured in VBM, VEGF, with the latter antimicrobials and 0.1% FAF-BSA. Incubations were at 37 °C, unless indicated otherwise. Experiments were best started with pHAECs confluent for 5 days or longer. Separate cells from individuals (2-36 years old) were used during the course of the experiments, with similar results. Incubations were 24 ± 5 hours. For cell-associated studies at 37 ºC, cells were washed 3X with mHBSS at 0 ºC. Imaging studies demonstrated that the vast majority of Dil-lipoproteins were within the cell under these conditions. For intracellular LDL levels, pHAECs were incubated at 0 °C for 5 minutes with ice cold 400 units/ml sodium heparin in mHBSS, washed twice with the same buffer, then two more times with ice cold mHBSS, to remove surface LDL. In experiments using excess unlabeled lipoproteins, the volume represented 3% or less of the culture medium. Lipids were extracted as described below or the cells were fixed with 4% paraformaldehyde in PBS prior to microscopy.

Lipoprotein purification. All procedures were performed between 0 and 4 °C. Centrifugations and dialysis were performed at 4 °C. Lipoproteins were isolated from freshly drawn human blood anticoagulated with Na_2EDTA. The blood was centrifuged at 4,000 g for 30 minutes to obtain the plasma. To the plasma, butylated hydroxytoluene (BHT) in DMSO was added to 45 µM (0.01%). Then, sequential density ultracentrifugations [57] were performed to obtain d<1.006 (VLDL), d=1.019-1.063 (LDL), or d=1.12-1.21 (HDL3) g/ml. Ultracentrifugations were done using type Ti70 rotor at 50,000 rpm for 20 (VLDL and LDL) or 48 hours (HDL3). The lipoproteins were dialyzed through ~4 kD molecular weight cut-off membrane in 3 successions against ~180 times dialysis buffer (DB): 10 mM Tris-HCl, 150 mM NaCl, 0.3 mM Na_2EDTA, pH 7.5, with deionized water, in the dark, each lasting about 24 hours. After concentration using 3 kD MWCO centrifugal filters, the LDL was filtered through 0.2 µm membrane under sterile conditions. Lipoprotein contents were routinely verified by western blotting and coomassie staining. In addition, the

cholesterol and cholesteryl ester contents were verified to be consistent with previous publications [58-60]. Lipoprotein concentrations represent the protein content throughout the manuscript.

Cellular cholesterol determination. Lipids were extracted into hexane: isopropanol (1:1) at room temperature for 3 hours, then the solvent was evaporated at room temperature using centrivap concentrator (Labconco). The extracted lipids were redissolved in isopropanol. To determine the unesterified cholesterol content, the lipids in isopropanol were mixed with 10 times volumes of cholesterol assay buffer (CAB): 0.1% FAF-BSA, 2 mM sodium taurocholate, 50 mM Tris-HCl, pH 7.5, 0.3 mM Na_2EDTA, 5% isopropanol, and 250 mM sucrose, with freshly added 0.5 U/ml horseradish peroxidase (HRP) and 0.02 U/ml cholesterol oxidase in the presence of 30-50 μM scopoletin [61] (excitation 360, emission 460). The CAB was kept at room temperature to ensure full solubilization of the lipids. This enzymatic approach is a modification of a previously published procedure [62]. Total cholesterol was determined as above, with the addition of 0.1 U/ml cholesteryl esterase. Readings were obtained after incubation at 37 °C for 20 minutes. Cholesteryl esters were determined by subtracting unesterified cholesterol from the total cholesterol. The residual cell matter after lipid extraction was lysed with 0.1 M NaOH, 0.1% SDS, for protein reading using BCA assay kit.

^{3}H-cholesteryl ester ([^{3}H]CE) generation. 40 μCi of ^{3}H-cholesterol in ethanol was added in 10 μl aliquots to 40 ml of 0.2 μm filtered human serum under sterile conditions. The mixture was incubated at 37 °C for 48 hours in the dark. ^{3}H-cholesteryl esters and other lipids were extracted into chloroform: methanol [63]. After evaporation of the solvent, the residue was dissolved in a minimal volume of chloroform. Subsequently, ^{3}H-cholesteryl esters were purified using serial bond elut columns with several hexane passages, as previously described [64].

Western blotting. pHAECs were lysed with lysis buffer (150 mM NaCl, 2% Triton X-100, 20 mM HEPES, pH 7.4, 2% protease inhibitor and 2% phosphatase inhibitor by sonication at 2 setting (Fisher sonic dismembrator model 100) on ice for 10 seconds. After centrifugation at 16,000 g x 10 minutes at 4 °C, equal volume of loading buffer (8 M urea, 2% SDS, 125 mM Tris-HCl, pH 7.0, 5% glycerol, 10% beta-mercaptoethanol, 0.06% bromphenol blue) was mixed with the supernatant at room temperature. This was followed by electrophoresis, electrical transfer to PVDF membrane, and immunoblotting.

Dil- and/or [^{3}H]CE-Lipoproteins. Generation of Dil-labeled lipoproteins was obtained essentially as previously described [65], with minor modifications. In brief, a mixture of about 2 mg HDL3, LDL, or VLDL protein and 7 ml of human lipoprotein deficient serum was mixed with ~1 mg [^{3}H]CE and/or 0.5 mg Dil in 10 μl DMSO aliquots under sterile conditions. After covering with foil, the mixture was incubated at 37 °C for 21 hours. The lipoproteins were repurified as described above, after adjusting to their respective densities with solid KBr. Combined Dil, [^{3}H]CE-LDL radioactivity was 8.1 dpm/μg protein.

LDL iodination with Na^{125}I. About 2 mg LDL protein in PBS and 10 iodination beads were incubated with Na^{125}I for 10 minutes at room temperature in glass vials. The transformation was stopped with 50 mM , and 100 μM BHT in DMSO (0.01%). The labeled LDL was

washed with desalting columns, then passed through centrifugal filters, 3 kD MWCO, against dialysis buffer.

^{125}I-LDL cell surface binding. Confluent pHAECs on plastic dishes were cultured with 0 or 5 ng/ml TNF in 15% serum for 48 hours. Treatment continued with serum-free medium in the continued presence of TNF for 3 hours to deplete surface-bound LDL. Subsequently, ^{125}I-LDL was added to 15 µg/ml for 1 hour at 37 °C. The cells were then washed two times with PBS at room temperature, then chilled on ice. Afterwards, serum-free medium at 4 °C with 0 (buffer) or 300 µg/ml LDL was added. Following additional incubation at 4 °C for 1 hour, the radioactivity released to the medium was taken as surface-releasable ^{125}I-LDL.

LDL oxidation. LDL was dialysed against PBS at 4 °C, passed through 0.2 µm filter, and incubated at 37 °C for 22 hours with 5 µM $CuSO_4$ in PBS under sterile conditions. After adding BHT in DMSO (0.005%) to 1 µM and Na_2EDTA to 10 mM, the mixture was dialysed against dialysis buffer, and finally filtered through 0.2 µm membrane under sterile conditions.

TBARS Assay. 2-thiobarbituric assay to estimate the extent of LDL oxidation was determined essentially as previously described [66], with some modifications. oxLDL was mixed successively with 3 volumes of 0.67% 2-thiobarbituric acid in 50 mM NaOH and 20% trichloroacetic acid, each containing 1 mM Na_2EDTA, respectively. After heating at 55 °C for 1 hour, the mixture was centrifuged at 16,000 g x 30 seconds. Fluorescence (530 nm excitation, 590 nm emission) reading in the supernatant was determined. Malonaldehyde bis-(dimethyl acetal) was used as a malondialdehyde (MDA) precursor for determining malondialdehyde standards. Care should be taken when performing this procedure, as volatile products are generated at 100 °C. Standards up to 5 nmol/ul were used. oxLDL was measured at 54 nmol MDA equivalents/ mg protein.

Depletion of apoE from Dil-lipoproteins. Normal mouse IgG agarose or apoE3 agarose beads were washed 4X with dialysis buffer under sterile conditions. Subsequently, Dil-HDL3 or Dil-LDL was added to the beads in dialysis buffer. Following 24 hour incubation at 4 °C, the unbound lipoproteins were sterile filtered through 0.2 µm membranes.

Cell surface biotinylation. Serum media from confluent pHAECs were replaced with 400 U/ml sodium heparin in mHBSS on ice for 5 minutes, washed 2X with the the same buffer, then 1X with ice cold mHBSS. After 1X wash with ice cold 1X PBS, the cells were treated with EZ Link Sulfo NHS Biotin in 1X PBS for 30 minutes at 4 °C. Unreacted biotin reagent was quenched with 100 mM glycine in PBS at 4 °C for 5 minutes. Following 1X wash with ice cold mHBSS, the cells were lysed on the plates with lysis buffer as indicated above under Western blotting on ice for 30 minutes, followed by sonication at 2 setting for 10 seconds on ice. Aliquots of the supernatant was mixed with 1 volume of 2X urea loading buffer without heating. The rest of the supernatant was mixed with strepavidin mag sepharose (pre-washed 2X with incubation buffer: 1% BSA, 0.1% NaN3, 0.1% Triton X-100). After 10X dilution with the incubation buffer containing 0.5% each of protease inhibitor and phosphate inhibitor cocktails, the suspension was

swirled for 12 hrs at 4 °C. The beads were washed 4X with precipitation buffer, then mHBSS at 4 °C. After 1X urea loading buffer (1:1 volume of lysis buffer and 2X urea loading buffer), with 1% protease and phosphatase inhibitors, respectively, the samples were heated at 95 °C for 5 minutes to solubilize the bound biotinylated proteins.

Dextran-Mn separation of intact and non-intact ^{125}I-LDL. Intact ^{125}I-LDL was separated from non-intact LDL using dextran sulfate, Mn^{2+} procedure essentially as previously described [67]. To the BL medium, d<1.21 g/ml FBS was added to 15% to produce a visible precipitate, mixed, then dextran sulfate and MnCl were added to 65 mg/ml and 0.2 M, respectively. After incubation for 20 minutes at room temperature, the mixtures were centrifuged at 5000 g x 5 minutes at 4 °C. The pellet was redissolved with 20 mg/ml dextran sulfate before scintillation counting.

Immunofluorescence. Confluent pHAECs in serum medium were incubated with 0 or 100 ng/ml TNF for 24 hours. Subsequently, the media were replaced with serum-free medium containing 10 µg/ml normal goat IgG or LDLR Ab (R&D Systems) in the continued presence of 0 or 100 ng/ml TNF for 0, 5, 30, or 120 minutes at 37 ºC. Afterwards, the cells were chilled on ice and further incubated at 4 ºC for 1 hour. Then the cells were washed 2X with ice-cold mHBSS, fixed with ice-cold methanol at -20 ºC for 10 minutes, washed once with mHBSS at room temperature, then blocked with 50% human serum in mHBSS, 0.1% sodium azide (blocking buffer) for 1 hour at room temperature. Afterwards, it was replaced with 4 µg/ml red antigoat antibody in blocking buffer. Following 30 minute incubation at room temperature, the medium was replaced with mHBSS, washed 3X with blocking buffer (10 minutes each at room temperature), fixed with 4% paraformaldehyde in PBS, followed by fluorescence microscopy.

Fluorescence Microscopy. Fluorescence microscopy was performed using Keyence phase-contrast fluorescence microscope. Images were analyzed using CellProfiler [68]. Confocal microscopy was performed using glass bottom dishes (Cellvis).

STATISTICAL ANALYSIS. Data are reported as averages ± standard deviation. n = number of independent experiments performed on separate days. Most experiments were performed with an average of 3 or greater replicates. Analysis of variance, followed by Tukey post hoc testing was done by using statpages.info website.

ABBREVIATIONS: ACAT, acyl-CoA cholesterol acyltransferase; AP, apical; apo(X) , apolipoprotein X; BHT, butylated hydroxytoluene; BL, basolateral; CAB, cholesterol assay buffer, DB, dialysis buffer; DiL, 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FAF-BSA, fatty acid-free bovine serum albumin; FBS, fetal bovine serum; HDL3, high density lipoprotein 3; HRP, horse radish peroxidase; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LRP, ldlr- related protein; mHBSS, modified Hanks' balanced salt solution; PBS, phosphate buffered saline; PCSK9, proprotein convertase subtilisin/kexin type 9; pHAECs, primary human aortic endothelial cells; RAP, receptor associated protein; SDS, sodium dodecyl sulfate;

TMTU, tetramethylthiourea; TNF, tumor necrosis factor alpha; VBM, vascular basal medium; VEGF, vascular endothelial growth factor kit

Results

TNF enhances cholesterol accumulation and LDL binding to pHAECs. Atherosclerotic lesion is characterized by release of inflammatory cytokines, among which is TNF. To evaluate the effect of TNF on pHAECs cholesterol content, confluent pHAECs were treated with or without TNF and the cellular cholesterol content was measured. As is shown in **Figure 1A,** TNF significantly increased unesterified cholesterol, and to a greater degree, esterified cholesterol (**Figure 1B**). To evaluate whether TNF affects the ability of LDL to bind to the cells, ^{125}I-LDL binding was performed. **Figure 1C** shows that TNF enhanced ^{125}I-LDL surface releasable ^{125}I-LDL at 4 °C, after the cells were pre-treated at 37 °C. The amount released increased in the presence of 20 folds unlabeled LDL. This indicates that the ^{125}I-LDL bound to some native LDL receptor. Further, cell-associated ^{125}I-LDL was also higher on TNF-treated pHAECs (**Figure 1D**).

