Tumor necrosis factor induces cholesterol accumulation in human arterial endothelial cells through LDL receptor surface redistribution

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ABSTRACT

Tumor necrosis factor (TNFα) is an important mediator of inflammatory processes involved in atherosclerotic development. Here, the effect of TNFα on confluent primary human aortic endothelial cell (pHAEC) cholesterol content and low density lipoprotein (LDL) trafficking were investigated. TNFα, up to 100 ng/ml, did not cause a significant change in cell number, but altered the cell morphology dose-dependently. It induced LDL cholesteryl ester accumulation in a time- and concentration-dependent manner. This increase was accompanied by enhanced ^{125}I-LDL plasma membrane binding. Dil-LDL binding to pHAECs pretreated with TNFα was suppressed by excess unlabeled LDL, but not oxidized LDL. Moreover, the multi-valent metal chelator, 1,10-phenathroline (PhenA), but not reducing agent, tetramethylthiourea (TMTU), inhibited TNFα-induced dil-LDL accumulation. Among receptor inhibitors against scavenger receptor B1 (SR-B1), LDL receptor (LDLR), LDL receptor related proteins (LRPs), using BLT-1, PCSK9, and RAP, respectively, only PCSK9 had a significant effect on dil-LDL uptake. Further, specific antibody against LDLR completely blocked LDL internalization. TNFα did not significantly alter total LDLR protein, but increased surface LDLR. On pHAECs grown on transwell inserts, TNFα did not enhance apical to basolateral LDL cholesterol release. It is concluded that TNFα induces LDLR surface localization and that LDLR does not promote apical to basolateral LDL transport across pHAECs.

INTRODUCTION

TNFα is a 17 kD soluble cytokine released from an insoluble membrane precursor by tumor necrosis factor converting enzyme (TACE) (1-3). It is capable of inducing apoptosis (4-6) or proliferation (5,7,8) of cells, depending on the accompanying presence of specific growth factors (9-12). It has been reported that TNFα is pro-atherogenic (13). However, it has been suggested that TNFα receptor expression in macrophages is anti-atherogenic(14), while the receptor expression in resident arterial wall cells is pro-atherogenic (15).

Serum concentration of TNFα increases with severity of atherosclerotic lesion (16,17) and age (18). It has been reported to be about 200 folds higher in the atherosclerotic lesion than in the circulating blood (19). In atherosclerotic lesions, macrophages (20) and smooth muscle cells (20,21) have been reported to express TNFα. 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) (22,23). In addition, it induces expression of monocyte chemoattractant protein I (MCP-1) (24), which stimulates subendothelial entry of lymphocytes and monocytes (25,26).

Arterial endothelial cells regulate the flux of lipoproteins through the arterial wall. Their ability to control the influx of proatherogenic lipoproteins, particularly LDL (27), is expected to have important implications on the progression of atherosclerosis. Unlike macrophages (28,29) and smooth muscle cells (28,30), normal confluent endothelial cells are resistant to cholesterol accumulation (31). However, fibro-fatty atherosclerotic lesions contain endothelial foam cells with intact monolayer (32-34).

To understand the possible mechanisms underlying cholesterol accumulation in contact-inhibited endothelial cells, confluent pHAECs were treated with TNFα in the presence of LDL. I present evidence that native LDL can induce cellular cholesterol accumulation in the presence of TNFα. It will be shown that TNFα alteration of pHAEC LDL uptake facilitates greater cholesterol accumulation in the cells.

RESULTS

TNFα induces cholesterol accumulation and promotes LDL binding to pHAECs.

Atherosclerotic lesion is characterized by release of inflammatory cytokines, among which is TNFα. To evaluate the effect of TNFα on pHAECs cholesterol content, confluent pHAECs were treated with or without TNFα and the cellular cholesterol content was measured. As is shown in **Fig. 1A-C**, TNFα significantly promoted increased in cellular total cholesterol, primarily in the form of cholesteryl esters (**Fig. 1B**).

The exact concentration of TNFα in atherosclerotic lesion is expected to vary, depending on the stage and severity of the disease (16,18,19). Thus, the dose-dependent effect of TNFα on pHAEC cholesterol content was measured. As is shown in **Fig. 1D**, in the presence of LDL, TNFα induced cholesterol accumulation in pHACs from 1 to 100 ng/ml. The amount increased as the TNFα concentration increased. To determine whether the increase in cellular cholesterol content was due to increased LDL binding, surface bound (**Fig. 1E**) and cell-associated (**Fig. 1F**) ^{125}I-LDL were measured. Plasma membrane dissociable ^{125}I-LDL values were measured at 4 °C after treatment of the pHAECs with ^{125}I-LDL at 37 °C. In **Fig. 1E**, TNFa increased surface dissociable ^{125}I-LDL. The amount dissociable was increased further in the presence of 20 folds excess unlabeled LDL. This was statistically significant when compared to cells without TNFα pretreatment.

TNFα-induced LDL binding does not require LDL oxidation. TNFα has been reported to promote superoxide and hydrogen peroxide release (35,36). Hence, experiments were performed to determine whether oxidative modification of LDL is a prerequisite to TNFa-induced LDL binding. To visualize TNFα-induced LDL binding to pHAECs, dil-labeled LDL (dil-LDL) was used to perform binding studies in the presence of the endocytosis inhibitor, latrunculin A (37). As is shown in **Fig. 2**, binding of dil-LDL in the presence of latrunculin A was significantly suppressed by excess unlabeled LDL, but not oxidized LDL. It should be noted that latrunculin A also caused the cells to partially detach from the plastic dishes and assume a spherical structure.

LDL accumulation in pHAECs is multi-valent metal chelator dependent, but reducing agent independent. To further evaluate the potential role of oxidative modification of LDL in facilitating LDL

accumulation induced by TNFα, pHAECs were pretreated with the metal chelator, PhenA or reducing agent, TMTU. Phen A does not have a reducing group, but TMTU is primarily a reducing agent, but capable of metal chelation at higher concentrations (38,39). As is shown in **Fig. 3**, PhenA, but not TMTU, suppressed dil-LDL accumulation with or without TNFα during a 24 hour incubation. It can also be seen in **Fig. 3** that TNFα did not cause a significant change in cell number and density in the absence of the metal chelator. As expected, Phen A caused some cells to detach. This detachment was more pronounced in the presence of the chelator.

TNFα-induced LDL uptake does not require LDLR-related (LRP) proteins. Having determined that LDL accumulation induced by TNFα is independent of LDL oxidation, experiments were performed to test the contribution of potential native LDL receptors. LDL have been reported to bind LDLR (40) and SR-B1 (41,42). It is also expected to bind LRP, in an apolipoprotein E (apoE)-dependent fashion (43). Although apoB is the primary apoprotein of LDL, a significant amount of apoE can be detected (44). As can be seen in **Fig. 4**, neither SR-B1 inhibition with BLT-1 (45) nor LRP inhibition with RAP (46,47) affected LDL uptake in pHAECs treated with or without TNFα. On the other hand, downregulation of LDLR with PCSK9 (48) significantly suppressed LDL uptake. Interesting, PCSK9 enhanced morphological changes induced by TNFa (**Fig. 4**).

