HMGB1 binds to modified LDL and enhances its uptake by macrophages

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Warmest regards,
Khulan Lkhamsuren

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LIST OF ABBREVIATED TERMS

HMGB1	high mobility group box 1
DAMP	damage associated molecular pattern
LDL	low density lipoprotein
mLDL	modified low density lipoprotein
oxLDL	oxidized low density lipoprotein
VLDL	very low density lipoprotein
HDL	high density lipoprotein
SR-A1	class A1scavenger receptor
RAGE	receptor for advanced glycation end products
TLR4	toll like receptor 4
LOX-1	lectin-like oxidized LDL receptor-1

ABSTRACT

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Atherosclerosis is an inflammatory cardiovascular disease which is commonly described as lipid accumulation in the intimal region of the arterial wall that eventually causes myocardial infarction and stroke. The high mobility group box 1 (HMGB1) is a nuclear protein which plays important role in immunity and inflammation. HMGB1 is elevated in atherosclerotic lesions which indicated its possible involvement in the progression of atherosclerosis. In this study, we observed that HMGB1 directly binds to LDL and modified LDL, but not to HDL. Moreover, modified LDL uptake by THP-1 macrophages was increased time- and dose-dependent manner in the presence of HMGB1. Addition of soluble CD36 scavenger receptor protein reversed the HMGB1 effect on modified LDL uptake. Further, HMGB1 bound to CD36 and CD36 overexpression enhanced the HMGB1 effect on modified LDL uptake. Collectively, our data indicates that HMGB1 bound to modified LDL and increased their uptake by macrophage through CD36 scavenger receptor.

Key words: HMGB1, oxidized LDL, CD36, atherosclerosis

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I. INTRODUCTION

Atherosclerosis is an inflammatory cardiovascular disease which is initiated by lipid accumulation in the intimal region of the arterial wall, eventually causing myocardial infarction and stroke. Despite the enormous amount of studies has been done regarding pathogenesis of atherosclerosis, there has been no efficient therapy found yet. Plaque in the artery wall is believed to be initiated by apolipoprotein B (ApoB)-containing low density lipoprotein (LDL) retention in the arterial intima and retained LDL can bind to proteoglycans and possibly undergoes modification. Modified and accumulated LDL then recruit the circulating monocytes which differentiate into macrophages and uptake the LDL by different mechanisms depending on the nature of modified LDL. These lipid-laden macrophages are called foam cells, a type of macrophages formed as a result of negative feedback following lipoprotein uptake, thus become packed with excessive amount of ingested lipids. Foam cells persist in the all stages of atherosclerosis[1]. Currently pathogenesis of this aggressive uptake of lipoprotein by macrophage is believed to be the "response-to-retention hypothesis of early atherosclerosis". This hypothesis suggests that LDL's long retention time allows it to be modified and processed within the intima and those modified LDL become the target of macrophage clearance and are identified as danger-associated molecular pattern (DAMP)[2].

In vitro oxidation model of LDL is reported to closely resemble the biological particles found in the lesion[3].

Scavenger receptors are a wide range of receptor family with large repertoire of ligands including modified and unmodified endogenous proteins and lipoproteins, as well as a number of conserved microbial structures, such as bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA)[4-6]. Scavenger receptor's main role is to identify and remove unwanted substances through endocytosis. Other functions of these multifunctional receptors include cell adhesion[7-9] and antigen presentation[10]. Scavenger receptors which can identify oxidation specific epitopes of oxLDL are CD36, SR-A1, MARCO, SR-B1, LOX-1 and SR-PSOX and their roles in atherosclerosis have been studied extensively[11-15]. Although their role in intracellular cholesterol accumulation was shown by numerous *in vitro* experiments[16], the *in vivo* studies are less clear[17]. However, more and more convincing reports are emerging on pro-atherogenic role of CD36 on the surface of macrophage[18-22]. When CD36 is exposed to oxLDL it forms a complex with TLR4 and TLR6 and triggers a sterile inflammatory response, eventually activating NF-ƙB[23,24]. Moreover, macrophages harvested from *Cd36*-null mice were defective in oxLDL uptake and atherosclerosis prone (*LDL receptor*-null or *Apo-E*-null) mice with CD36 deficiency developed less atherosclerotic lesion than *LDL receptor*-null or Apo-E deficient mice when the mice were on high fat diet[25-27]. CD36 also mediates cytoskeletal rearrangement which induces macrophage to trap in atherosclerotic plaque[28] and activates platelets[29-31] which participate in the thrombus formation after the plaque rupture and progression of inflammatory process during atherosclerosis[32]. It was also suggested that chemokine signaling enhances CD36 responsiveness toward oxLDL and accelerates foam cell formation, as integrin activated by chemokine signaling induced CD36 clustering and effectively altered the threshold for cells to engage with oxLDL[33]. These reports suggested that CD36 scavenger receptor could be the main mechanism which macrophage utilizes to bind and uptake modified LDL.