ACAT inhibitor does not prevent TNF-induced LDL cholesterol accumulation. It has been reported that TNF induces cholesteryl ester accumulation in monocytes through enhanced ACAT activity in the presence of oxLDL [69]. To evaluate the extent to which enhanced ACAT activity is responsible in raising pHAEC cholesterol in the presence of LDL, the cells were treated with or without ACAT inhibitor, Sandoz 58-035. As is reported in **Figure 2A-B**, inhibition of ACAT activity slightly raised the unesterified cholesterol, and significantly suppressed cholesteryl ester accumulation. Despite this inhibition, the TNF-induced total cholesterol accumulation, now mainly in the unesterified form, was not prevented (**Figure 2A,C**). ACAT inhibition also reduced the total cholesterol in the cells, with or without TNF stimulation (**Figure 2C**). This is a known effect of ACAT inhibition. The higher elevated unesterified cholesterol is more likely to be effluxed from the cells than the esterified one [70].

LDL oxidation is not required for TNF-induced LDL accumulation. Having demonstrated that TNF promoted LDL binding to pHAECs (Figure 1), the requirement for oxidative modification of LDL was investigated. Particularly, TNF has been reported to promote release of the reactive oxygen species, superoxide and hydrogen peroxide [71, 72]. Hence, experiments were performed to determine whether oxidative modification of LDL is a prerequisite to TNF-induced LDL binding. To visualize TNF-induced LDL binding and subsequent internalization, Dil-LDL was used. Internalized Dil-LDL was significantly increased through TNF pre-treatment (**Figure 3A-B**). It can also be seen in **Figure 3A-B** that excess unlabeled native LDL blocked binding and internalization of Dil-LDL, with and without TNF treatment. This demonstrates that native LDL components, and therefore receptors, are required for Dil-LDL binding and internalization. Excess unlabeled oxLDL, on the other hand, did not prevent Dil-LDL intracellular accumulation in control and TNF-treated cells. Instead, there was a tendency of oxLDL to enhance intracellular Dil-LDL with or without TNF.

TNF induces massive DiI over [^{3}H]CE lipid accumulation from LDL. The lipids, DiI and ^{3}H-cholesteryl esters ([^{3}H]CE), are stably fixed within LDL. To evaluate the ability of pHAECs to retain LDL hydrolysable [^{3}H]CE or the non-hydrolysable DiI, the cells were treated with increasing concentration of TNF in the presence of [^{3}H]CE-LDL or DiI-LDL. As is shown in **Figure 4A**, ^{3}H-cholesterol accumulation derived from [^{3}H]CE-LDL was increased by about 2 folds with increasing TNF concentration. On the other hand, DiI accumulation increased by about 50 folds (**Figure 4B**). Next, cells were treated with increasing DiI-LDL concentration without (**Figure 4C**) or with TNF (**Figure 4D**). As is shown in **Figure 4C**, 400 µg/ml DiI-LDL increased intracellular DiI level by about 50 folds compared to that at 1 µg/ml under control condition. Compared to Ctrl at 400 µg/ml DiI-LDL, TNF increased the intracellular DiI by over 200 folds (**Figure 4D**).

Lysosomal inhibitor enhances TNF-induced LDL lipid accumulation. The lowering of pH facilitates disintegration and degradation of internalized LDL by low pH-dependent lysosomal enzymes. This degradation is suppressed by the pH-raising compound, chloroquine [73]. To evaluate whether a similar phenomenon occurs in pHAECs, the cells were treated in the presence or absence of chloroquine. The presence of chloroquine caused the DiI to accumulate circumferentially within the cells, presumably in defective lysosomes (**Figure 5A**). As can be seen in **Figure 5A-B**, chloroquine greatly enhanced DiI-LDL accumulation without TNF. The presence of TNF further increased the amount of chloroquine-induced cellular DiI-LDL. Chloroquine also increased ^{3}H-cholesterol level from [^{3}H]CE-LDL. However, it was not as pronounced as in the case of DiI (**Figure 5C**).

ApoE is not required for TNF-induced DiI-LDL accumulation. Apolipoprotein B (apoB) is the major protein in LDL [74]. However, variable amounts of apoE are also present in LDL [75]. Since apoE plays a major role in binding several lipoprotein receptors of the LDLR family [76, 77], the effect of apoE depletion on TNF-induced DiI-LDL intracellular levels was investigated. The successful depletion of apoE from DiI-HDL3 and DiI-LDL can be seen in **Figure 6A**. That both HDL3 and LDL contain apoE, while apoA1 and apoB are present in significant amounts only in HDL3 and LDL, respectively, is apparent in the figure. In **Figure 6B**, it can be seen that the isolation procedure did not inhibit the ability of TNF to induce DiI-LDL accumulation. Further, apoE depletion had no effect in this regard.

TNF-induced DiI-LDL accumulation is blocked by specific LDLR antibody. Having found evidence that TNF promotes cholesterol accumulation in pHAECs through enhanced endocytosis of native LDL, I next investigated what members of the LDLR family are responsible. As can be seen in **Figure 7A**, the pan-LDLR family blocker PCSK9 [78-80], but not RAP [81], suppressed the DiI-LDL accumulation. The LDLR family members are abundantly expressed in hepatocytes. Thus, to evaluate whether RAP activity is measurable in hepatocytes, the cells were treated RAP (**Figure 7B**). The results show that the failure of RAP to inhibit DiI-LDL internalization is specific to pHAECs. Since RAP has been reported to be ineffective against LDLR [82], the data point to LDLR as the most likely candidate.

To investigate the contribution of LDLR in mediating TNF-induced DiI-LDL accumulation, an LDLR antibody was investigated for its specificity and function in pHAECs treated with or without TNF. As can

d a single protein consistent with the expected migration

position of LDLR. It can be seen that incubation of TNF-treated cells with the antibody induced an upward migratory shift in LDLR protein to a larger extent than in control cells. Attempts to directly detect the LDLR antibody were unsuccessful, suggesting that the shift is due to another interaction or modification.

HDL3 critically depends on non-LDLR mechanisms for uptake, especially in the absence of apoE [83]. Thus, to further evaluate the specificity of the LDLR antibody, intracellular uptake of DiI-HDL3 was measured. As can be seen in **Figure 7D**, intracellular DiI-HDL3 levels was significantly blocked by excess unlabeled HDL3, whereas that of LDL only had a mild effect. Further, the LDLR antibody did not block intracellular DiI-HDL3 to a considerable level. In contrast, the LDLR antibody blocked intracellular DiI-LDL accumulation (**Figure 7E**). This blockage was greater than 99% in TNF-treated cells.

TNF upregulates cell surface LDLR protein. Having determined that LDLR is responsible for TNF-induced LDL accumulation in pHAECs, I next tested whether total and surface LDLR is changed by TNF. As can be seen **in Figure 8A,D**, TNF-induced upregulation of the LDLR protein total and surface by about 90% and 138%, respectively. ABCA1 (Figure 8A, B) and ICAM-1 (Figure 8A, C) proteins, both of which have been reported to be induced by TNF treatment [29, 84, 85], were found to be significantly increased as well. A low molecular weight form of ABCA1, however, was primarily induced in TNF-treated cells. In contrast, scavenger receptor B1 (SR-B1) was downregulated by TNF (Figure 8A, E). Similar findings have been reported on hepatocytes [86]. Figure 8A also shows that the intracellular proteins GAPDH were undetectable at the cell surface. Although caveolin-1 has been reported to be closely associated with the inner plasma membrane [87, 88], it was not detected in the cell surface assay. This indicates that outer plasma membrane proteins were selectively labeled.

TNF promotes rapid association of LDLR with its antibody. To evaluate the consequence of the increase in cell surface LDLR induced by TNF on the association and entry of LDLR antibody, immunofluorescence studies were performed. As can be seen in **Figure 9A**, TNF did not enhance association of control antibody with the cells, but significantly induced association with the specific LDLR antibody by 30 minutes at 37 °C. This indicates that TNF did not promote non-selective endocytic processes. The surface distribution of LDLR (arrows) and the internalization of the surface LDLR (arrow heads) in cells treated with TNF can be seen in **Figure 9B**.

The total amount of surface LDLR bound by the antibody at 37 °C can be seen to increase with time. At 0, 5, 30, and 120 minutes, there was about 30, 50, 7, and 2 folds, respectively, more surface LDLR antibody in TNF-treated cells compared to control cells at the corresponding time (**Figure 9C-D**). This demonstrates that LDLR antibody rapidly binds surface LDLR in the case of TNF-treated cells, plateauing by about 2 hours.

Anti-oxidant suppresses TNF-induced DiI-LDL accumulation. TNFα signaling cascade involves multiple mediators, among which are the transcription factor, NF-κB, and apoptosis signal-regulating kinase 1 (ASK1), an upstream mediator of JNK and p38 [89], that facilitate apoptosis. To evaluate whether these

DiI-LDL accumulation, experiments were performed in the

presence of their respective inhibitors. As can be seen in **Figure 10A**, the inhibitors did not suppress the action of TNFα. In contrast, the anti-oxidant, TMTU, inhibited this effect with increasing doses (**Figure 10B**).

TNF does not affect AP to BL release of degraded LDL protein. To address the effect of increased LDLR-mediated uptake of LDL on apical to basolateral LDL protein transport, the pHAECs were grown to confluence on transwell inserts, as shown in **Figure 11A**. ^{125}I-LDL detected in the BL medium was either non-intact (**Figure 11B**) or intact (**Figure 11C**). In **Figure 11B**, it is shown that unlabeled LDL competitor reduced the amount of non-intact ^{125}I-LDL, indicating release in a receptor-dependent manner. This was TNF-independent. In contrast, the unlabeled competitor had no effect on the intact ^{125}I-LDL measured in the BL medium (**Figure 11C**). TNFα tended to enhance BL release of intact ^{125}I-LDL, consistent with enhanced cell-cell permeability. The overall data thus indicate that the LDLR protein cargo is not efficiently trafficked in a polarized manner in pHAECs.

TNF does not affect AP to BL LDL lipid release. To determine whether LDLR facilitates AP to BL LDL lipid transport, the pHAECs on transwell inserts were incubated with LDL colabeled with DiI and ^{3}H-cholesteryl esters (**Figure 12A-B**). As can be seen in the figures, TNF dose-dependently increased the accumulation of DiI-LDL on the pHAECs grown on transwell inserts, in the same manner as those grown on plastic dishes. Similar to pHAECs on plastic dishes, the LDLR antibody significantly blocked the TNFα-induced uptake of DiI-LDL by pHAECs grown on transwell inserts by over 99%. The corresponding uptake of ^{3}H-cholesteryl esters is shown in **Figure 12C**. The ^{3}H-cholesteryl ester accumulation induced by TNF is blocked in the presence of antibody against LDLR. Surprisingly, LDLR blockage had no effect on the amount of measurable ^{3}H-choleseryl ester detectable in the BL medium (**Figure 12D**). This is consistent with the BL DiI-LDL, whose value was independent of TNF treatment (**Figure 12E**).

Discussion

Native LDL modification due to oxidation [90], aggregation [91], or complex formation with proteoglycans [92-95] is known to promote cholesterol accumulation in macrophages. In contrast, confluent arterial endothelial cells are resistant to cholesterol accumulation induced by any of these lipoproteins. The results demonstrate that this can be altered in the presence of TNF. Thus, LDL modification is not an absolute requirement for LDL-induced cholesterol or lipid accumulation. The data suggest that pHAECs, like macrophages, may be susceptible to changes related to excess cholesterol loading.

Excess cholesterol in arterial endothelial cells is likely to lead to endothelial dysfunction due, in part, to space and membrane disruption [96]. General upregulation of the LDLR receptor function through inhibition of PCSK9 have been reported to benefit against coronary artery disease [97, 98]. Notwithstanding, PCSK9 also downregulates other receptors in the LDLR family [79, 80], and binds the scavenger receptor, CD36, concurrent with thrombosis [97, 99]. Thus, the effect of increasing LDLR in the athero-protective effects of PCSK9, beyond lowering serum LDL concentration, is more complex.

LR protein [80], specific blocking antibody against LDLR did

not cause a major change in the LDLR protein (Figure 7). Instead, it induced a shift in the protein, which indicates that LDLR may signal under appropriate circumstances. Of significance, LDLR has been implicated in non-LDL uptake mechanisms. For example, it has been reported to have anti-apoptotic and anti-inflammatory properties [100]. Hence, this receptor plays important roles beyond plasma LDL regulation. Indiscriminate upregulation may have subtle negative consequences.