TNFα-induced LDL uptake is LDLR-dependent. To test the possibility that TNFa-induced LDL uptake was LDLR dependent, as suggested in **Fig. 4**, pHAECs were pretreated with control or LDLR antibody as shown in **Fig. 5**. As the data in **Fig. 5A** shows, the control antibody had no effect on dil-LDL uptake, but polyclonal antibody against LDLR completely blocked dil-LDL uptake. As is indicated in **Fig. 5B**, the LDLR antibody was specific for LDLR. Additionally, it can be seen in **Fig. 5B** that treatment of pHAECs with TNFα enhanced LDLR-antibody interaction, leading to formation of higher molecular weight complex. This complex is resistant to 1% SDS, 4M urea, and 5% beta mercaptoethanol that were present in lysate. In **Fig. 5C**, it is shown that TNFα did not significantly alter the total LDLR in the cells.

TNFα promotes increase in plasma membrane LDLR. Results from **Fig. 1E-F** and **Fig. 5B-C** suggested that TNFα increased the amount of LDLR present on the pHAEC surface. To further confirm this possibility, experiments were performed at 4 °C to determine the relative amount of LDLR present with or without TNFα pretreatment. As is shown in **Fig. 6**, there was no surface fluorescence in the absence of LDLR antibody. TNFα increasd the amount of high affinity surface LDLR by about 4 folds. This high affinity LDLR receptors were not significantly altered in cells pre-treated with 80 µg/ml LDL for 2 hours. The morphological changes induced by TNFα can be readily seen in Fig. 6. While the untreated pHAECs appear as irregular cobble-stone structures at confluence, TNFα-treated cells appear elongated, with fibroblast/smooth muscle cell type morphology.

TNFα does not promote AP to BL transport 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 **Fig. 7A**. ^{125}I-LDL detected in the BL medium was either non-intact (**Fig. 7B**) or intact (**Fig. 7C**). In **Fig. 7B**, it is shown that unlabeled LDL competitor reduced the amount of non-intact ^{125}I-LDL in a TNFα-independent manner. In contrast, the unlabeled competitor had no effect on the intact ^{125}I-LDL measured in the BL medium (**Fig. 7C**).

LDLR does not promote AP to BL LDL cholesterol release in pHAECs. To determine whether LDLR facilitates AP to BL transport, the pHAECs on transwell inserts were incubated

with LDL colabeled with Dil and ^{3}H-cholesteryl esters (**Fig. 8**). As can be seen in **Fig. 8A**, TNFα dose-dependently increased the accumulation of dil-LDL on the pHAECs grown on transwell inserts, in the same manner as those grown on plastic dishes. Similar to pHAECs on plastic dishes, LDLR antibody significantly blocked uptake of dil-LDL by pHAECs grown on transwell inserts. The corresponding uptake of ^{3}H-cholesteryl esters is shown in **Fig. 8B**. It is shown that the ^{3}H-cholesteryl ester accumulation induced by TNFα 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 (**Fig. 8C**). This is consistent with the BL dil-LDL, whose value was independent of TNFα treatment (**Fig. 8D**).

DISCUSSION

Native LDL modification due to oxidation (49,50), aggregation (51), or complex formation with proteoglycans (52-54) is known to promote cholesterol accumulation in macrophages. Although confluent arterial endothelial cells are resistant to cholesterol accumulation induced by lipoproteins, I show here that this can be altered in the presence of TNFα. Thus, LDL modification is not an absolute requirement for LDL-induced cholesterol accumulation.

As measured by cholesterol (**Fig. 1A-D**) and Dil fluorescence (**Figs. 3-5, 8**), TNFα dose-dependently caused several folds increase in pHAECs intracellular LDL content. Results shown in **Fig. 5A** demonstrated that this was LDLR dependent. If LDLR served to promote AP to BL LDL transport, then a significant amount of Dil fluorescence and ^{3}H-cholesteryl ester LDL would have been detectable in the BL medium of pHAECs treated with TNFα compared to the untreated. Instead, there was no difference in the BL labels between these two groups, with or without LDLR antibody (**Fig. 8C-D**). There was no significant difference in non-intact BL ^{125}I-LDL between control and TNFα-treated cells (**Fig. 7B**). The finding that non-intact BL^{125}I-LDL was reduced in presence of excess unlabeled LDL suggested that it represented LDL degraded within the cells. On the other hand, intact BL ^{125}I-LDL was not affected by excess unlabeled LDL. This indicates that the intact BL ^{125}I-LDL represents LDL that passed between the pHAECs through non-selective transport process (**Fig. 7C**). The degradation of LDL protein through LDLR is consistent with what have been found for macrophages (55,56).

Interestingly, bovine brain microvascular endothethial cells have been reported to promote apical to basolateral transport of LDL through LDLR (57). It thus appears that LDLR trafficking in brain microvascular endothelial cells is different from human aortic endothelial cells. Additionally, it has been reported that AP to BL LDL transport occurs in vivo across arterial endothelial cells (58). My findings suggest that this transport is probably not through LDLR. SR-BI, on the other hand, have been reported to bind LDL (41,59,60). Since, SR-B1 have been shown to cause AP to BL transport of HDL in bovine aortic endothelial cells (61), it is possible that it can do the same for LDL in human aortic endothelial cells. However, the results (**Fig. 5A-B**), indicate that LDLR is the dominant receptor utilized by pHAECs for LDL uptake.

EXPERIMENTAL PROCEDURES

Materials. Agilent Technologies (Santa Clara, CA): Bond elut column, 12256060. Alfa Aesar (Haverhill, MA)*:* 1,10-phenanthroline (PhenA, A16254), tetramethylthiourea (TMTU, L13392). ATCC (Manassas, VA)*:* pHAECs (PCS-100-011), vascular basal medium (VBM, PCS-100-030), and vascular endothelial growth factor kit (VEGF, PCS-100-041). Caymen Chemical (Ann Arbor, MI)*:* PCSK9 (20631). Enzo Life Sciences (Farmingdale, NY)*:* Latrunculin A (BML-T119), receptor associated protein (RAP, BML-SE552-0100). Pall Corporation (Port Washington, NY): Centrifugal filter

(MAP003C36). Perkin Elmer (Waltham, MA): [1,2-3H(N)]-Cholesterol (NET139001). ProSci Inc (Poway, CA): Tumor necrosis factor alpha (TNFα, 96-734). R&D Systemss (Minneapolis, MN): anti-goat (NL001), Fetal bovine serum (FBS, S11550), LDLR antibody (AF2148), normal goat IgG (AB-108-C). *Sigma-Aldrich* (St. Louis, MO): BLT-1 (373210), Dil (468495), fatty acid free bovine serum albumin (FAF-BSA, A8806), modified Hanks balanced salt solution (mHBSS, H8264), phosphatase inhibitor (P0044), protease inhibitor (P8340). Spectrum Labs (Cincinnati, OH): Dialysis membrane, 131204. Thermo Fisher Scientific (Waltham, MA): Antibiotic-antimycotic (15240112), desalting columns (89890), iodination beads (28665).