High mobility group box 1 (HMGB1) is a nuclear protein which binds to DNA and facilitates DNA-activity associated functions and plays important role in immunity and inflammation[34]. HMGB1 functions as a pro-inflammatory mediator[35,36] and is considered to be a DAMP molecule. HMGB1 is constitutively and ubiquitously expressed in most cells and is secreted passively by necrosis and actively during inflammation[37,38]. It was reported that HMGB1 participates in various cellular activities such as, autophagy[39], apoptosis[40, 41], as well as, macrophage migration, dendritic cell activation and maturation[45-47]. Structurally HMGB1 consists of two similar DNA-binding domains, boxes A and B, and C-terminal domain of acidic tail. Pro-inflammatory effect of HMGB1 was shown to be present in its box B[48]. Recently it has been also reported that depending on the redox state of its three cysteine residues, extracellular HMGB1 can have different biological functions. HMGB1 is elevated in human atherosclerotic lesions, especially in the nuclei and in the cytoplasm of macrophages and smooth muscle cells near the intima and in the areas adjacent to the necrotic core[42,49,50] which indicates its involvement in atherosclerosis. There are several evidences which prove that involvement. HMGB1 induces vascular smooth muscle cell migration and proliferation[42,43] and endothelial cell activation[44], which are critical steps during atherosclerosis. Moreover, rHMGB1 shown to activate vascular endothelial cells leading to expression and secretion of ICAM-1, VCAM-1, E-selectin, G-CSF, RAGE, TNFα, MCP-1, IL-8, plasminogen activator inhibitor 1, and tissue plasminogen activator[44]. Further, HMGB1 promoted proliferation of smooth muscle cells from atherosclerotic plaques, migration to the intimal layer, as well as expression of C-reactive protein, MMP2, MMP3 and MMP9[42, 50]. More notably, when neutralizing HMGB1 antibody was administered to Apo-E deficient mice fed with high-fat diet, the mice had 55% less atherosclerotic lesion formation when compared to control mice on the same diet[51]. Although these previous studies suggested HMGB1's engagement in atherosclerosis, its specific role relating to lipoproteins, lipid uptake and foam cell formation remains unknown. In this study, I observed that HMGB1 directly binds to LDL and oxidized LDL and increases their uptake by macrophage through CD36 scavenger receptor.

II. MATERIALS AND METHODS

1. Cell culture

THP-1 cells and RAW 264.7, HEK293T cells were cultured in RPMI 1640 and Dulbecco's Modified Eagle Medium (DMEM) media respectively with supplementation of 10% fetal bovine serum (FBS) and penicillin/streptomycin as complete media. All cultures were maintained to approximately 90% confluency in a humidified atmosphere of 5% CO_2–95% air at 37 ℃. THP-1 cells were differentiated by 0.5μM phorbol 12-myristate 13-acetate (PMA) in complete media for 3 hours. After differentiation cells were washed in RPMI 1640 twice, seeded and used for an experiment the next day.

2. Surface Plasmon Resonance (SPR)

SPR was measured using a Biacore instrument. Briefly, CM5 sensor chips were activated. HMGB1 was coupled to the chip in 10 mM sodium acetate buffer (pH 4). The surface of the CM5 dextran sensor chip was activated with 0.2 M *N*-ethyl-*N'*-(3-diethylamino-propyl)-carbodiimide and 0.05 M *N*-hydroxysuccinimide. To evaluate binding, various concentrations of LDL, VLDL, HDL and cholesterol were added. Samples were passed over the sensor chip at a flow rate of 10 μl/min. Response unit (RU) values for samples were calculated by subtracting the control values using BIA evaluation 3.0 software (BIAcore).

3. Streptavidin-biotin conjugated protein binding

HMGB1 protein was biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo, Rockford, IL, USA). Biotinylated HMGB1 or biotin as a control was let bind to 3μm of streptavidin labeled microsphere (Sigma Aldrich, St. Louis, MO, USA) in PBS and washed. Acetylated LDL conjugated with Alexa 488 was then incubated at room temperature for two hours. The beads were washed and imaged using confocal microscope FluoView™ 1000 (Olympus, Japan).

4. Oxidized LDL preparation

Native LDL (Alfa Aesar, CAT#BT-903, Rockfold, IL, USA) was dialyzed with dialysis cassette membrane (pore size: 10,000 MWCO) in 0.9% NaCl (buffer : nLDL = 1000 : 1) solution for 24 hours, periodically changing the buffer 1-2 times, to remove EDTA which can act as an antioxidant. Dialyzed LDL was then oxidized with 5μM $CuO_4S \cdot 5H_2O$ (Sigma Aldrich, CAT# 12849) for 24 hours in the dark at 37℃ with gentle shaking at 100rpm. Oxidation was stopped with addition of 0.1mM EDTA and oxidized LDL was stored in 4℃ for up to 2 months. Copper ions were removed by dialysis for 24 hours in 0.9% NaCl. Oxidation and purity of LDL was checked by relative electrophoretic mobility (REM) in 1% agarose gel in barbital buffer (0.05 M sodium barbital and 0.01 M barbital, pH8.6). Migration distance of the oxLDL was compared to that of native LDL and expressed as REM (1.2±0.5, arbitrary units). The oxidation level of oxLDL was also checked with OXI-TEK TBARS assay (Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the kit's manual. OxLDL TBARS value was 55.5 whereas native LDL's was 4.0.

5. Oil Red O staining

PMA differentiated THP-1 cells were seeded in 4-well chamber glass slides with density of $1x10^5$ per well. The cells were maintained in RPMI medium with 10% FBS for 24 hours before the experiment. Then the cells were incubated with 50μg/ml oxLDL for 24 hours in the presence or absence of HMGB1. HMGB1 was pre-incubated with oxLDL for 30 minutes at 37℃.After washing with PBS twice the cells were fixed with 4% paraformaldehyde for 10 minutes at 4℃, then stained with 3mg/ml Oil red O dye for 2 minutes at 37℃ in the dark. Then the cells were rinsed briefly with 60% isopropanol and with PBS three times. The images were taken by light microscope.

6. CD36 plasmid transfection

HEK293T cells were seeded on 8-well chamber glass slide a day before transfection with a density of 0.5 x 10^5 cells per well. WT CD36 plasmid was transfected using PolyExpress™ in vitro DNA transfection reagent (Excellgen, Rockville, MD, USA) according to the manufacturer's instructions. Briefly, 3μl Polyexpress and 1μg DNA complex was treated to the cells for 5 hours in Opti-MEM (Gibco, Thermo Fisher Scientific, USA) media. The plasmid expression was checked after 24 hours post-transfection.

7. FACS analysis

HEK293T cells were seeded in 6-well plate with 4 x 10^5 cell density a day before transfection. Transfected cells were harvested and washed in FACS buffer (0.2% BSA in PBS) and incubated with CD36 antibody (Abcam, Cambridge, UK) in FACS buffer on ice for 30 minutes. After washing twice, the cells were resuspended in FACS buffer containing secondary antibody (anti-mouse IgGAM-FITC) and incubated on ice for 30 minutes. After washing twice, cells were resuspended in FACS buffer containing fixation solution (4% paraformaldehyde), then either directly analyzed with BD FACS Verse™ machine or kept in dark at 4℃ for later use.