The results from this report indicate that pHAECs have efficient mechanism(s) of getting rid of LDL-derived cholesterol. TNF induced about 25 folds intracellular Dil-LDL compared to [^{3}H]CE-LDL. Thus it appears that pHAECs are normally protected against LDL-derived cholesterol accumulation by keeping cell surface LDLR down, combined with efficient efflux mechanisms. The TNF-induction of ABCA1 (Figure 8), which mediates cholesterol efflux, suggest that ABCA1 may be involved. However, the significance of an apparently truncated form (Figure 8A) is unclear. The tendency of TNF to induce ABCA1 expression in some cell types has also been published by others. Specifically, it has been reported that TNF enhances cholesterol efflux and ABCA1 mRNA and protein through NF-kB signaling [85] in macrophages, and possibly adipocytes [101]. However, as is often characteristic of the complex nature of TNF signaling, it has also been reported to downregulate ABCA1 in hepatocytes [102], Caco-2 intestinal cell[103], and osteocytes [104].

TNF is a pleiotropic autocrine and paracrine mediator important in multiple signaling cascades that range from activating immune cells to fight viruses, bacteria, and cancer cells, to promoting entry of monocytes and lymphocytes into atherosclerotic lesions [4, 105, 106]. The latter process is recognized to be pro-atherogenic. TNF has also been reported to play an important role in the current COVID-19 pandemic [107]. Thus, nonspecific disruption of TNF activity in the animal interferes with many important biological functions [108]. It has also been reported that TNF upregulates LDLR in hepatocytes [102]. My finding that TNF upregulates surface LDLR function in pHAECs implies that it is possible to regulate this process selectively, while leaving the beneficial functions of TNF intact.

It has been reported that AP to BL LDL transport occurs in vivo across arterial endothelial cells [109]. Findings from this report suggest that this transport is probably not through LDLR, as lysosome inhibition (Figure 5) significantly promoted Dil-LDL accumulation with or without TNF. SR-BI, on the other hand, has been reported to faciliate AP to BL transport of LDL in an LDLR-independent manner [110]. However, there is some controversy about the role of LDLR in trafficking ligand across the brain endothelium. Thus, it has been reported that LDLR mediates transcytosis of LDL and its ligand in brain microvascular endothelial cells [111] in vitro. However, studies using animal models suggest that LRP and very low density lipoprotein receptor (VLDLR), not LDLR, are responsible for the transcytosis [112]. Likewise, the binding of LDLR to RAP is controversial. On one hand, it has been reported to bind LDLR [113], while others report the opposite [82]. The data suggest that if it binds LDLR, it does not interfere with the ability of LDLR to subsequently internalize LDL.

Conclusions

In summary, the action of TNF on LDL uptake in pHAECs is due to cell surface upregulation of LDLR, combined with downregulation of SR-B1. Because SR-B1 is also a receptor for LDL, its downregulation promotes preferential binding and internalization through LDLR. Unlike SR-B1 which does not promote net LDL cholesterol accumulation in cells [112], LDLR is a potent inducer of LDL-derived cholesterol storage due to trafficking of its cargo to lysosomes.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this manuscript

Competing interests: The author declares that he has no competing interests

Funding: This work was supported through institutional grants: Meharry Clinical and Translation Research Center (MeTRC) 5U54MD007593-09, RCMI 5U54MD007586-33, as well as SC1HL101431. Confocal microscopy core was supported through NIH grants MHD007586 and S10RR025497.

Authors' contributions: EUO performed all aspects of the manuscript

Acknowledgments. The author thanks the Meharry core facility staff for assistance in the using the equipment.

References

1. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B: **An endotoxin-induced serum factor that causes necrosis of tumors**. *Proc Natl Acad Sci U S A* 1975, **72**(9):3666-3670.
2. Balkwill F: **Tumour necrosis factor and cancer**. *Nat Rev Cancer* 2009, **9**(5):361-371.
3. Holbrook J, Lara-Reyna S, Jarosz-Griffiths H, McDermott M: **Tumour necrosis factor signalling in health and disease**. *F1000Res* 2019, **8**.
4. Mercogliano MF, Bruni S, Elizalde PV, Schillaci R: **Tumor Necrosis Factor alpha Blockade: An Opportunity to Tackle Breast Cancer**. *Front Oncol* 2020, **10**:584.
5. Kriegler M, Perez C, DeFay K, Albert I, Lu SD: **A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF**. *Cell* 1988, **53**(1):45-53.
6. Jue DM, Sherry B, Luedke C, Manogue KR, Cerami A: **Processing of newly synthesized cachectin/tumor necrosis factor in endotoxin-stimulated macrophages**. *Biochemistry* 1990, **29**(36):8371-8377.

7. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S *et al*: **A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells**. *Nature* 1997, **385**(6618):729-733.
8. Sedger LM, McDermott MF: **TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future**. *Cytokine Growth Factor Rev* 2014, **25**(4):453-472.
9. Hiscott J, Marois J, Garoufalis J, D'Addario M, Roulston A, Kwan I, Pepin N, Lacoste J, Nguyen H, Bensi G *et al*: **Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop**. *Mol Cell Biol* 1993, **13**(10):6231-6240.
10. Paliogianni F, Raptis A, Ahuja SS, Najjar SM, Boumpas DT: **Negative transcriptional regulation of human interleukin 2 (IL-2) gene by glucocorticoids through interference with nuclear transcription factors AP-1 and NF-AT**. *J Clin Invest* 1993, **91**(4):1481-1489.
11. Laster SM, Wood JG, Gooding LR: **Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis**. *J Immunol* 1988, **141**(8):2629-2634.
12. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Jr., Shepard HM: **Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro**. *Science* 1985, **230**(4728):943-945.
13. Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P: **Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo**. *Proc Natl Acad Sci U S A* 1987, **84**(15):5277-5281.
14. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM *et al*: **Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis**. *Nature* 1996, **380**(6569):75-79.
15. Sluss HK, Barrett T, Derijard B, Davis RJ: **Signal transduction by tumor necrosis factor mediated by JNK protein kinases**. *Mol Cell Biol* 1994, **14**(12):8376-8384.
16. Aoki H, Kang PM, Hampe J, Yoshimura K, Noma T, Matsuzaki M, Izumo S: **Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes**. *J Biol Chem* 2002, **277**(12):10244-10250.
17. Scheurich P, Thoma B, Ucer U, Pfizenmaier K: **Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-alpha: induction of TNF receptors on human T cells and TNF-alpha-mediated enhancement of T cell responses**. *J Immunol* 1987, **138**(6):1786-1790.
18. Vilcek J, Palombella VJ, Henriksen-DeStefano D, Swenson C, Feinman R, Hirai M, Tsujimoto M: **Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors**. *J Exp Med* 1986, **163**(3):632-643.
19. Bruggeman LA, Drawz PE, Kahoud N, Lin K, Barisoni L, Nelson PJ: **TNFR2 interposes the proliferative and NF-kappaB-mediated inflammatory response by podocytes to TNF-alpha**. *Lab Invest* 2011, **91**(3):413-425.
20. Jelinek DF, Lipsky PE: **Enhancement of human B cell proliferation and differentiation by tumor** *J Immunol* 1987, **139**(9):2970-2976.

21. Ligresti G, Aplin AC, Zorzi P, Morishita A, Nicosia RF: **Macrophage-derived tumor necrosis factor-alpha is an early component of the molecular cascade leading to angiogenesis in response to aortic injury**. *Arterioscler Thromb Vasc Biol* 2011, **31**(5):1151-1159.
22. Chu SC, Tsai CH, Yang SF, Huang FM, Su YF, Hsieh YS, Chang YC: **Induction of vascular endothelial growth factor gene expression by proinflammatory cytokines in human pulp and gingival fibroblasts**. *J Endod* 2004, **30**(10):704-707.
23. Maloney JP, Gao L: **Proinflammatory Cytokines Increase Vascular Endothelial Growth Factor Expression in Alveolar Epithelial Cells**. *Mediators Inflamm* 2015, **2015**:387842.
24. Quispe R, Michos ED, Martin SS, Puri R, Toth PP, Al Suwaidi J, Banach M, Virani SS, Blumenthal RS, Jones SR *et al*: **High-Sensitivity C-Reactive Protein Discordance With Atherogenic Lipid Measures and Incidence of Atherosclerotic Cardiovascular Disease in Primary Prevention: The ARIC Study**. *J Am Heart Assoc* 2020, **9**(3):e013600.
25. Yuan S, Carter P, Bruzelius M, Vithayathil M, Kar S, Mason AM, Lin A, Burgess S, Larsson SC: **Effects of tumour necrosis factor on cardiovascular disease and cancer: A two-sample Mendelian randomization study**. *EBioMedicine* 2020, **59**:102956.
26. Rus HG, Niculescu F, Vlaicu R: **Tumor necrosis factor-alpha in human arterial wall with atherosclerosis**. *Atherosclerosis* 1991, **89**(2-3):247-254.
27. Paolisso G, Rizzo MR, Mazziotti G, Tagliamonte MR, Gambardella A, Rotondi M, Carella C, Giugliano D, Varricchio M, D'Onofrio F: **Advancing age and insulin resistance: role of plasma tumor necrosis factor-alpha**. *Am J Physiol* 1998, **275**(2):E294-299.
28. Byl B, Roucloux I, Crusiaux A, Dupont E, Deviere J: **Tumor necrosis factor alpha and interleukin 6 plasma levels in infected cirrhotic patients**. *Gastroenterology* 1993, **104**(5):1492-1497.
29. Nagashima S, Mendes MC, Camargo Martins AP, Borges NH, Godoy TM, Miggiolaro A, da Silva Deziderio F, Machado-Souza C, de Noronha L: **Endothelial Dysfunction and Thrombosis in Patients With COVID-19-Brief Report**. *Arterioscler Thromb Vasc Biol* 2020, **40**(10):2404-2407.
30. Liu YN, Peng YL, Liu L, Wu TY, Zhang Y, Lian YJ, Yang YY, Kelley KW, Jiang CL, Wang YX: **TNFalpha mediates stress-induced depression by upregulating indoleamine 2,3-dioxygenase in a mouse model of unpredictable chronic mild stress**. *Eur Cytokine Netw* 2015, **26**(1):15-25.
31. Branen L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S: **Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice**. *Arterioscler Thromb Vasc Biol* 2004, **24**(11):2137-2142.
32. Canault M, Peiretti F, Mueller C, Kopp F, Morange P, Rihs S, Portugal H, Juhan-Vague I, Nalbone G: **Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus**. *Atherosclerosis* 2004, **172**(2):211-218.
33. Ohta H, Wada H, Niwa T, Kirii H, Iwamoto N, Fujii H, Saito K, Sekikawa K, Seishima M: **Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice**. *Atherosclerosis* 2005, **180**(1):11-17.

34. Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, van Vlijmen BJ: **Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice**. *Cardiovasc Res* 2005, **66**(1):179-185.
35. Oberoi R, Vlacil AK, Schuett J, Schosser F, Schuett H, Tietge UJF, Schieffer B, Grote K: **Anti-tumor necrosis factor-alpha therapy increases plaque burden in a mouse model of experimental atherosclerosis**. *Atherosclerosis* 2018, **277**:80-89.
36. Barath P, Fishbein MC, Cao J, Berenson J, Helfant RH, Forrester JS: **Detection and localization of tumor necrosis factor in human atheroma**. *Am J Cardiol* 1990, **65**(5):297-302.
37. Barath P, Fishbein MC, Cao J, Berenson J, Helfant RH, Forrester JS: **Tumor necrosis factor gene expression in human vascular intimal smooth muscle cells detected by in situ hybridization**. *Am J Pathol* 1990, **137**(3):503-509.
38. Zhang X, Bishawi M, Zhang G, Prasad V, Salmon E, Breithaupt JJ, Zhang Q, Truskey GA: **Modeling early stage atherosclerosis in a primary human vascular microphysiological system**. *Nat Commun* 2020, **11**(1):5426.
39. Zeiher AM, Fisslthaler B, Schray-Utz B, Busse R: **Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells**. *Circ Res* 1995, **76**(6):980-986.
40. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA: **Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant**. *Proc Natl Acad Sci U S A* 1994, **91**(9):3652-3656.
41. Deshmane SL, Kremlev S, Amini S, Sawaya BE: **Monocyte chemoattractant protein-1 (MCP-1): an overview**. *J Interferon Cytokine Res* 2009, **29**(6):313-326.
42. Schreyer SA, Peschon JJ, LeBoeuf RC: **Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55**. *J Biol Chem* 1996, **271**(42):26174-26178.
43. Schreyer SA, Vick CM, LeBoeuf RC: **Loss of lymphotoxin-alpha but not tumor necrosis factor-alpha reduces atherosclerosis in mice**. *J Biol Chem* 2002, **277**(14):12364-12368.
44. Zhang L, Peppel K, Sivashanmugam P, Orman ES, Brian L, Exum ST, Freedman NJ: **Expression of tumor necrosis factor receptor-1 in arterial wall cells promotes atherosclerosis**. *Arterioscler Thromb Vasc Biol* 2007, **27**(5):1087-1094.
45. Xanthoulea S, Thelen M, Pottgens C, Gijbels MJ, Lutgens E, de Winther MP: **Absence of p55 TNF receptor reduces atherosclerosis, but has no major effect on angiotensin II induced aneurysms in LDL receptor deficient mice**. *PLoS One* 2009, **4**(7):e6113.
46. Blessing E, Bea F, Kuo CC, Campbell LA, Chesebro B, Rosenfeld ME: **Lesion progression and plaque composition are not altered in older apoE-/- mice lacking tumor necrosis factor-alpha receptor p55**. *Atherosclerosis* 2004, **176**(2):227-232.
47. Chandrasekharan UM, Mavrakis L, Bonfield TL, Smith JD, DiCorleto PE: **Decreased atherosclerosis in mice deficient in tumor necrosis factor-alpha receptor-II (p75)**. *Arterioscler Thromb Vasc Biol* 2007, **27**(3):e16-17.
48. Ross R, Wight TN, Strandness E, Thiele B: **Human atherosclerosis. I. Cell constitution and he superficial femoral artery**. *Am J Pathol* 1984, **114**(1):79-

93.