Cell culture and incubations. pHAECs (passages 3-7) 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, unless indicated otherwise. Incubations were at 37 °C, unless indicated otherwise.

LDL purification. All procedures were performed between 0 and 4 °C. Centrifugations and dialysis were performed at 4 °C. LDL was 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 (62) were performed (after adding solid KBr) to obtain d<1.019 and 1.019-1.063 g/ml (LDL), respectively, floating lipoproteins. Ultracentrifugations were done using type Ti70 rotor at 50,000 rpm for 20 hours. The LDL was 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, 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. The protein contents of the isolated LDL 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 (63-65). LDL concentrations represent the protein content throughout the manuscript.

Cell cholesterol determination. pHAECs treated with or without TNFα were washed two times with PBS containing 400 units/ml sodium heparin and once with PBS, at room temperature, to remove surface LDL. After extraction of neutral lipids with hexane: 2-propanol (1:1), the solvent was evaporated. The residual cell matter was lysed with 0.1 M NaOH, 0.1% SDS, for protein reading. The lipids were redissolved with cholesterol assay buffer (CAB), 0.1 mM sodium phosphate, 5 mm sodium taurocholate, 2% Triton X-100, 0.5 mM polyethylene glycol 8000. Total and free cholesterol content were determined enzymatically using CAB in the presence of approximately 1 mM scopoletin (66) (excitation 360, emission 460), a modification of a previously published procedure (67). Cholesteryl esters were calculated by subtracting the cholesterol content in the absence of cholesterol esterase from that in its presence.

^{3}H-cholesteryl ester ($C^{3H}E$) 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. After covering with foil, the mixture was incubated at 37 °C for 48 hours. The ^{3}H-cholesteryl esters generated were isolated using serial bond elut columns with several hexane passages, as previously described (68).

Western blotting. pHAECs were lysed with lysis buffer (0.2 % SDS, 2% Triton X-100, PBS, pH 7.5, 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 C^{3H}E-LDL. Generation of Dil-labeled LDL was obtained essentially as previously described (69), with minor modifications. In brief, a mixuture of about 2 mg LDL protein and 7 ml of human lipoprotein deficient serum was mixed with ~1 mg C^{3H}E and/or 0.5 mg Dil in 20 µl DMSO aliquots under sterile conditions. After covering with foil, the mixture was incubated at 37 °C for 21 hours. LDL was repurified as described above, after adjusting to 1.063 g/ml with solid KBr. Dil, C^{3H}E-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 reaction was stopped with 50 mM each of unlabeled sodium iodide and sulfite, 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 DB.

^{125}I-LDL cell curface binding. Confluent pHAECs on plastic dishes were cultured with 0 or 5 ng/ml TNFα in 15% serum for 48 hours. Treatment continued with serum-free medium in the continued presence of TNFα for 3 hours. 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 DB, and finally filtered through 0.2 µm membrane under sterile conditions.

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 (70). To the BL medium, d<1.21 g/ml FBS was added to 15%, mixed, and the 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.

STATISTICAL ANALYSIS. Data are reported as averages ± standard deviation, where indicated. Analysis of variance, followed by Tukey post hoc testing was done by using statpages.info website.

DATA AVAILABILITY. All data are contained in the manuscript.

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CONFLICT OF INTEREST

The author declares that he has no conflicts of interest with the contents of this article

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FOOTNOTES

Abbreviations: AP, apical; apoE , apolipoprotein E; 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; 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; PhenA, 1,10-phenanthroline; pHAECs, primary human aortic endothelial cells; RAP, receptor associated protein; SDS, sodium dodecyl sulfate; TMTU, tetramethylthiourea; TNFα, tumor necrosis factor alpha; VBM, vascular basal medium; VEGF, vascular endothelial growth factor kit;

FIGURE LEGENDS

Figure 1. TNFα enhances cholesterol accumulation and LDL binding to pHAECs. A-C, Confluent pHAECs in serum-free, growth factor-containing medium were treated with 20 µg protein/ml human LDL in the presence (+) or absence (-) of 100 ng/ml TNFα for 72 hours. Free cholesterol (FC), cholesteryl esters (CE), and total cholesterol (TC) were measured as described under Experimental Procedures. **D**, Confluent pHAECs were treated with 20 µg/ml LDL and 0.25 µCi/ml ^{3}H-cholesterol in the presence of the indicated concentration of TNFα for 48 hours. After washing 3 times with 450 units/ml heparin in PBS, the lysate radioactivity was measured by scintillation counting. **E**, Confluent pHAECs were pre-treated with 0 or 5 ng/ml TNFα. After 3 hour pre-equilibration in serum-free medium, the cells were incubated with ^{125}I-LDL for 1 hour. Subsequent to washing and chilling to 4 °C, the amount of radioactivity released to the medium in the absence (-) or presence (+) of excess unlabeled LDL (unLDL) was measured, as detailed under Experimental Procedures. It should be noted that the binding is temperature dependent. Little binding occurs at 4 °C. **F**, The experiment was performed as in E, and the amount of radioactivity associated with the cell was determined. Each dot represents a single replicate from one (D) and two (A-C, E-F) independent experiments performed in triplicates. The averages are indicated as bars.

Figure 2. LDL oxidation is not required for TNFα-induced LDL binding. Confluent pHAECs were incubated with 100 ng/ml TNFα in 15% serum medium for 18 hours. This was replaced with serum-free medium containing 4 µg/ml of the endocytosis inhibitor, latrunculin A, in the continued presence of TNFα for 1 hour. Subsequently, equal volume of 5 µg /ml dil-LDL in the serum-free medium with 1% control buffer, 250 µg/ml LDL, or 250 µg/ml oxLDL was added. After 2 hours, the media were replaced with mHBSS, and the fluorescence was determined using phase-contrast microscopy. The images are from a single experiment, representative of 4 per group. All images are of the same magnification.

Figure 3. Lipid-soluble reducing agent does not inhibit TNFα-induced LDL accumulation. Confluent pHAECs in 15% serum were treated with DMSO control (0.1% DMSO), or 0.1% DMSO containing PhenA or TMTU for 18 hours. The media was then replaced with serum-free, growth-factor containing medium, with 10 µg/ml diL-LDL in the continued presence of DMSO, PhenA, or TMTU, with 0 (Ctrl) or 100 ng/ml TNFα. Following 24 hours of incubation, the cells were washed once with mHBSS before phase-contrast fluorescence microscopy. The images are from a single experiment, representative of two independent experiments performed in duplicates. All images are of the same magnification.

Figure 4. Effect of receptor antagonists on LDL uptake. Confluent pHAECs in 15% serum were incubated with 0 (Ctrl) or 100 ng/ml TNFα for 24 hrs. The cells were then pretreated in the presence of 0.05% DMSO alone (DMSO), or with 50 µM BLT-1, 12.5 µg/ml human PCSK9, or 21 µg/ml human RAP for 1 hour. Subsequently, 8 µg/ml dil-LDL was added. After further incubation for 4 hours, the media were removed, and the cells were washed once with mHBSS, followed by fluorescence phase-contrast microscopy. The images are from a single experiment, representative of two independent experiments in duplicates or greater. All images are of the same magnification.