8. Confocal microscopy

Cells were grown on chamber slides. After washing with PBS, cells were fixed with 4% paraformaldehyde for 10 minutes in the dark, and then either permeabilized with 0.1% Triton X-100 in PBS for 5 minutes for endogenous molecule staining or directly blocked with PBS containing 10% FBS for 30 minutes. Next, for the experiments with immunostaining (regarding Nile Red staining, refer to number 10 in Materials and Methods section.), the cells were incubated with primary antibodies for 1 hour at room temperature, and stained with secondary antibodies for 1 hour at room temperature as well. Cells were washed with PBS with Tween-20 between each step. Chamber was removed and the glass

slides were mounted with Vecta Shield (Vector Laboratories, Inc., CA, USA) mounting solution containing DAPI. Lastly, the cells were analyzed using confocal microscopy FluoView™ 1000(Olympus, Japan) with an oil-immersed objective. The images were quantified using ImageJ software.

9. Amplex® Red cholesterol assay

OxLDL with different concentrations were incubated withHMGB1 coated microplate for 3 hours at room temperature in the dark. After washing with PBS two times, cholesterol content of oxLDL bound toHMGB1 was evaluated by the Amplex® Red Cholesterol assay kit (Invitrogen, Rockfold, IL, USA) according to the manufacturer's instructions. Briefly, Amplex Red reagent (1 U/ml horseradish peroxidase, 1 U/ml cholesterol oxidase, and 1 U/ml cholesterol esterase) are added to 50μl sample in 96 well plates. After incubation at 37℃ for 60 minutes in the dark, the fluorescence was measured using a microplate reader at 530/25 nm excitation, and 590/35 nm emission wavelengths. The total cholesterol (TC) content was determined by measuring the cholesterol concentration following digestion with cholesterol esterase. To measure free cholesterol (FC), cholesterol esterase is omitted from the assay. Each sample is triplicated in the assay and at least three independent experiments were performed.

10. Nile Red lipid staining

PMA-differentiated THP-1 cells were incubated with 30 μg/ml LDL (Alfa Aesar, CAT#BT-903), 30 μg/ml oxLDL (Alfa Aesar, CAT#BT-910), and 30 μg/ml highly-oxLDL (Alfa Aesar, CAT#BT-910X) for 24 hours. After washing, the cells were fixed with 4% paraformaldehyde for 10 min in the dark, and then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes for endogenous molecule staining. Next, the cells were stained with 100 ng/ml Nile Red for 10 minutes in the dark and washed three times with PBS.

11. Immunoprecipitation (IP)

Protein G Dynabeads® (Novex by Life Technologies AS, Oslo, Norway) were washed twice with PBS with 0.05% Tween 20 and incubated with anti-HMGB1 antibody (Abcam, Cambridge, UK cat#ab18256) for 4 hours at room temperature with rotation. After washing beads were incubated with HMGB1 (AnRT Co. Seoul, Korea) together with either his-tagged CD36 recombinant protein (ATGen, Seoul, Korea) or whole cell lysate (WCL) of THP-1 for overnight at 4℃ with rotation. Then the beads were washed and the immunoprecipitated protein complexes were eluted and subjected to Western blot analysis with anti-CD36 antibody (Abcam, Cambridge, UK cat# ab17044).

12. Western blot

Cells were lysed with RIPA buffer and centrifuged for 10 minutes at 14,000 rpm at 4℃, and supernatants were collected. After the supernatants were subjected to BCA assay for protein concentration optimization (for IP this step was skipped),loading buffer [0.3125 M Tris- HCl pH 6.8, sodium dodecyl sulfate (SDS) 10% (w/v), 0.5 M dithiothreitol (DTT), 0.5% bromophenol blue] (Noble Bioscience, INC) was added and boiled at 100℃ for 10 minutes, and loaded to 10% SDS-PAGE gel. Then the size separated proteins were transferred to a 0.2 μm nitrocellulose transfer membrane at 330 mA for 2 hours. The membrane was then blocked with 5% skim milk in TBS-T (Tris buffered saline with 0.1% Tween 20) for 1 hour and followed by incubation with specific primary antibody with dilution factor of at 4℃ overnight with gentle rocking and HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibody for 1 hour. After washing the membrane with TBS-T for three times with each washing lasting 10 minutes, the protein bands were visualized using ECL (Ab Frontier, Seoul, Korea) chemiluminescence detection kit.

III. RESULTS

1. Binding of HMGB1 to LDL

In order to test the binding of HMGB1 to lipoproteins, SPR assay was used. CM5 chip was fixed with HMGB1, and various concentrations of LDL, VLDL, HDL, and cholesterol were flowed over the chip. As shown in Figure 1A-D, HMGB1 bound to LDL and VLDL in dose-dependent manner, but not to HDL and cholesterol. Then the binding between HMGB1 and acetylated LDL (acLDL) was assessed using HMGB1-coated beads. Coating of microbeads with HMGB1 was confirmed (Figure 1E, red color). When the microbeads were treated with acLDL for 2 hours, the binding of acLDL to HMGB1-coated microbeads was dose-dependently increased (Figure 1E). When I tested the binding of HMGB1 to oxidized LDL (oxLDL) using Amplex® Red Cholesterol assay, the binding was increased in a dose-dependent manner of both molecules (Figure 1F). Moreover, the binding of oxLDL to HMGB1-coated plate was inhibited by free HMGB1 treatment (Figure 2), suggesting the specific binding between HMGB1 and oxLDL.