49. Hoff HF, Morton RE: **Lipoproteins containing apo B extracted from human aortas. Structure and function**. *Ann N Y Acad Sci* 1985, **454**:183-194.
50. Mahley RW, Innerarity TL, Weisgraber KH, Fry DL: **Canine hyperlipoproteinemia and atherosclerosis. Accumulation of lipid by aortic medial cells in vivo and in vitro**. *Am J Pathol* 1977, **87**(1):205-226.
51. Vlodavsky I, Fielding PE, Fielding CJ, Gospodarowicz D: **Role of contact inhibition in the regulation of receptor-mediated uptake of low density lipoprotein in cultured vascular endothelial cells**. *Proc Natl Acad Sci U S A* 1978, **75**(1):356-360.
52. Poole JC, Florey HW: **Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits**. *J Pathol Bacteriol* 1958, **75**(2):245-251.
53. Simionescu M: **Implications of early structural-functional changes in the endothelium for vascular disease**. *Arterioscler Thromb Vasc Biol* 2007, **27**(2):266-274.
54. Joris I, Zand T, Nunnari JJ, Krolikowski FJ, Majno G: **Studies on the pathogenesis of atherosclerosis. I. Adhesion and emigration of mononuclear cells in the aorta of hypercholesterolemic rats**. *Am J Pathol* 1983, **113**(3):341-358.
55. Zhang Y, Vittinghoff E, Pletcher MJ, Allen NB, Zeki Al Hazzouri A, Yaffe K, Balte PP, Alonso A, Newman AB, Ives DG *et al*: **Associations of Blood Pressure and Cholesterol Levels During Young Adulthood With Later Cardiovascular Events**. *J Am Coll Cardiol* 2019, **74**(3):330-341.
56. Boren J, Chapman MJ, Krauss RM, Packard CJ, Bentzon JF, Binder CJ, Daemen MJ, Demer LL, Hegele RA, Nicholls SJ *et al*: **Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel**. *Eur Heart J* 2020, **41**(24):2313-2330.
57. Havel R, Eder H, Bragdon J: **The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum**. *J Clin Invest* 1955, **34**(9):1345-1353.
58. Skipski VP, Barclay M, Barclay RK, Fetzer VA, Good JJ, Archibald FM: **Lipid composition of human serum lipoproteins**. *Biochem J* 1967, **104**(2):340-352.
59. Kuchinskiene Z, Carlson LA: **Composition, concentration, and size of low density lipoproteins and of subfractions of very low density lipoproteins from serum of normal men and women**. *J Lipid Res* 1982, **23**(5):762-769.
60. Mahley RW, Innerarity TL, Rall SC, Jr., Weisgraber KH: **Plasma lipoproteins: apolipoprotein structure and function**. *J Lipid Res* 1984, **25**(12):1277-1294.
61. Andreae WA: **A sensitive method for the estimation of hydrogen peroxide in biological materials**. *Nature* 1955, **175**(4463):859-860.
62. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC: **Enzymatic determination of total serum cholesterol**. *Clin Chem* 1974, **20**(4):470-475.
63. Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification**. *Can J Biochem Physiol* 1959, **37**(8):911-917.

64. Kaluzny MA, Duncan LA, Merritt MV, Epps DE: **Rapid separation of lipid classes in high yield and purity using bonded phase columns**. *J Lipid Res* 1985, **26**(1):135-140.

65. Pitas RE, Innerarity TL, Weinstein JN, Mahley RW: **Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy**. *Arteriosclerosis* 1981, **1**(3):177-185.

66. Parthasarathy S, Steinbrecher UP, Barnett J, Witztum JL, Steinberg D: **Essential role of phospholipase A2 activity in endothelial cell-induced modification of low density lipoprotein**. *Proc Natl Acad Sci U S A* 1985, **82**(9):3000-3004.

67. Burstein M, Scholnick HR, Morfin R: **Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions**. *J Lipid Res* 1970, **11**(6):583-595.

68. McQuin C, Goodman A, Chernyshev V, Kamentsky L, Cimini BA, Karhohs KW, Doan M, Ding L, Rafelski SM, Thirstrup D *et al*: **CellProfiler 3.0: Next-generation image processing for biology**. *PLoS Biol* 2018, **16**(7):e2005970.

69. Lei L, Xiong Y, Chen J, Yang JB, Wang Y, Yang XY, Chang CC, Song BL, Chang TY, Li BL: **TNF-alpha stimulates the ACAT1 expression in differentiating monocytes to promote the CE-laden cell formation**. *J Lipid Res* 2009, **50**(6):1057-1067.

70. An S, Jang YS, Park JS, Kwon BM, Paik YK, Jeong TS: **Inhibition of acyl-coenzyme A:cholesterol acyltransferase stimulates cholesterol efflux from macrophages and stimulates farnesoid X receptor in hepatocytes**. *Exp Mol Med* 2008, **40**(4):407-417.

71. Satriano JA, Shuldiner M, Hora K, Xing Y, Shan Z, Schlondorff D: **Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-alpha and immunoglobulin G. Evidence for involvement of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase**. *J Clin Invest* 1993, **92**(3):1564-1571.

72. Ding AH, Nathan CF, Stuehr DJ: **Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production**. *J Immunol* 1988, **141**(7):2407-2412.

73. Trinh MN, Brown MS, Goldstein JL, Han J, Vale G, McDonald JG, Seemann J, Mendell JT, Lu F: **Last step in the path of LDL cholesterol from lysosome to plasma membrane to ER is governed by phosphatidylserine**. *Proc Natl Acad Sci U S A* 2020, **117**(31):18521-18529.

74. Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Ala-Korpela M: **Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL**. *Biochim Biophys Acta* 2000, **1488**(3):189-210.

75. Mendivil CO, Rimm EB, Furtado J, Sacks FM: **Apolipoprotein E in VLDL and LDL with apolipoprotein C-III is associated with a lower risk of coronary heart disease**. *J Am Heart Assoc* 2013, **2**(3):e000130.

76. Holtzman DM, Herz J, Bu G: **Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease**. *Cold Spring Harb Perspect Med* 2012, **2**(3):a006312.

77. Zhao N, Liu CC, Qiao W, Bu G: **Apolipoprotein E, Receptors, and Modulation of Alzheimer's Disease**. *Biol Psychiatry* 2018, **83**(4):347-357.

78. Shan L, Pang L, Zhang R, Murgolo NJ, Lan H, Hedrick JA: **PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide**. *Biochem Biophys Res Commun* 2008, **375**(1):69-73.

79. Poirier S, Mayer G, Benjannet S, Bergeron E, Marcinkiewicz J, Nassoury N, Mayer H, Nimpf J, Prat A, Seidah NG: **The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2**. *J Biol Chem* 2008, **283**(4):2363-2372.

80. Canuel M, Sun X, Asselin MC, Paramithiotis E, Prat A, Seidah NG: **Proprotein convertase subtilisin/kexin type 9 (PCSK9) can mediate degradation of the low density lipoprotein receptor-related protein 1 (LRP-1)**. *PLoS One* 2013, **8**(5):e64145.

81. Ruiz J, Kouiavskaia D, Migliorini M, Robinson S, Saenko EL, Gorlatova N, Li D, Lawrence D, Hyman BT, Weisgraber KH *et al*: **The apoE isoform binding properties of the VLDL receptor reveal marked differences from LRP and the LDL receptor**. *J Lipid Res* 2005, **46**(8):1721-1731.

82. Herz J, Goldstein JL, Strickland DK, Ho YK, Brown MS: **39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor**. *J Biol Chem* 1991, **266**(31):21232-21238.

83. Kraemer FB, Shen WJ, Patel S, Osuga J, Ishibashi S, Azhar S: **The LDL receptor is not necessary for acute adrenal steroidogenesis in mouse adrenocortical cells**. *Am J Physiol Endocrinol Metab* 2007, **292**(2):E408-412.

84. He P, Gelissen IC, Ammit AJ: **Regulation of ATP binding cassette transporter A1 (ABCA1) expression: cholesterol-dependent and - independent signaling pathways with relevance to inflammatory lung disease**. *Respir Res* 2020, **21**(1):250.

85. Gerbod-Giannone MC, Li Y, Holleboom A, Han S, Hsu LC, Tabas I, Tall AR: **TNFalpha induces ABCA1 through NF-kappaB in macrophages and in phagocytes ingesting apoptotic cells**. *Proc Natl Acad Sci U S A* 2006, **103**(9):3112-3117.

86. Khovidhunkit W, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR: **Regulation of scavenger receptor class B type I in hamster liver and Hep3B cells by endotoxin and cytokines**. *J Lipid Res* 2001, **42**(10):1636-1644.

87. Simon L, Campos A, Leyton L, Quest AFG: **Caveolin-1 function at the plasma membrane and in intracellular compartments in cancer**. *Cancer Metastasis Rev* 2020, **39**(2):435-453.

88. Hubert M, Larsson E, Vegesna NVG, Ahnlund M, Johansson AI, Moodie LW, Lundmark R: **Lipid accumulation controls the balance between surface connection and scission of caveolae**. *Elife* 2020, **9**.

89. Ogier JM, Nayagam BA, Lockhart PJ: **ASK1 inhibition: a therapeutic strategy with multi-system benefits**. *J Mol Med (Berl)* 2020, **98**(3):335-348.

90. Xia X, Xu Q, Liu M, Chen X, Liu X, He J, Hu T, Yu C, Huang H, Liu S *et al*: **Deubiquitination of CD36 by** *Cell Death Dis* 2020, **11**(8):636.

91. Ruuth M, Nguyen SD, Vihervaara T, Hilvo M, Laajala TD, Kondadi PK, Gistera A, Lahteenmaki H, Kittila T, Huusko J *et al*: **Susceptibility of low-density lipoprotein particles to aggregate depends on particle lipidome, is modifiable, and associates with future cardiovascular deaths**. *Eur Heart J* 2018, **39**(27):2562-2573.
92. Hurt E, Camejo G: **Effect of arterial proteoglycans on the interaction of LDL with human monocyte-derived macrophages**. *Atherosclerosis* 1987, **67**(2-3):115-126.
93. Salisbury BG, Falcone DJ, Minick CR: **Insoluble low-density lipoprotein-proteoglycan complexes enhance cholesteryl ester accumulation in macrophages**. *Am J Pathol* 1985, **120**(1):6-11.
94. Hurt E, Bondjers G, Camejo G: **Interaction of LDL with human arterial proteoglycans stimulates its uptake by human monocyte-derived macrophages**. *J Lipid Res* 1990, **31**(3):443-454.
95. Puig N, Montolio L, Camps-Renom P, Navarra L, Jimenez-Altayo F, Jimenez-Xarrie E, Sanchez-Quesada JL, Benitez S: **Electronegative LDL Promotes Inflammation and Triglyceride Accumulation in Macrophages**. *Cells* 2020, **9**(3).
96. Malhotra P, Gill RK, Saksena S, Alrefai WA: **Disturbances in Cholesterol Homeostasis and Non-alcoholic Fatty Liver Diseases**. *Front Med (Lausanne)* 2020, **7**:467.
97. Silverstein RL: **PCSK9 (Proprotein Convertase Subtilisin/Kexin 9) Goes "DAMP"**. *Circulation* 2021, **143**(1):62-64.
98. Adorni MP, Zimetti F, Lupo MG, Ruscica M, Ferri N: **Naturally Occurring PCSK9 Inhibitors**. *Nutrients* 2020, **12**(5).
99. Qi Z, Hu L, Zhang J, Yang W, Liu X, Jia D, Yao Z, Chang L, Pan G, Zhong H *et al*: **PCSK9 (Proprotein Convertase Subtilisin/Kexin 9) Enhances Platelet Activation, Thrombosis, and Myocardial Infarct Expansion by Binding to Platelet CD36**. *Circulation* 2021, **143**(1):45-61.
100. Sun R, Peng M, Xu P, Huang F, Xie Y, Li J, Hong Y, Guo H, Liu Q, Zhu W: **Low-density lipoprotein receptor (LDLR) regulates NLRP3-mediated neuronal pyroptosis following cerebral ischemia/reperfusion injury**. *J Neuroinflammation* 2020, **17**(1):330.
101. Zhao SP, Dong SZ: **Effect of tumor necrosis factor alpha on cholesterol efflux in adipocytes**. *Clin Chim Acta* 2008, **389**(1-2):67-71.
102. Ma KL, Ruan XZ, Powis SH, Chen Y, Moorhead JF, Varghese Z: **Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice**. *Hepatology* 2008, **48**(3):770-781.
103. Field FJ, Watt K, Mathur SN: **TNF-alpha decreases ABCA1 expression and attenuates HDL cholesterol efflux in the human intestinal cell line Caco-2**. *J Lipid Res* 2010, **51**(6):1407-1415.
104. Wehmeier KR, Kurban W, Chandrasekharan C, Onstead-Haas L, Mooradian AD, Haas MJ: **Inhibition of ABCA1 Protein Expression and Cholesterol Efflux by TNF alpha in MLO-Y4 Osteocytes**. *Calcif Tissue Int* 2016, **98**(6):586-595.
105. Zhao S, Jiang J, Jing Y, Liu W, Yang X, Hou X, Gao L, Wei L: **The concentration of tumor necrosis factor-alpha determines its protective or damaging effect on liver injury by regulating Yap activity**.