Figure 5. Specific LDLR antibody blocks TNFα-induced LDL uptake. A, Confluent pHAECs were incubated with 0 (Ctrl) or 100 ng/ml TNFα in 15% serum for 24 hours. The latter was replaced with serum-free medium for 1 hour in the continued presence of TNFα, with no antibody (No Ab), 18 µg/ml normal goat IgG (Ctrl Ab), or goat LDLR IgG antibody (LDLR Ab). Subsequently, dil-LDL in the serum-free medium was added to 1.25 µg/ml. After 2 hours, the media were washed once with mHBSS, followed by phase contrast fluorescence microscopy. All images are of the same magnification. **B-C**, The cells in A were lysed for western blotting for the LDLR and GAPDH. The images are from a single experiment, representative of two independent experiments, performed in duplicates and triplicates.

Figure 6. TNFα induces surface localization of LDLR. Confluent pHAECs were cultured with 0 (Ctrl) or 100 ng/ml TNFα in 15% serum for 24 hours. The media were then replaced with serum-free solution in the continued presence TNFα, with (+) or without (-) 80 µg/ml LDL for 2 hours. After washing once with mHBSS, the cells were chilled on ice in mHBSS for 5 min. Subsequently, ice-cold 20 µg/ml of normal goat IgG or LDLR Ab in mHBSS, 0.5% FAF-BSA (BM) was added. After 1 hour at 4 °C, the cells were washed 2 times with ice-cold mHBSS, then incubated with ice cold 52 µg/ml fluorescent anti-goat antibody in BM for 1 hour at 4 °C. Microscopy was performed after washing two times with mHBSS at 0 °C. The images are from a single experiment, performed in duplicates per group. All images are of the same magnification.

Figure 7. TNFα does not affect AP to BL LDL transport of degraded LDL protein. A, Diagram of the experimental setup in B-C, in which pHAECs are grown to confluence on 3 µm polycarbonate filters, with ^{125}I-LDL in the AP medium. **B**, pHAECs were grown on 3 µm polycarbonate inserts for 5 days to confluence. The AP and BL media were subsequently replaced with fresh 15% serum, with 0 (Ctrl) or 5

ng/ml TNFα added to the BL side. After 48 hours, the AP media were replaced with serum-free media containing 10 µg/ml ^{125}I-LDL with vehicle buffer (no competitor) or 30 folds unlabeled LDL, in the continued presence of BL TNFα. After 3 hours, the BL media were collected, and non-intact ^{125}I-LDL was measured as described under Experimental Procedures. **C**, The experiment was performed as in B, followed by detection of intact BL ^{125}I-LDL. The bars are averages from 3 independent experiments performed in triplicates or more ± standard deviations.

Figure 8. TNFα does not affect AP to BL LDL cholesterol release. A, pHAECs were seeded to ~80% and grown to confluence for 18 days on 3 µm polycarbonate transwell inserts. Subsequently, the medium was replaced with 15% serum containing 0, 10, or 100 ng/ml TNFα on both sides for 19 hours. Afterwards, the media were replaced with serum-free media in the continued presence of TNFα. The AP medium also contained either 20 µg/ml normal goat IgG or goat LDLR antibody (LDLR Ab). Following a 2 hour incubation, LDL double labeled with ^{3}H-cholesteryl esters and Dil were added to 2.2 mg/ml. The cells were further incubated for 3 hours, then the cells on the inserts were washed two times with 450 units/ml sodium heparin in mHBSS, once with mHBSS, followed by microscopy. All images are of the same magnification. **B**, The cells in A were lysed after microscopy with 0.1% SDS, 0.1 M NaOH for ^{3}H radioactivity by scintillation counting. **C**, Following a 3 hour AP to BL transport as in A, the BL ^{3}H radioactivity was determined. **D**, The cells were treated for 3 or 24 hours (Hrs) as in A with 0 (Ctrl) or 100 ng/ml TNFα, and the BL fluorescence was determined. The images are from a single experiment, representative of 3 independent experiments performed in duplicates. The bars are averages ± standard deviations.