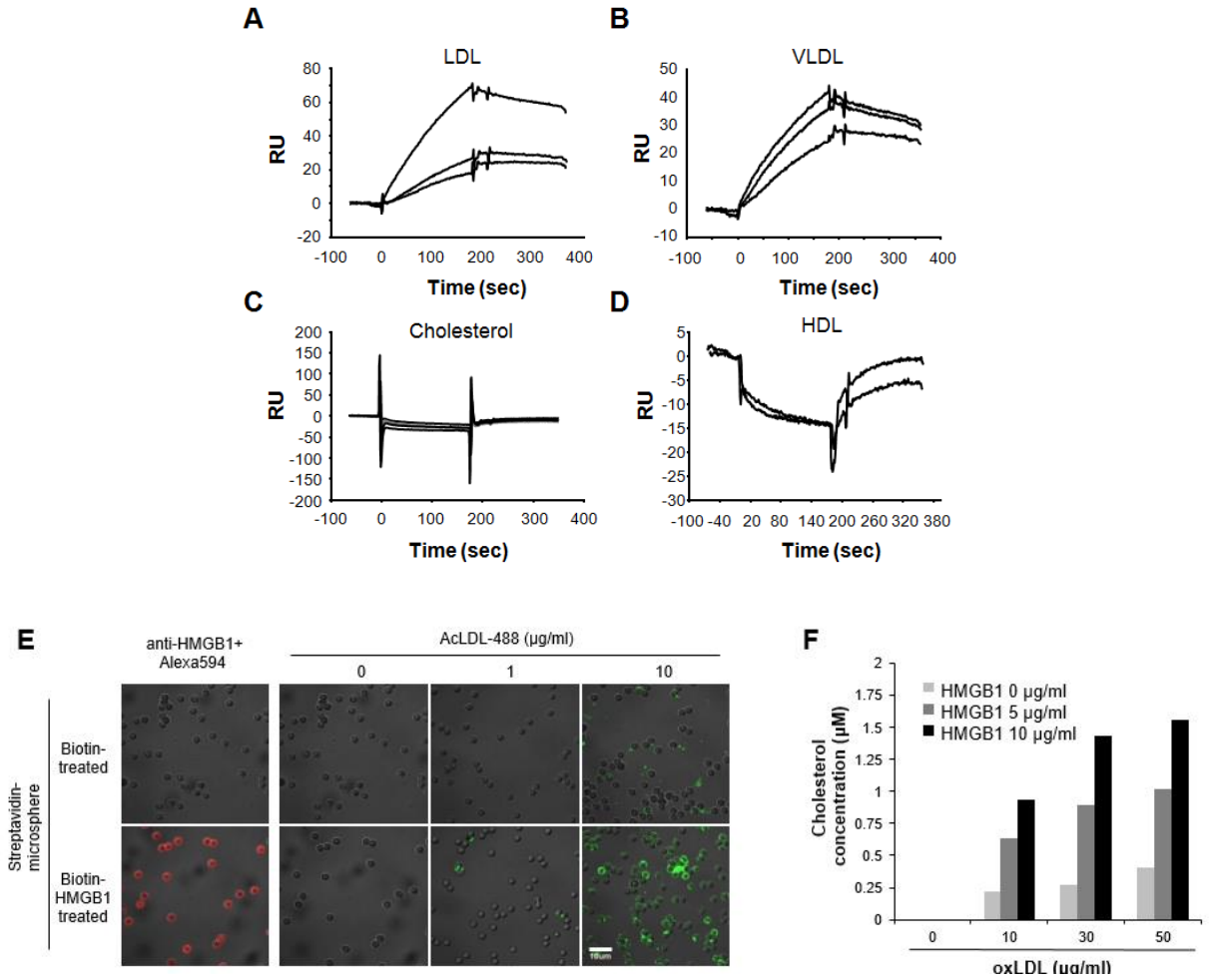


Figure 1. Binding of HMGB1 to LDL. (A-D) SPR assay of HMGB1 binding to LDL, VLDL, cholesterol, and HDL. Various concentrations were flowed over HMGB1-immobilized chips. LDL: 14.3, 7.14, 3.57 nM, VLDL: 4.17, 2.08, 1.04 nM, cholesterol: 650, 325, 162.5, 81.25 μM, and HDL: 571.4, 285.7 nM. (E) Streptavidin-microspheres were treated with biotinylated HMGB1, and stained with primary and secondary antibodies of anti-HMGB1 and Alexa594-conjugated anti-Ig antibodies (left panel). Microspheres were incubated with acetylated LDL conjugated with Alexa 488. (F) EIA plates were coated with HMGB1 protein, and then various concentrations (10, 30, 50 μg/ml) of oxLDL were added for 3 hours at room temperature. Bound cholesterol amount was detected using Amplex® Red cholesterol assay.