106. Gareb B, Otten AT, Frijlink HW, Dijkstra G, Kosterink JGW: **Review: Local Tumor Necrosis Factor-alpha Inhibition in Inflammatory Bowel Disease**. *Pharmaceutics* 2020, **12**(6).

107. Del Valle DM, Kim-Schulze S, Hsin-Hui H, Beckmann ND, Nirenberg S, Wang B, Lavin Y, Swartz T, Madduri D, Stock A *et al*: **An inflammatory cytokine signature helps predict COVID-19 severity and death**. *medRxiv* 2020.

108. Gerriets V, Bansal P, Goyal A, Khaddour K: **Tumor Necrosis Factor Inhibitors**. In: *StatPearls.* Treasure Island (FL); 2020.

109. Vasile E, Simionescu M, Simionescu N: **Visualization of the binding, endocytosis, and transcytosis of low-density lipoprotein in the arterial endothelium in situ**. *J Cell Biol* 1983, **96**(6):1677-1689.

110. Ghaffari S, Jang E, Naderinabi F, Sanwal R, Khosraviani N, Wang C, Steinberg BE, Goldenberg NM, Ikeda J, Lee WL: **Endothelial HMGB1 Is a Critical Regulator of LDL Transcytosis via an SREBP2-SR-BI Axis**. *Arterioscler Thromb Vasc Biol* 2021, **41**(1):200-216.

111. Molino Y, David M, Varini K, Jabes F, Gaudin N, Fortoul A, Bakloul K, Masse M, Bernard A, Drobecq L *et al*: **Use of LDL receptor-targeting peptide vectors for in vitro and in vivo cargo transport across the blood-brain barrier**. *FASEB J* 2017, **31**(5):1807-1827.

112. Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB, Holtzman DM, Zlokovic BV: **apoE isoform-specific disruption of amyloid beta peptide clearance from mouse brain**. *J Clin Invest* 2008, **118**(12):4002-4013.

113. Fisher C, Beglova N, Blacklow SC: **Structure of an LDLR-RAP complex reveals a general mode for ligand recognition by lipoprotein receptors**. *Mol Cell* 2006, **22**(2):277-283.

Figures

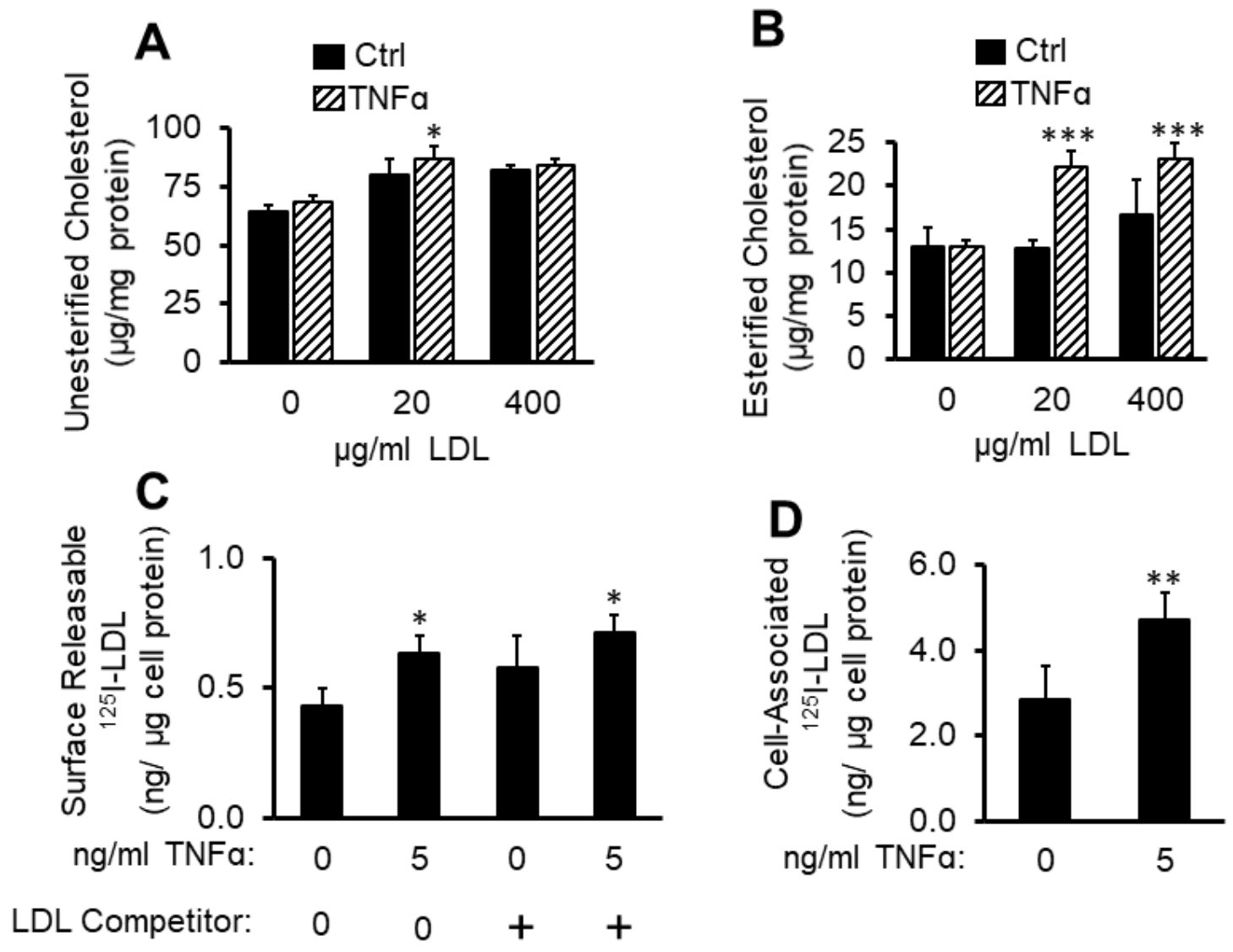

Figure 1

TNF enhances cholesterol accumulation and LDL binding to pHAECs. A-B, pHAECs were treated with the indicted concentration of human LDL without (Ctrl) or with 100 ng/ml TNF for 24 hours. Unesterified cholesterol and cholesteryl esters were determined as described under Materials and Methods. n= 5. C, Cells were pre-treated with 0 or 5 ng/ml TNF in serum medium as described under Materials and Methods. After incubation at 37 °C, the amount of 125I-LDL released to serum-free medium at 4 ºC without (0) or with 20 folds unlabeled LDL competitor (+) was determined as the surface 125I-LDL. D, The experiment was performed as in E, and the amount of radioactivity associated with the cell was determined. C-D, n =3. *,**,***, p< 0.05, 0.01, 0.001, relative to the corresponding Ctrl.

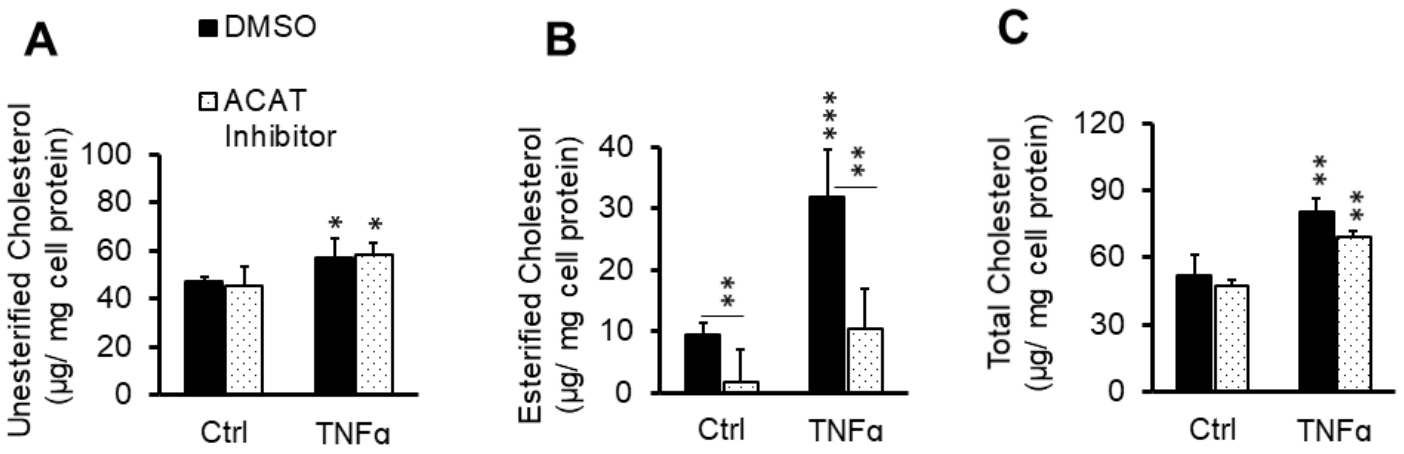

Figure 2

ACAT inhibitor does not prevent TNF-induced LDL cholesterol accumulation. Cells in serum-free medium were treated with 0 or 100 ng/ml TNF in the presence of 0.1% DMSO (DMSO) or 10 µg/ml ACAT inhibitor (Sandoz 58-035) and 100 µg/ml LDL for 24 hrs. After washing with heparin as described under Materials and Methods, the cellular cholesterol content was determined. n = 3. *,**,***, p< 0.05, 0.01, 0.001, relative to corresponding Ctrl or as indicated with bars.

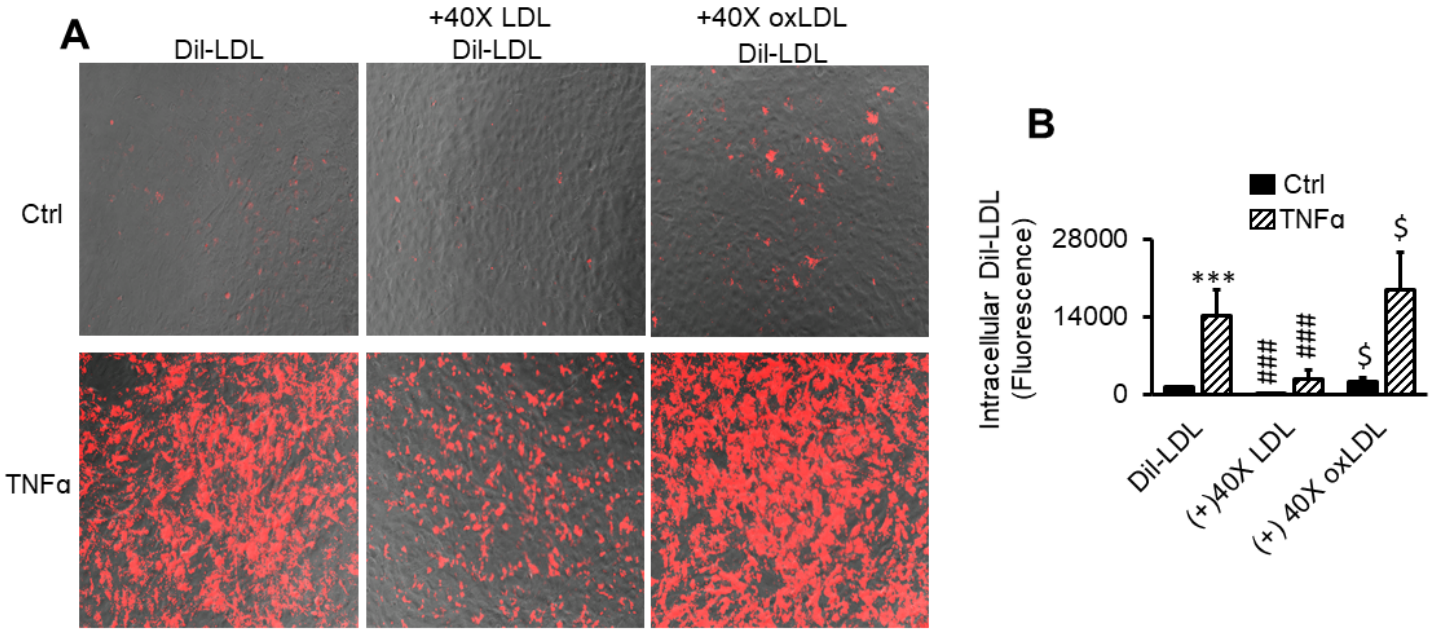

Figure 3

LDL oxidation is not required for TNF-induced LDL accumulation. A-B, Cells were treated with 0 (Ctrl) or 100 ng/ml TNF (TNF) for 24 hrs in serum medium. The media were replaced with serum-free media in the continued presence of TNF for 2 hrs to remove surface lipoproteins. Finally, 5 µg/ml Dil-LDL in serum-free medium (Dil-LDL) ± 40 times LDL (+40X LDL) or oxLDL (+40X oxLDL) was added, followed by 3 hour incubation. The images are 20X magnifications. n = 3. ***, p< 0.001 vs. Ctrl. $, ###, p<0.05, 0.001, relative to Dil-LDL alone.