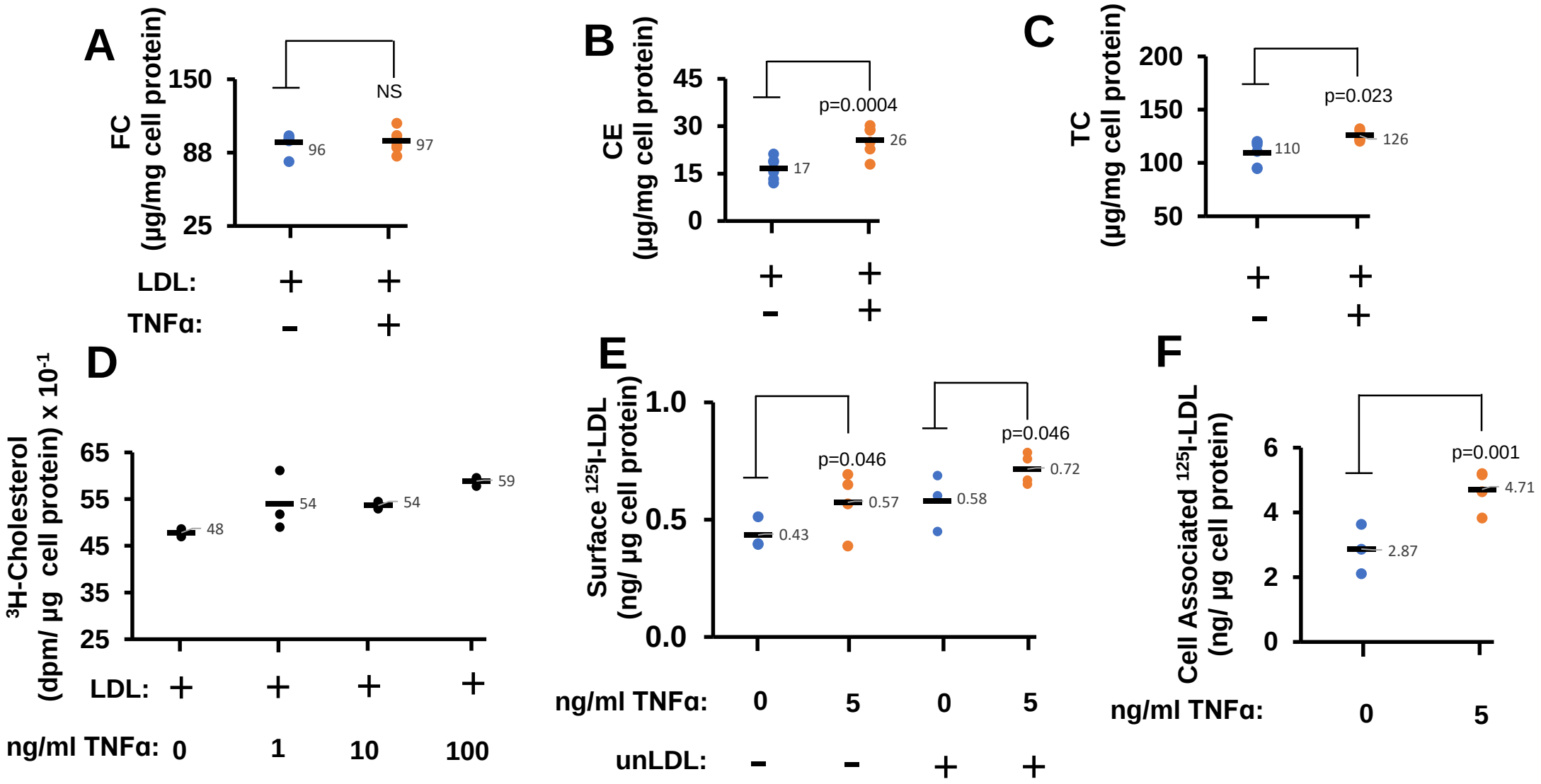
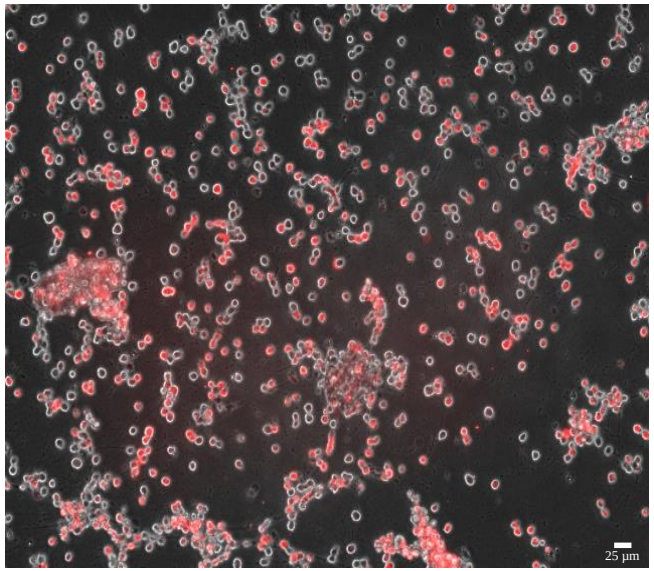
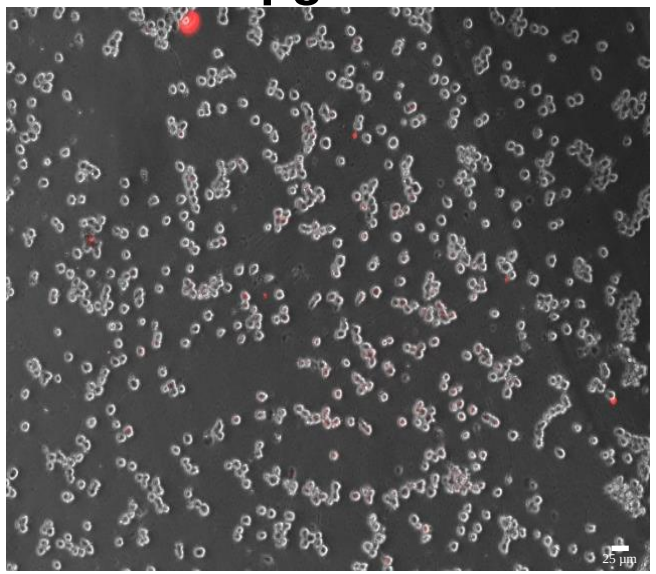

A
FC (µg/mg cell protein)
150
88
25
NS
96
97
LDL: + +
TNFα: - +
B
CE (µg/mg cell protein)
45
30
15
0
p=0.0004
17
26
+ +
- +
C
TC (µg/mg cell protein)
200
150
100
50
p=0.023
110
126
+ +
- +
D
3H-Cholesterol (dpm/ µg cell protein) x 10-1
65
55
45
35
25
48
54
54
59
LDL: + + + +
ng/ml TNFα: 0 1 10 100
E
Surface 125I-LDL (ng/ µg cell protein)
1.0
0.5
0.0
p=0.046
p=0.046
0.43
0.57
0.58
0.72
ng/ml TNFα: 0 5 0 5
unLDL: - - + +
F
Cell Associated 125I-LDL (ng/ µg cell protein)
6
4
2
0
p=0.001
2.87
4.71
ng/ml TNFα: 0 5