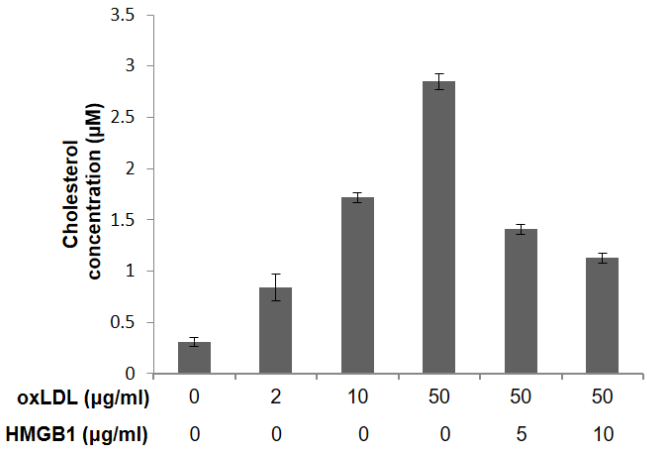
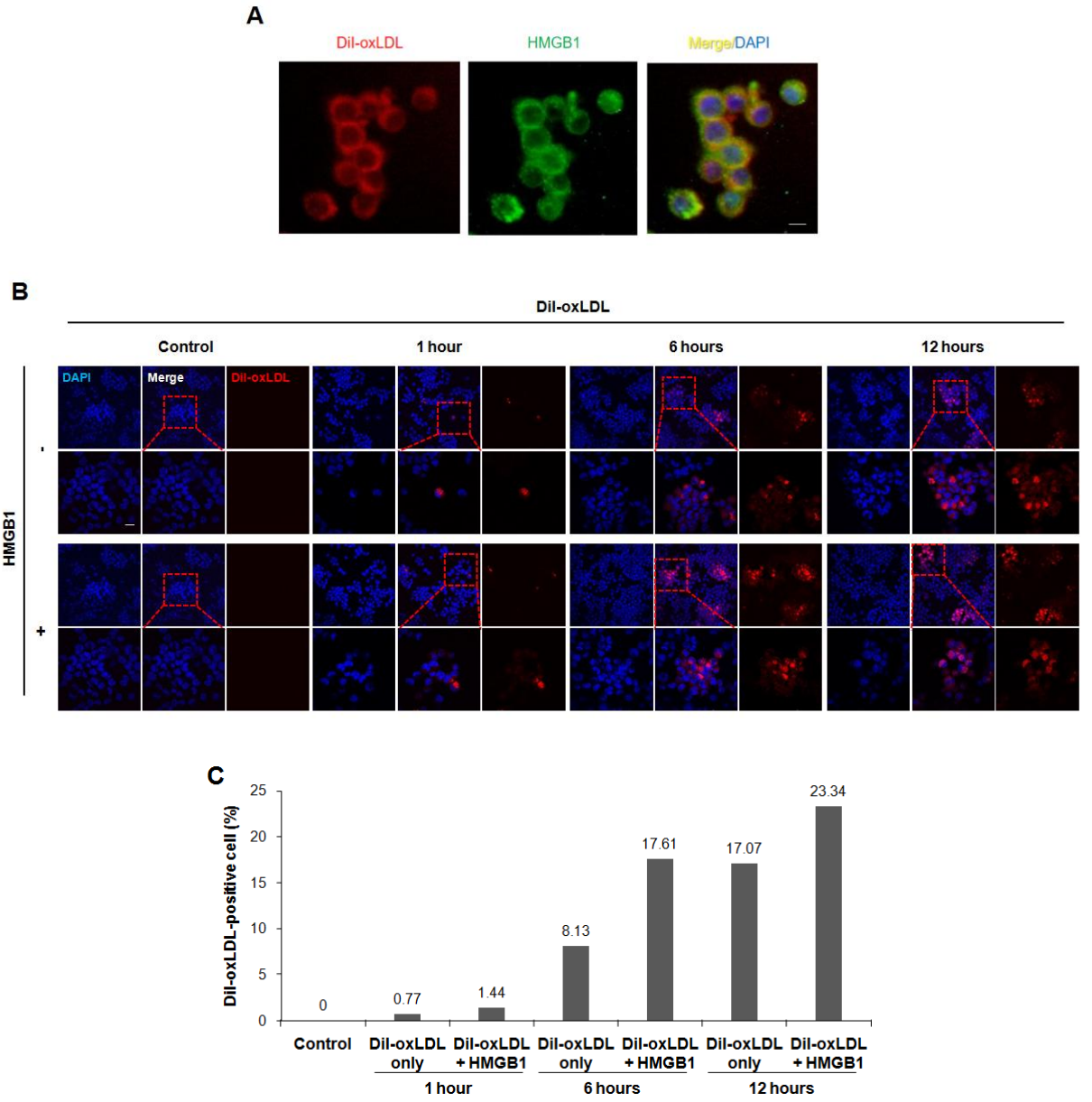


Figure 2. Inhibition assay of HMGB1 to oxLDL binding. HMGB1 (10 μg/ml) was immobilized on EIA plate and treated with oxLDL without or with free HMGB1. Cholesterol content of bound oxLDL was measured by Amplex® Red Cholesterol assay. N=3.

2. HMGB1 mediated increase of oxidized LDL uptake in macrophage

Next I asked that what can be the role of HMGB1 binding to oxLDL. As previous studies demonstrated HMGB1's pro-atherogenic role, I examined the effect of HMGB1 on oxLDL uptake by macrophages. When RAW264.7 macrophages were treated with pre-incubated mixture of DiI-oxLDL (oxLDL with red fluorescent tag) and HMGB1 for 4 hours, co-localization of the two molecules at the cell membrane could be observed (Figure 3A). After PMA-differentiated THP-1 human monocytes were incubated with DiI-oxLDL with and without HMGB1, DiI-oxLDL uptake was increased in a dose- and time-dependent manner in HMGB1 added cells (Figure 3B-E). Similar results were obtained when THP-1 cells were incubated with oxLDL in the presence or absence of HMGB1 and stained with Oil Red O (Figure 3F). In order to assess HMGB1 effect on differentially oxidized LDL, THP-1 cells were treated with LDL, oxLDL, and highly-oxidized LDL (HoxLDL) in the absence or presence of HMGB1 followed by Nile red staining. Lipid uptake was increased in the presence of HMGB1 (Figure 3G). These data suggested that HMGB1 binds to LDL and modified LDL and increases their uptake by macrophages.