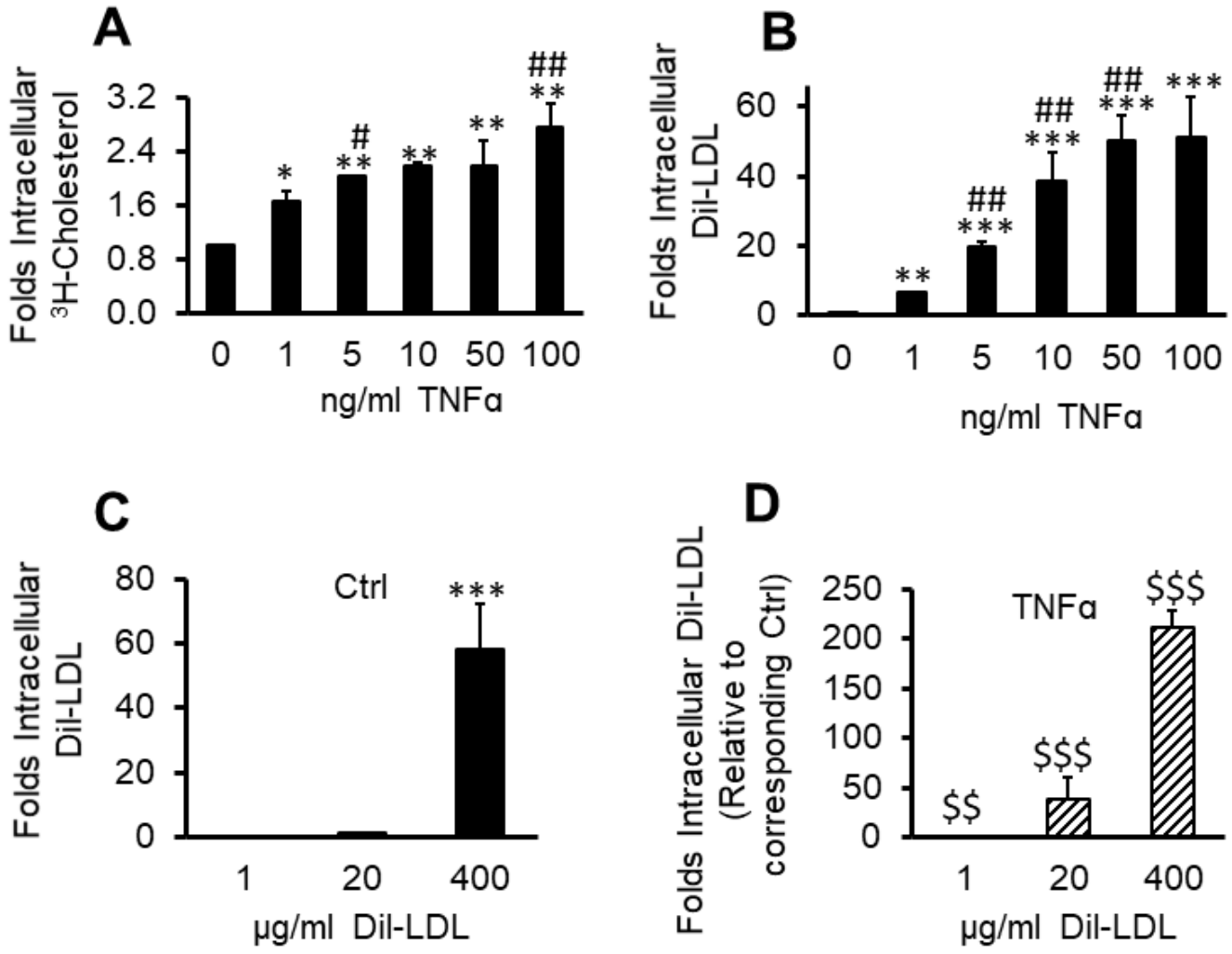

Figure 4

TNF induces massive Dil over [3H]CE lipid accumulation from LDL. pHAECs in serum medium were pre-treated with the indicated concentration of human TNF for 24 hrs. Subsequently, the cells were treated in the continued presence of the previous TNF concentration and 50 µg/ml [3H]CE-LDL (A) or 5 µg/ml Dil-LDL (B) without serum for 24 hrs. A, n =4, B, n =3. The cells were treated without (Ctrl), (C), or with 100 ng/ml TNF (D), as above, in the presence of increasing concentration of Dil-LDL. n = 5. *,**,*** ,p< 0.05, 0.01, 0.001, relative to 0 ng/ml TNF or 1 µg/ml Dil-LDL. #, ##, p< 0.05, 0.01, vs. previous TNF dose.

$, p<0.01, 0.001, relative to the corresponding Dil-LDL concentration under Ctrl condition.

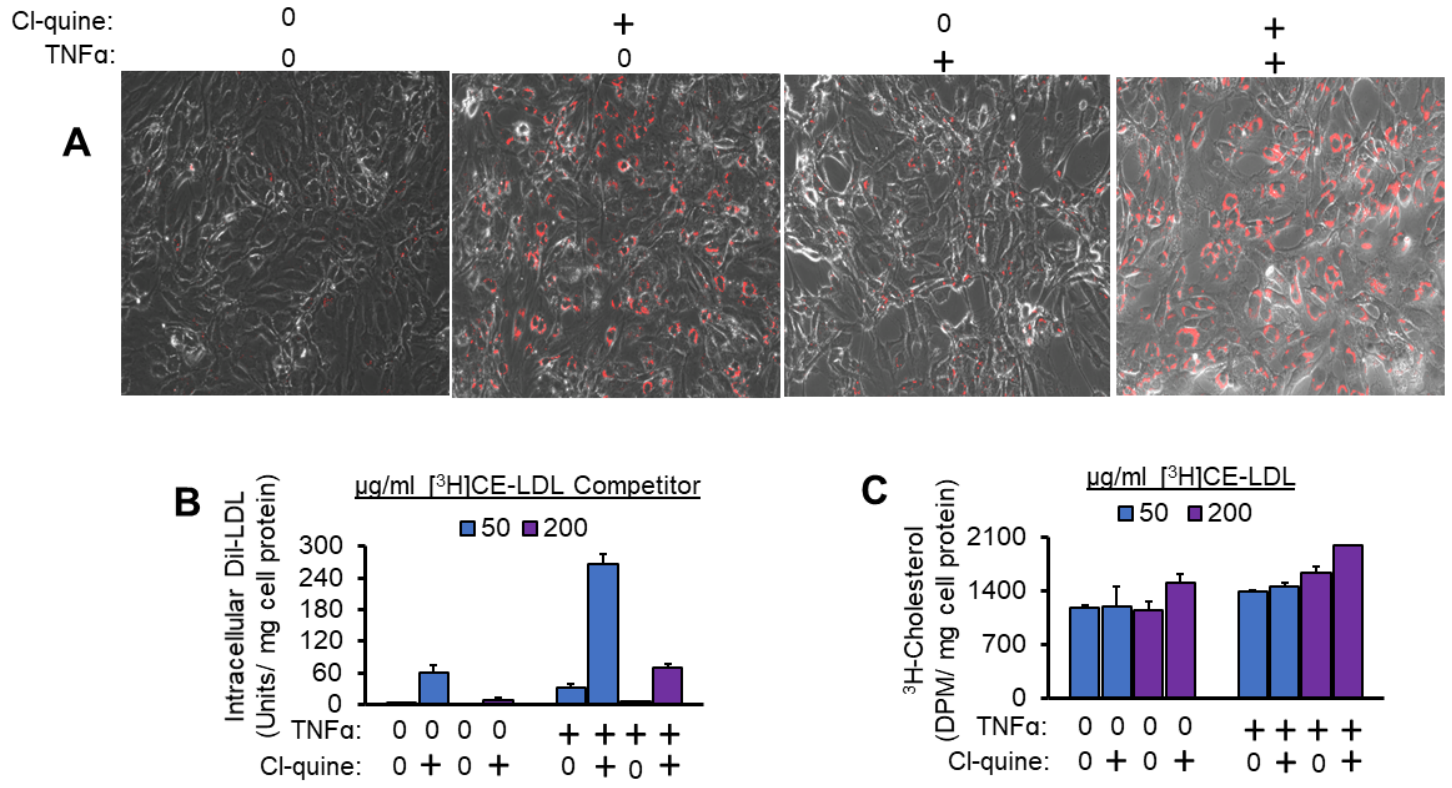

Figure 5

Lysosomal inhibitor enhances TNF-induced LDL lipid accumulation. The cells were pre-treated with 0 or 100 ng/ml TNF (+) for 24 hrs in the presence of serum. Subsequently, in the continued presence of TNF, the cells were treated with 0.5 µg/ml Dil-LDL, plus 50 or 200 µg/ml [3H]CE-LDL, and containing 0 or 50 µM chloroquine diphosphate (Cl-quine), +, a lysosomal blocker, without serum. After 24 hrs, the intracellular fluorescence (A-B) or radioactivity (C) was determined. The separation of #cells in A is an artificact of preparation prior to lysing the cells for radioactivity. The circumferential accumulation of the Dil-LDL in the presence of chloroquine is apparent (A). The images are 20X magnifications. n = 2.

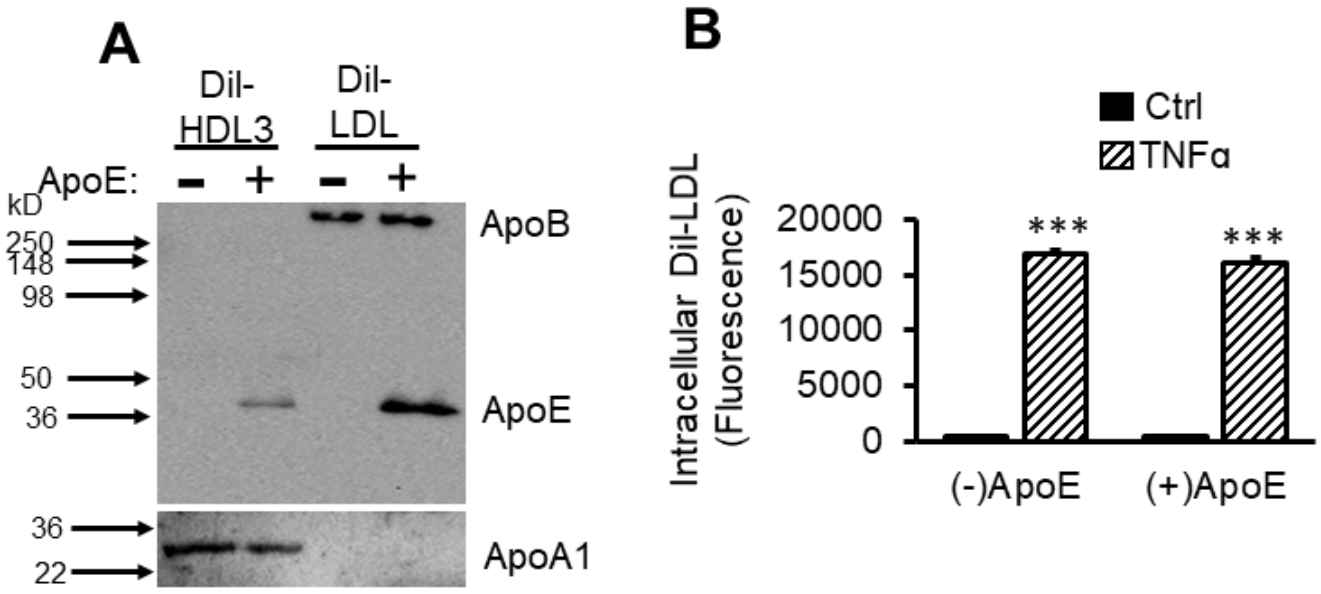

Figure 6

ApoE is not required for TNF-induced Dil-LDL accumulation. A, Dil-HDL3 or Dil-LDL was depleted of apoE (-), or apoE was retained (+) as described under Materials and Methods. The presence of apoB and apoE were determined by immunoblotting 0.5 and 1 μg/ml Dil-HDL3 and Dil-LDL, respectively. B, pHAECs were treated ± 100 ng/ml TNF in serum medium for 24 hrs. Following 2 hr incubation in serum free medium, in the continued presence of TNF, the cells were incubated with 2.5 μg/ml Dil-LDL without (-) or with apoE (+) for 3 hrs.