Figure 1

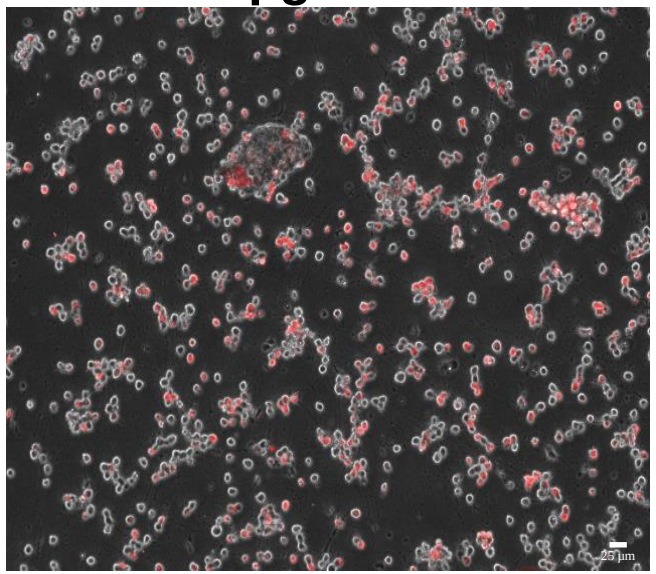
2.5 µg/ml dil-LDL + 1% control buffer



2.5 µg/ml dil-LDL +125 µg/ml LDL



2.5 µg/ml dil-LDL +125 µg/ml oxLDL



TNFα: + + +

Figure 2

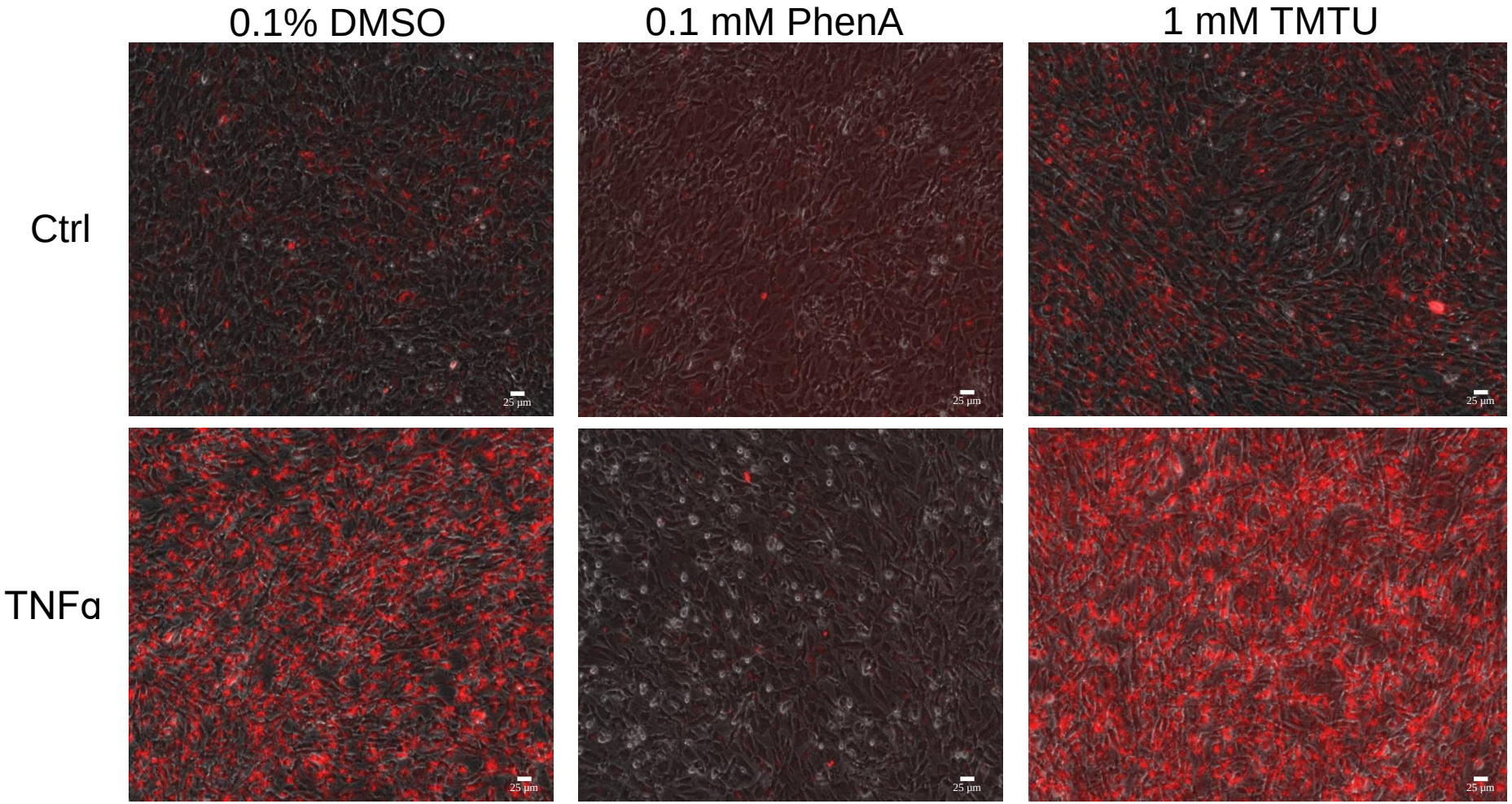


Figure 3

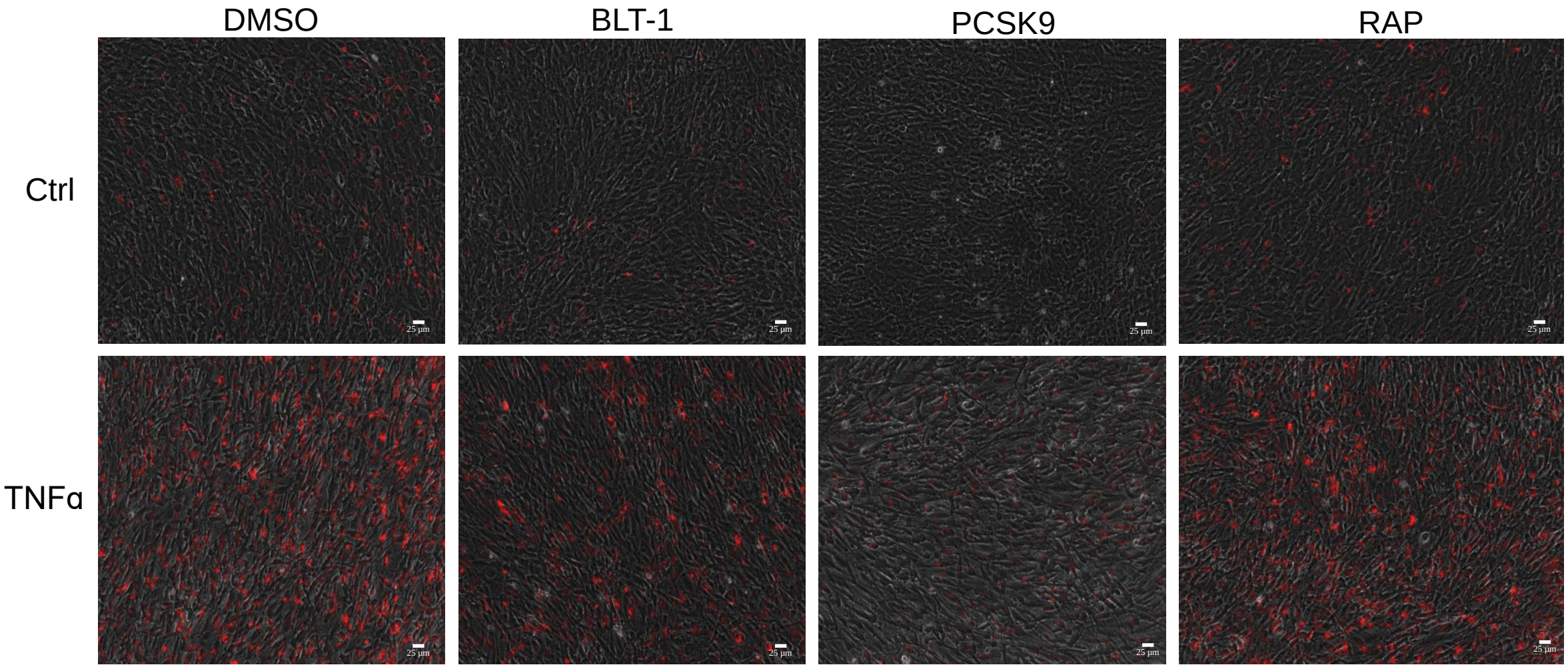
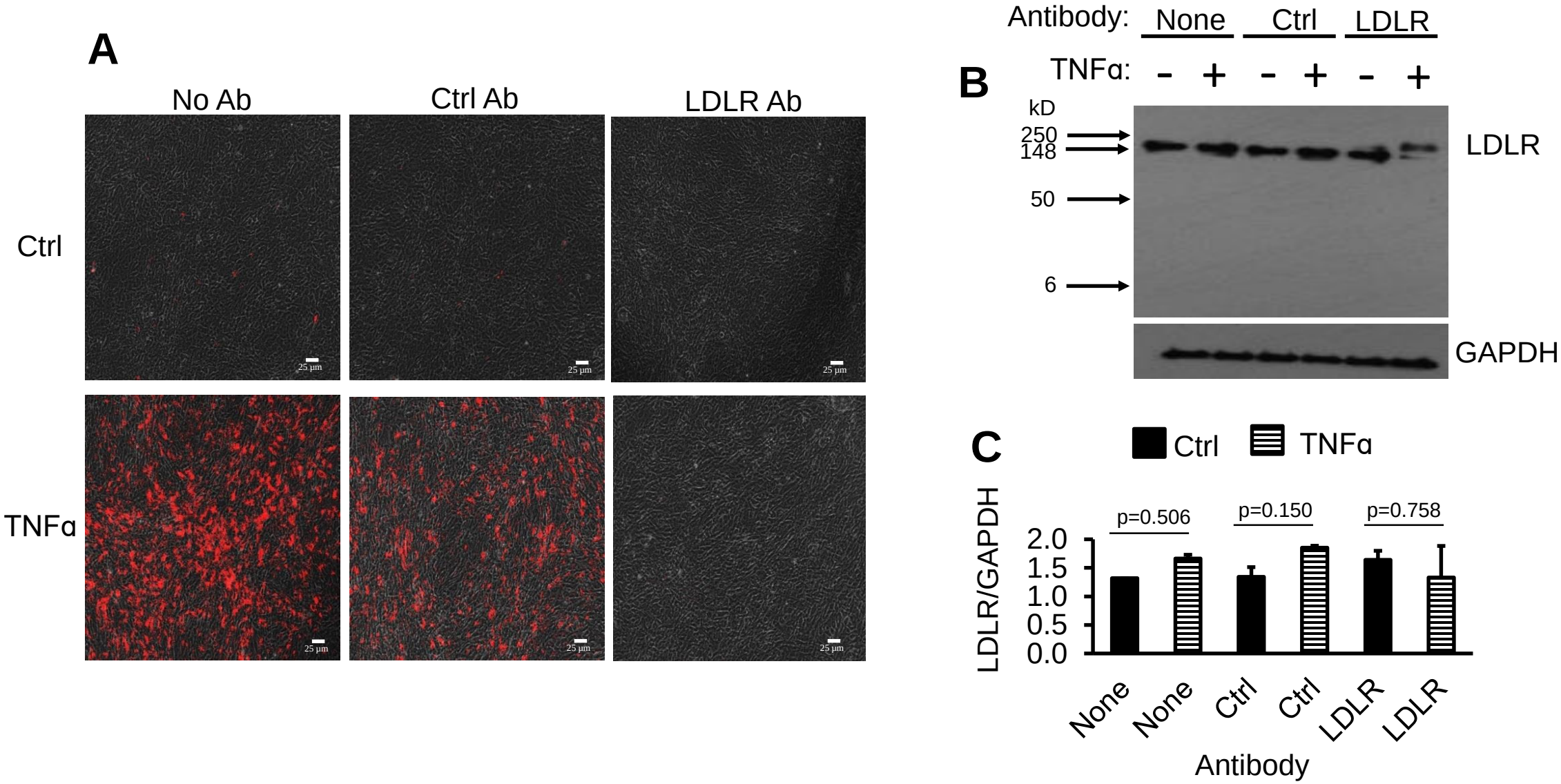