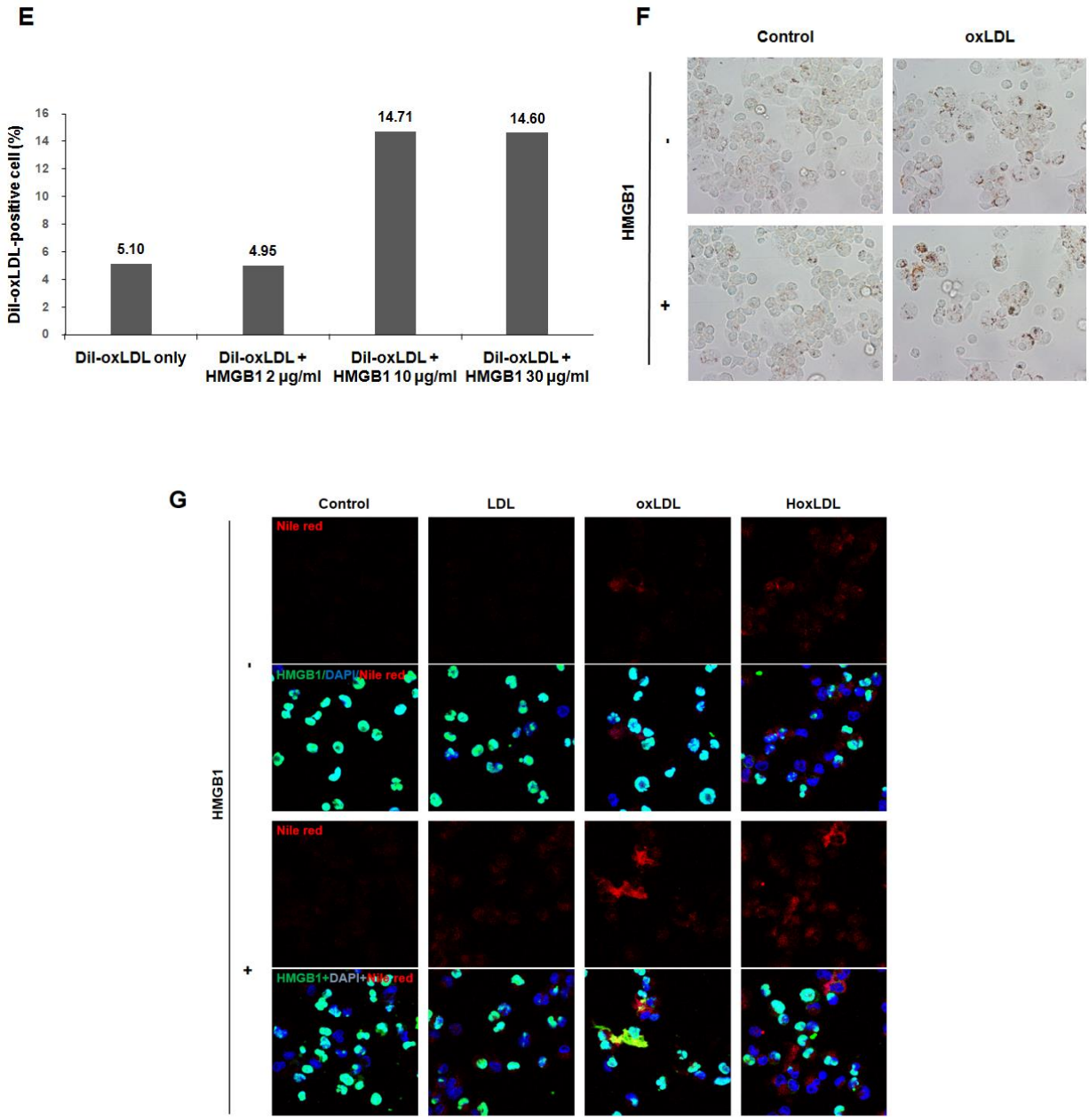
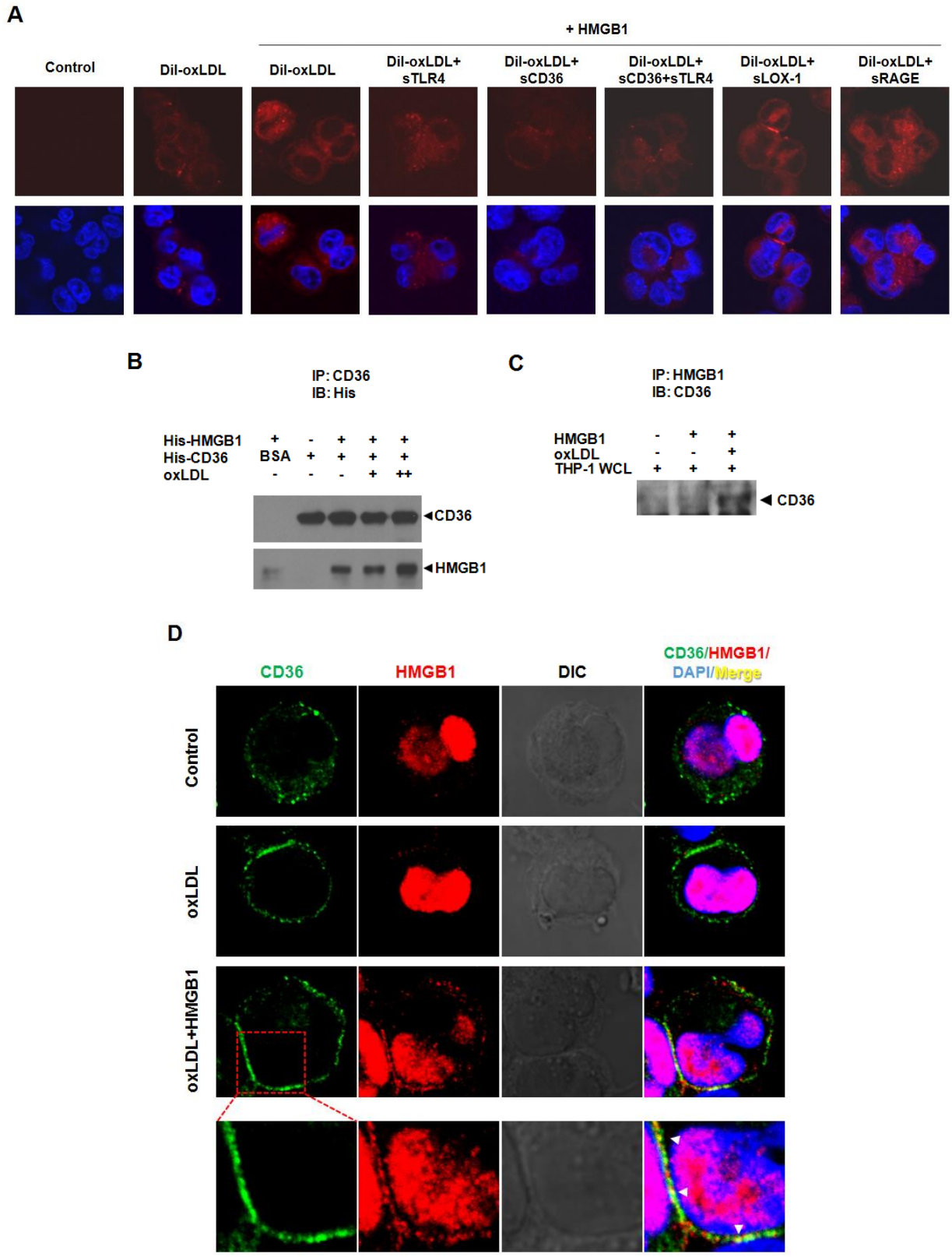