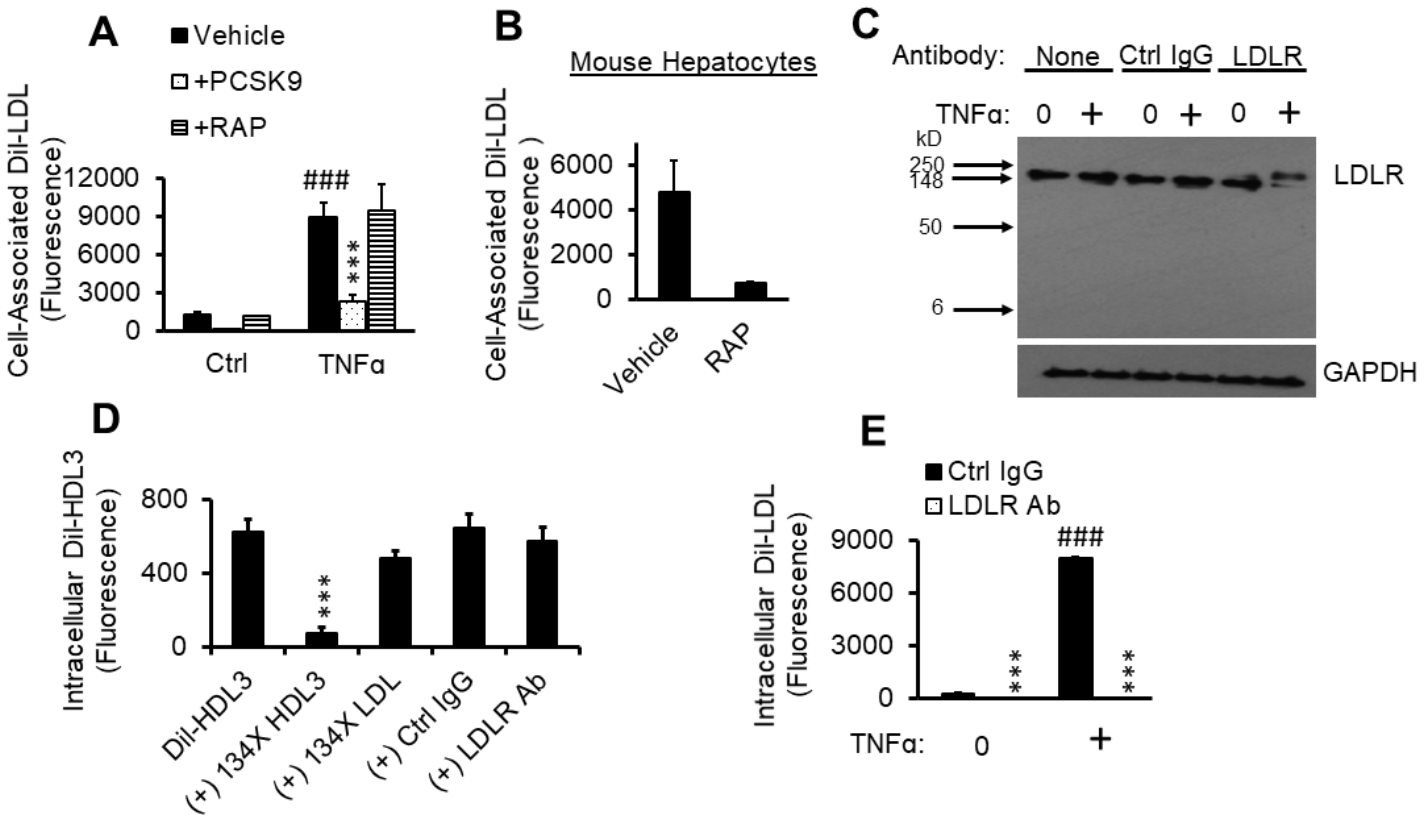

Figure 7

TNFα-induced Dil-LDL accumulation is blocked by specific LDLR antibody. A, pHAECs in serum medium were pre-treated with 0 (Ctrl) or 100 ng/ml TNFα for 24 hrs. Afterwards, the cells were cultured in the presence of vehicle, 12.5 μg/ml human PCSK9 (ApoER, LDLR, LRP, and VLDLR inhibitors), or 21 μg/ml human RAP (ApoER2, LRP, and VLDLR inhibitors) in serum-free medium for 1 hr. Lastly, 8 μg/ml Dil-LDL was added, followed by 4 hr incubation. n = 3. B, Mouse immorto hepatocytes were incubated in serum-free RPMI 1640 medium containing vehicle or RAP as above for 1 hr. Lastly, the hepatocytes were incubated for 2 hrs with Dil-LDL. n = 1. ###, ***, p< 0.001 vs Ctrl vehicle and TNFα vehicle, respectively. C, Cells were cultured in serum medium ± 100 ng/ml TNFα, followed by 3 hr incubation in serum-free medium in the continued presence of TNFα (+) without antibody (None), 18 μg/ml control IgG (Ctrl Ig) or LDLR antibody (LDLR), then immunoblotted for LDLR and GAPDH. n = 3. D, pHAECs were cultured in serum-free medium for 2 hrs, followed by incubation with 5 μg/ml Dil-HDL3 with (+) 134X unlabeled HDL3, LDL, 20 μg/ml control IgG (Ctrl IgG) or LDLR antibody (LDLR Ab), as indicated. n = 6. ***, p< 0.001 vs Dil-HDL3. E, Cells were cultured in serum medium with 0 or 100 ng/ml TNFα (+), followed by 2 hr incubation in serum-free medium in the continued presence of TNFα. Subsequently, 5 μg/ml Dil-LDL in the presence of 20 μg/ml control IgG (Ctrl IgG) or LDLR antibody (LDLR Ab) was added. Note that the values for LDLR Ab are very low compared to others. n = 8. ###, ***, p< 0.001 vs. corresponding 0 μg/ml TNFα and Ctrl IgG, respectively.

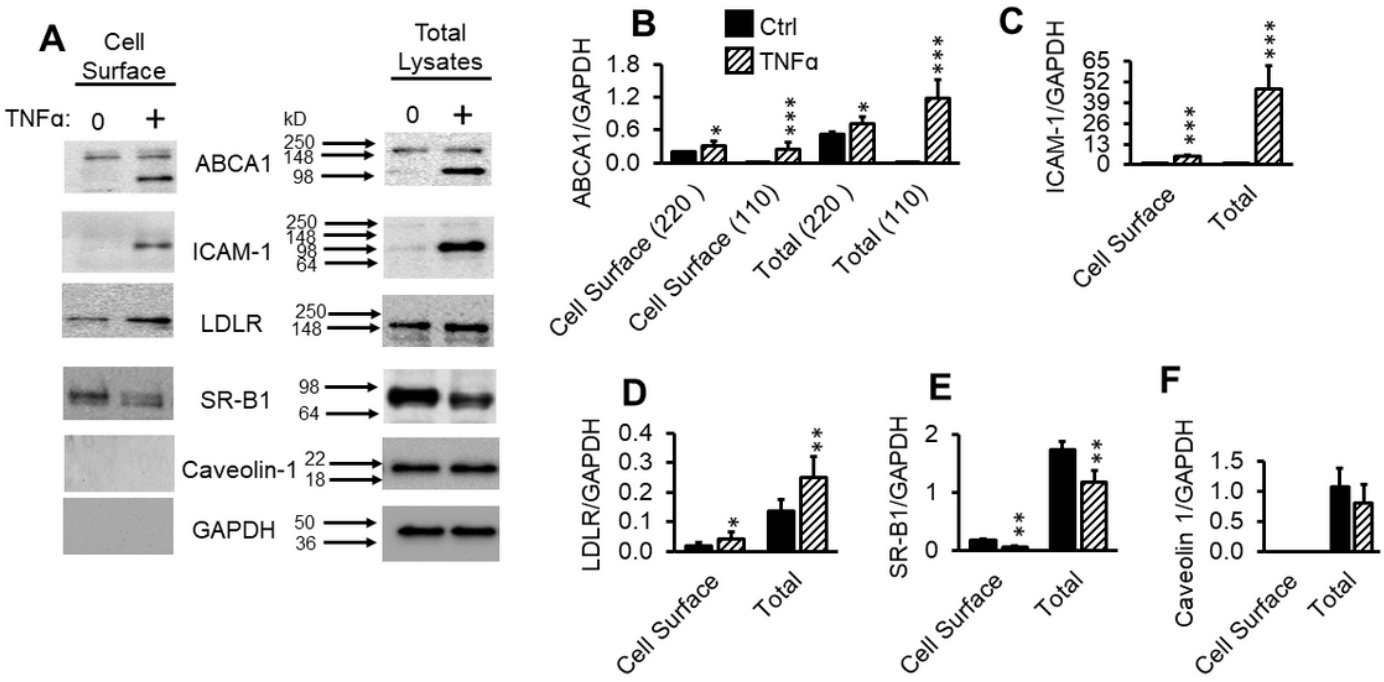

Figure 8

TNF upregulates cell surface LDLR protein. A-F, Confluent pHAECs in serum medium were pre-treated with 0 or 100 ng/ml TNF (+) for 24 hrs in serum medium. The cells were then treated and lysed for total (Total Lysates) and biotinylated cell surface (Cell Surface) proteins, as explained under Experimental Procedures. ABCA1 bands (A,B) at ~ 220 and 110 kD are indicated. n = 3. *,**,*** ,p< 0.05, 0.01, 0.001, vs corresponding Ctrl.