Figure 4


A
No Ab
Ctrl Ab
LDLR Ab
Ctrl
TNFα
25 µm
B
Antibody:
None
Ctrl
LDLR
TNFα:
- + - + - +
kD
250
148
50
6
LDLR
GAPDH
C
Ctrl
TNFα
LDLR/GAPDH
p=0.506
p=0.150
p=0.758
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1.5
1.0
0.5
0.0
None
None
Ctrl
Ctrl
LDLR
LDLR
Antibody

Figure 5

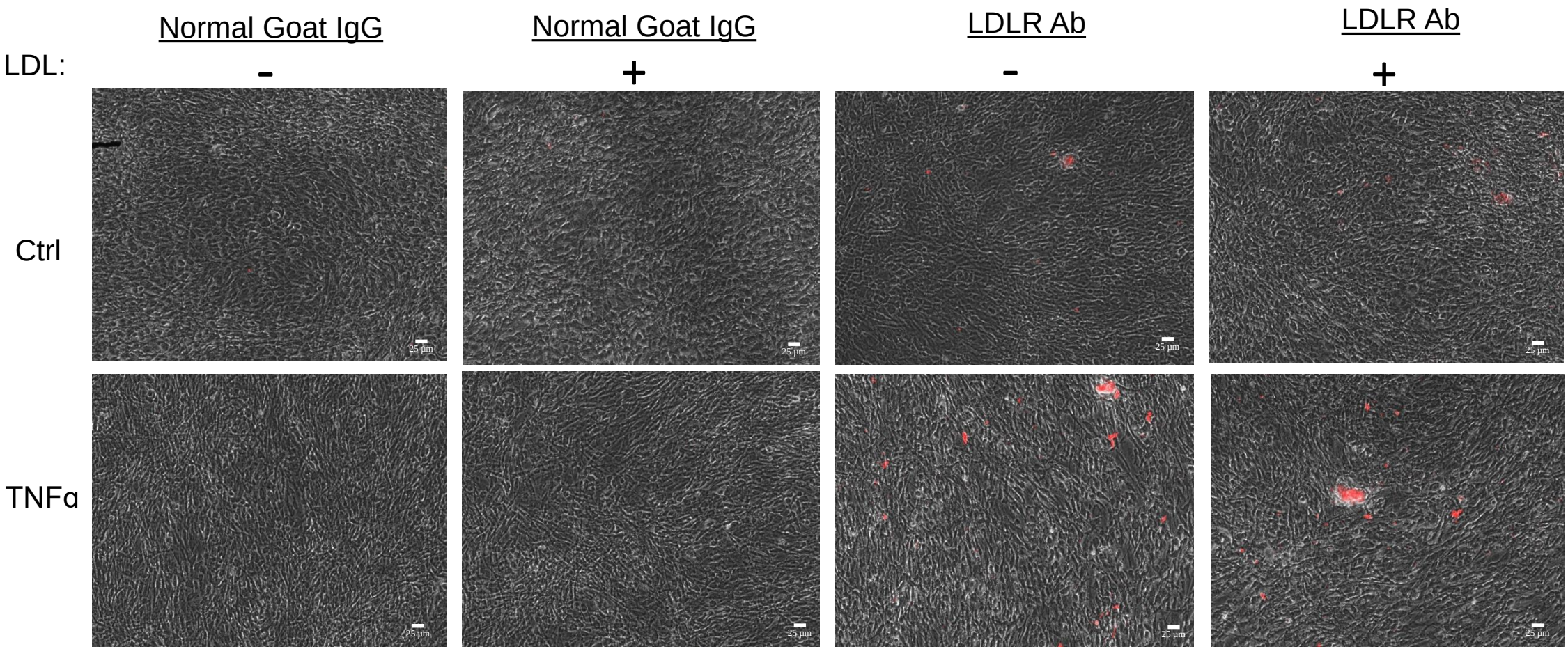
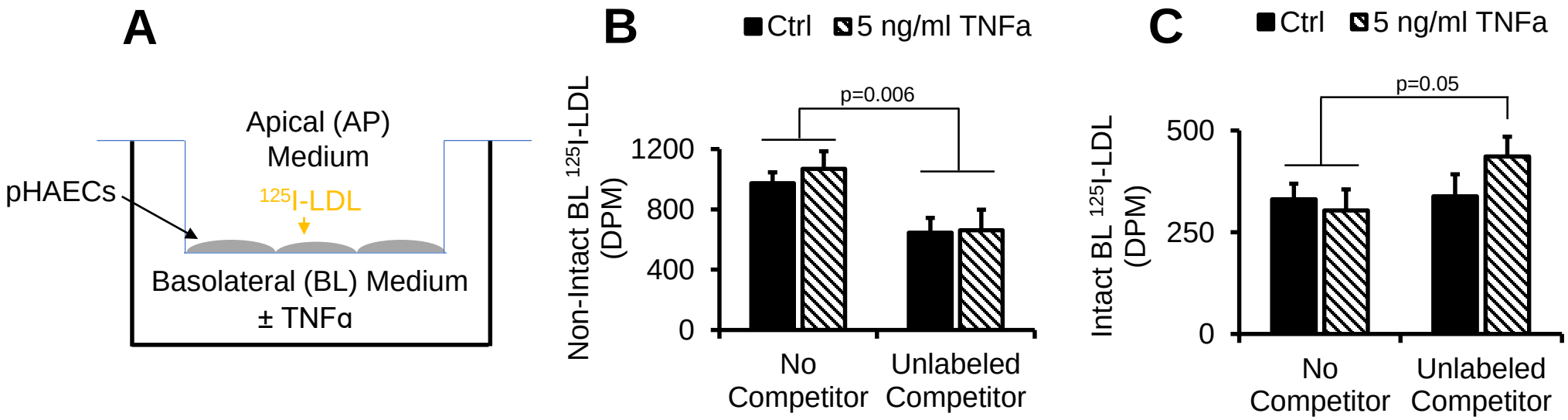