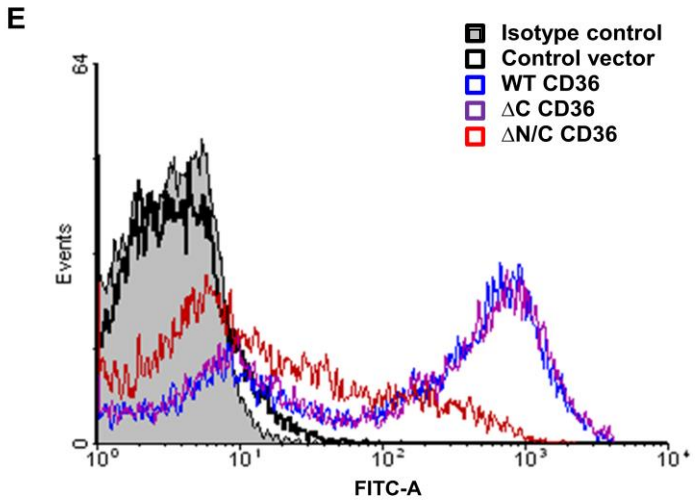
Figure 3. Uptake of modified LDL in macrophages. (A) RAW264.7 cells were incubated with a pre-incubated mixture of DiI-oxLDL(10μg/ml)and HMGB1 (50μg/ml)for 4 hours. Cell surface binding of both molecules could be observed. (B) THP-1 cells were incubated with 30 μg/ml DiI-oxLDL with or without 10 μg/ml HMGB1 for 1, 6, 12 hours. DiI-oxLDL uptake was observed. (C) Quantification of B. Percentage of DiI-oxLDL-positive cells of all imaged cells.(D) THP-1 cells were incubated with 30 μg/ml

DiI-oxLDL with or without 2, 10, 30 μg/mlHMGB1 for 6 hours. (C) Quantification of D. Percentage of DiI-oxLDL-positive cells of all imaged cells. (F) THP-1 cells were incubated with 50 μg/ml oxLDL for 24 hours with/out10μg/ml HMGB1, and Oil red O staining was performed. (G) THP-1 cells were treated with 30 μg/ml LDL, oxLDL, and high-oxLDL in the absence or presence of HMGB1 and stained with Nile red.

3. HMGB1 effect on oxLDL uptake is mediated by CD36 scavenger receptor

In order to find out which receptor/s is/are playing the key role in HMGB1 effect on oxLDL uptake, I pre-incubated HMGB1 and oxLDL with soluble receptors including receptor for advanced glycation end products (RAGE), toll like receptor 4 (TLR4), CD36 and lectin-like oxidized LDL receptor-1 (LOX-1). When these protein cocktails were treated to THP-1 cells, HMGB1-mediated increased oxLDL uptake was inhibited the most in the cells incubated with soluble CD36 and CD36/TLR4 (Figure 4A). This data suggested that CD36 is possibly the main mediator of HMGB1's effect on oxLDL uptake. Next I checked whether HMGB1directly interacts with CD36 scavenger receptor. HMGB1 and CD36 purified proteins bound each other (Figure 4B) and CD36 in whole cell lysate (WCL) of THP-1 cells was also observed to bind with HMGB1 in vitro (Figure 4C). Interestingly, the binding between two molecules were stronger in the presence of oxLDL (Figure 4B and C). After1 hour treatment of oxLDL and HMGB1, CD36 and HMGB1 co-localized at the cell plasma membrane (Figure 4D bottom lane). Moreover, when CD36 was over-expressed in the cell (Figure 4E), HMGB1 mediated increased oxLDL uptake was even more enhanced (Figure 4F). These data indicated that HMGB1binds to modified LDL and increases its uptake in macrophages via CD36 scavenger receptor.