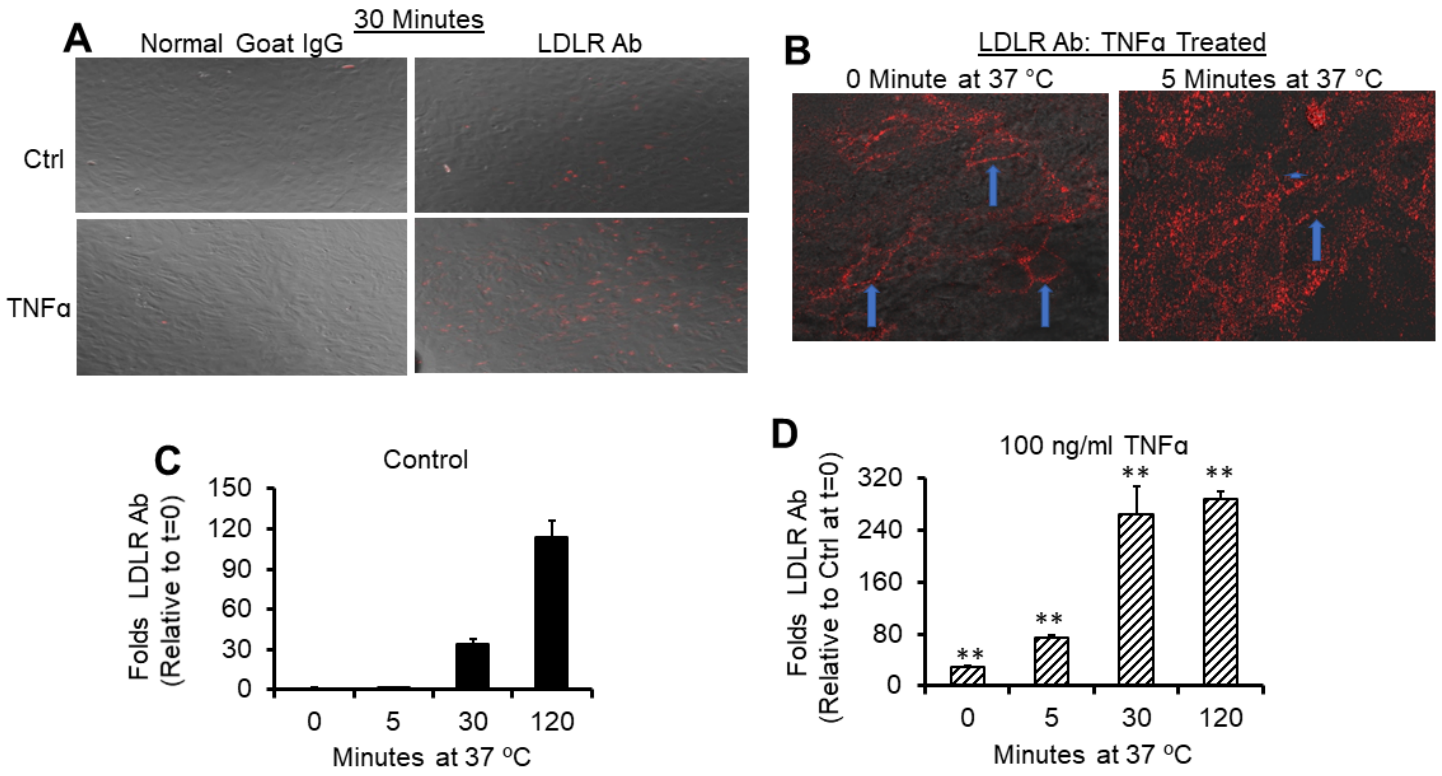

Figure 9

TNF promotes rapid association of LDLR with it antibody. pHAECs in serum medium were pre-treated with 0 (Ctrl) or 100 ng/ml TNF (TNF) for 24 hrs. Subsequently, in the continued presence of TNF, the media were replaced with 5 µg/ml control normal goat IgG or LDLR antibody (LDLR Ab) for 0, 5, 30, or 120 minutes at 37 °C. The surface-accessible LDLR was then determined as detailed under Experimental Procedures. A, 20X objective magnification of LDLR detected after 30 minutes. B, 40X confocal detection of LDLR in TNF-treated pHAECs at 0 and 5 minutes. The arrows show the membrane distribution of the LDLR in focus, while the arrowhead shows internalized LDLR. C, Surface-accessible LDLR as a function of time under control condition. D, As C, in the presence of TNF. nN = 4. **, p< 0.01 relative to control at the same time.

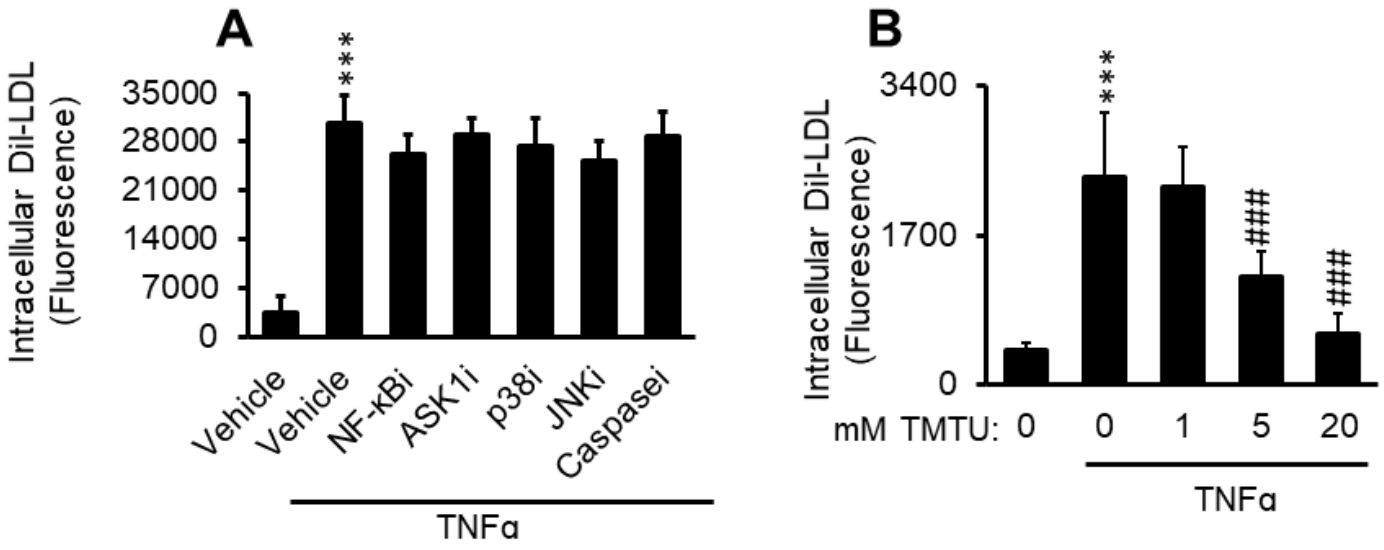

Figure 10

Anti-oxidant suppresses TNF⍺-induced DiI-LDL accumulation. A, pHAECs were pre-treated with 0.05% DMSO (Vehicle) or TNF⍺-cascade inhihitors, as indicated: NF-kBi (25 μM). Two different forms were tested. ASK1i (5 μM), p38i (1 μM), JNKi (5 μM), or pan-caspase inhibitor, Caspasei (5 μM) for 30 minutes in serum-free medium. Subsequently, serum-free 3 μg/ml DiI-LDL, with 100 ng/ml TNF⍺, as indicated, was added, followed by 24 hr incubation. n = 3. B, Cells were treated with 1% DMSO vehicle with 100 ng/ml TNF⍺ as indicated, in the presence of 0, 1, 5, or 20 mM TMTU and 3 μg/ml DiI-LDL. Subsequently, the cells were cultured for 24 hrs. n = 4. ***, ###, p< 0.001 vs. 0 μg/ml TNF⍺ and 0 mM TMTU in the presence of TNF⍺, respectively.

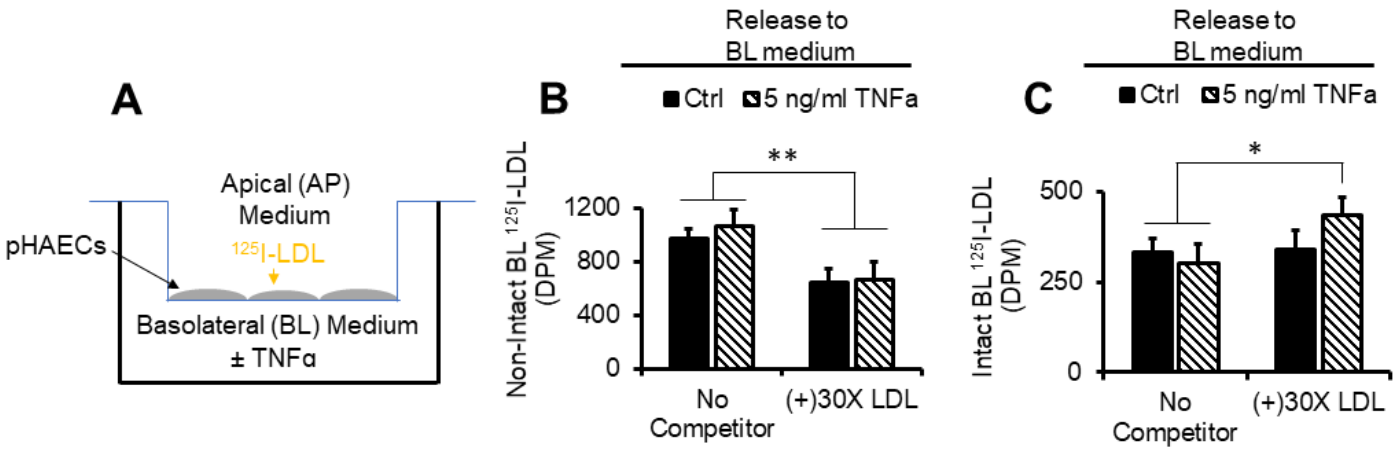

Figure 11

TNFα does not affect AP to BL release of degraded LDL protein. A, Diagram of the experimental setup in B-C, in which pHAECs were grown to confluence on 3 μm polycarbonate filters, with 125I-LDL in the AP medium. B, pHAECs in serum medium were pre-treated with 0 (Ctrl) or 5 ng/ml TNFα on the BL side for 48 hrs. Then 10 μg/ml 125I-LDL without serum was added to the AP side (No Competitor) or with 30 folds unlabeled LDL (+)30X LDL, in the continued presence of TNFα. After 3 hours, the BL media were collected, and non-intact 125I-LDL was measured as described under Experimental Procedures. C, The experiment was performed as in B, followed by detection of intact BL 125I-LDL. n =3.*,**, p< 0.05, 0.01, respectively,

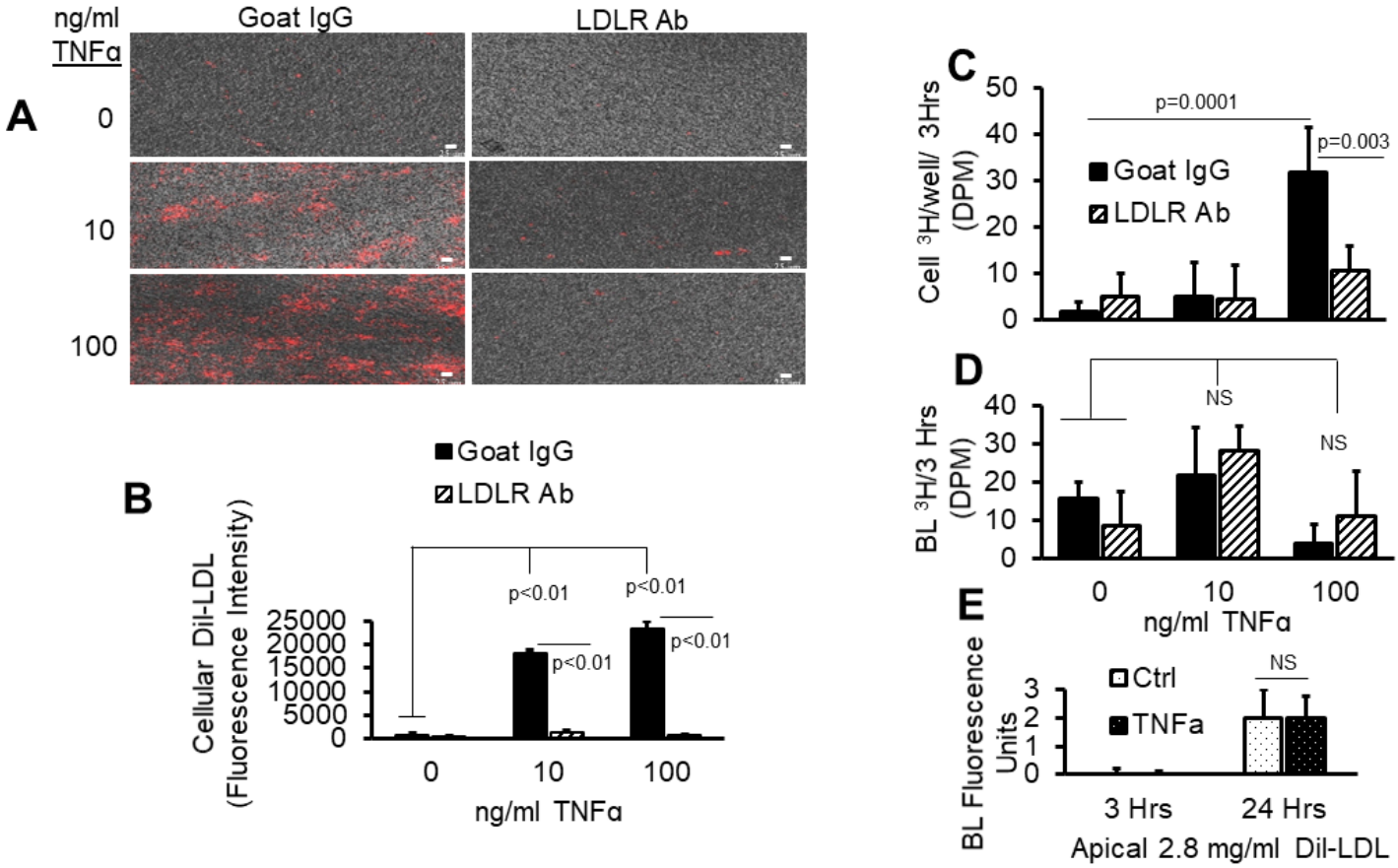

Figure 12

TNFα does not affect AP to BL LDL lipid release. A-D, pHAECs were pre-treated as in Figure 11 with 0, 10, or 100 ng/ml TNFα for 24 hrs in serum. Subsequently, 20 μg/ml control normal goat IgG (Goat IgG) or LDLR antibody (LDLR Ab) was added to the AP medium without serum for 2 hrs. Finally, 1.6 mg/ml double labeled DiI,[3H]CE-LDL was added to the AP medium for 3 hrs. After heparin wash, the intracellular fluorescence (A-B), 3H radioactivity (C), or medium BL 3H (D) was determined. E, The cells were treated for 3 or 24 hrs with 0 (Ctrl) or 100 ng/ml TNFα without serum in the presence of apical DiI-LDL, and the BL fluorescence was determined. n = 3.