Normal Goat IgG
Normal Goat IgG
LDLR Ab
LDLR Ab
LDL:
-
+
-
+
Ctrl
TNFα
25 µm

Figure 6


A
Apical (AP)
Medium
pHAECs
125I-LDL
Basolateral (BL) Medium
± TNFα
B
Ctrl
5 ng/ml TNFa
Non-Intact BL 125I-LDL
(DPM)
p=0.006
1200
800
400
0
No
Competitor
Unlabeled
Competitor
C
Ctrl
5 ng/ml TNFa
Intact BL 125I-LDL
(DPM)
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250
0
No
Competitor
Unlabeled
Competitor

Figure 7

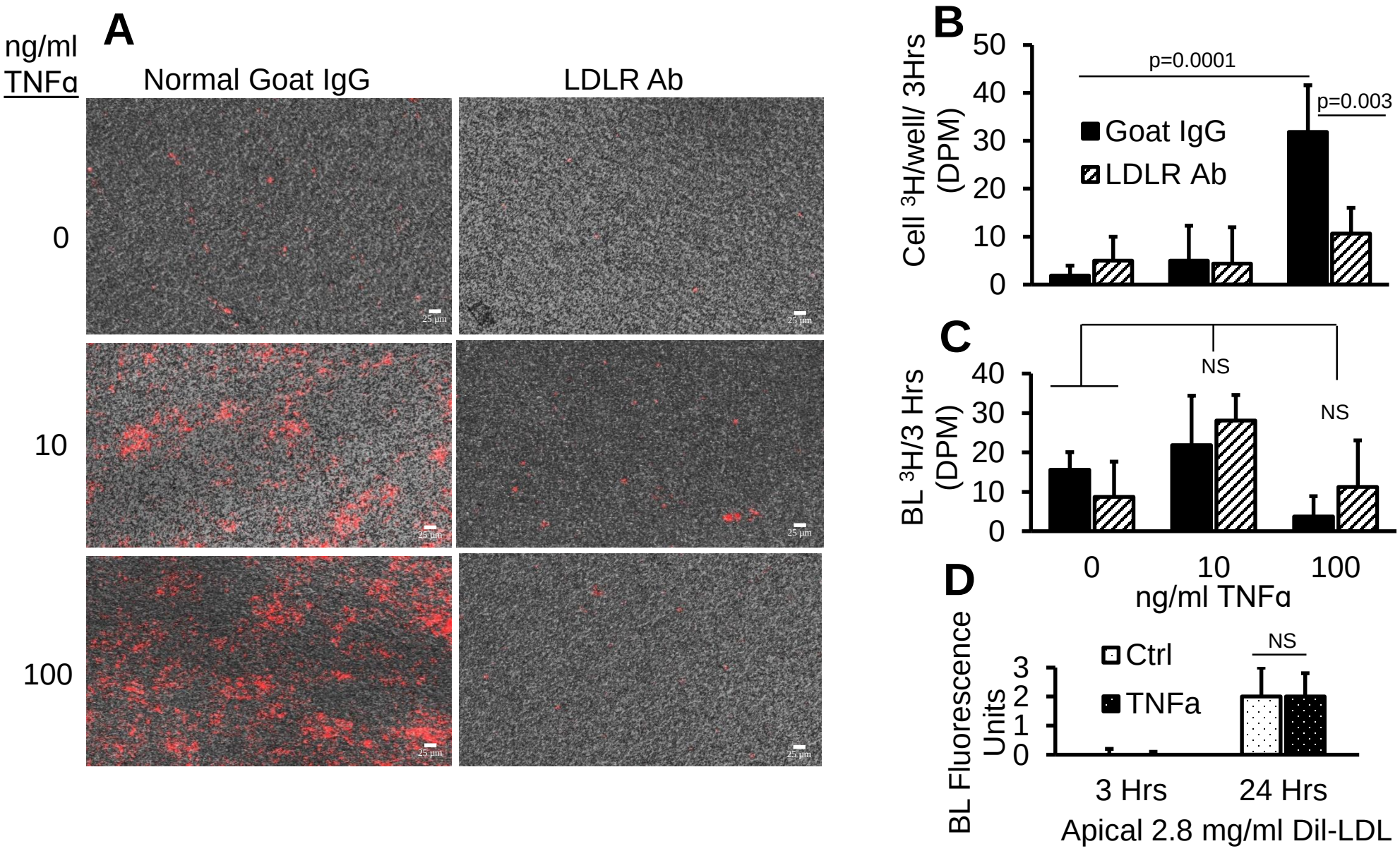
A
ng/ml TNFα
Normal Goat IgG
LDLR Ab
0
10
100
B
Cell ³H/well/ 3Hrs (DPM)
p=0.0001
p=0.003
Goat IgG
LDLR Ab
C
BL ³H/3 Hrs (DPM)
NS
NS
ng/ml TNFα
D
BL Fluorescence Units
Ctrl
TNFa
NS
3 Hrs
24 Hrs
Apical 2.8 mg/ml Dil-LDL

Figure 8