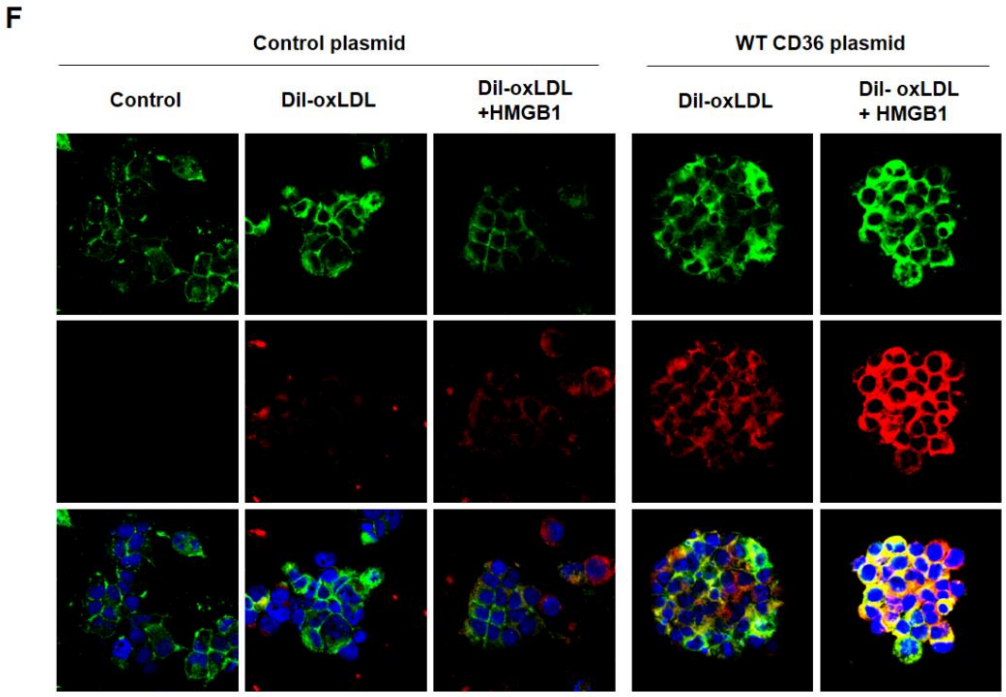


Figure 4. HMGB1 effect on oxLDL uptake is mediated by CD36 scavenger receptor. (A) 10 μg/ml DiI-oxLDL alone or preincubated with 10 μg/ml HMGB1 alone or with 10 μg/ml soluble receptor/s (sTLR4, sCD36, sCD36/TLR4, sLOX-1, sRAGE) for 1 hour at 37℃ were treated to THP-1 cells for 3 hours and the images were obtained by confocal microscope. (B) Protein G coupled beads coated with anti-HMGB1 antibody were incubated with CD36 and HMGB1 purified proteins for overnight. The precipitated proteins (with whole cell lysate (WCL) in case of (C)) were subjected to WB with anti-CD36

antibody and revealed binding between the two molecules. (D) THP-1 cells were treated with oxLDL with or without HMGB1 together for 1 hour and CD36 (green) and HMGB1 (red) were visualized using anti-CD36 and anti-HMGB1 antibodies, respectively. (E) HEK293T cells were transfected with CD36 plasmids, wild type (WT), C terminal truncated, N/C terminals truncated, and immunostained using anti-CD36 antibody and subjected to FACS analysis. (F) HEK293T cells transfected with WT CD36 plasmid or control pCMV vector were incubated with media only (control) or DiI-oxLDL with or without HMGB1 for 1 hour and lipid raft (green) was visualized using cholera toxin B-FITC; DiI-oxLDL (red).

IV. DISCUSSION

Cardiovascular diseases are responsible for 31% of all deaths in the world[59]. Although numerous studies have been done, pathogenesis and causes of atherosclerosis remain largely unknown, and no efficient therapy has been found yet. Chronic inflammation plays a key role in atherosclerosis progression. One of the main mediators of inflammation is HMGB1 which are secreted by apoptotic cells and activated macrophages and monocytes. Engagement of HMGB1 with atherosclerosis has been suggested in several previous studies, however, its role and involvement in excessive lipid uptake by macrophages, a hallmark of atherogenesis, is still unknown. In this study, I have observed that HMGB1 selectively binds to LDL, VLDL and modified LDL, but not to HDL. One of the possible functions of this binding, here we have shown that HMGB1 increased the lipid uptake by macrophages. When HMGB1 was pre-incubated with modified LDL, the formed complex was more aggressively uptaken by macrophages when compared to the uptake of modified LDL alone (Figure 3). Furthermore, among several LDL uptake mechanisms by macrophages, CD36 scavenger receptor and ligand internalization showed to be mediating the observed HMGB1 effect. HMGB1 and CD36 had a direct interaction (Figure 4B, C). Moreover, when soluble CD36 was added to inhibit the endogenous CD36 binding to HMGB1-oxLDL complex, HMGB1 effect on oxLDL uptake was decreased (Figure 4A). In addition, when CD36 was transiently over expressed by HEK293T cells, HMGB1 effect on lipid uptake was enhanced (Figure 4F). Taken together, these results indicated that the possible mechanism through which HMGB1 enhances the uptake of modified LDL can be CD36 scavenger receptor.

The binding of HMGB1 to LDL and VLDL, but not to HDL has significance in that HDL is known as a “good cholesterol” and LDL, VLDL – as “bad cholesterol”. The reason why would HMGB1 selectively bind to the “bad” and problematic molecule and not to “good” and harmless molecule, certainly caught our attention. HMGB1 not only bound to LDL, but also to modified LDLs including oxLDL and acLDL. Scavenger receptors, such as, CD36 recognizes oxidation specific epitopes of oxLDL.

Whether HMGB1 also recognizes oxLDL in a similar fashion is a subject of future studies. Further, structurally HMGB1 consists of two similar domains: anti-inflammatory A box and pro-inflammatory B box. In one of our ongoing experiments, we are using the separate A and B box peptides for oxLDL binding studies which can give us deeper knowledge in HMGB1 and oxLDL interaction.

Here, we have also showed that HMGB1 directly interacted with CD36 scavenger receptor. Although we have examined whether HMGB1 had an effect on CD36 protein expression, different doses of HMGB1 did not alter CD36 expression level (data not shown). Notably, HMGB1 and CD36 binding was stronger in the presence of oxLDL (Fig. 4 B and C) which indicated the three molecules' synergistic action. It was reported that oxLDL binding to CD36 was not inhibited by acLDL[52] and a stable expression of CD36 in fibroblasts resulted in oxLDL, but not LDL or acLDL binding[53]. Other studies, however, reported that CD36 can also bind acLDL[54,55]. Therefore, whether HMGB1's effect on increased acLDL is mediated by CD36 can be controversial and needs further investigation. Chemokines are reported to cause CD36 clustering, thus increased its responsiveness toward oxLDL via integrin activation[33]. In our study HMGB1 has been shown to have a similar action towards CD36 in response to oxLDL. As shown in Figure 4D, oxLDL increased CD36 clustering, confirming the previous reports, and interestingly, HMGB1 addition further increased that clustering. One of the underlying mechanisms behind this might be that HMGB1 also activates integrin, increasing its affinity and valency[56]. This HMGB1 mediated increased CD36 aggregation at cell membrane might have boosted the responsiveness of CD36 to oxLDL. Different ligands induce different CD36 downstream signaling. Molecular mechanisms underlying CD36 mediated oxLDL uptake include Jun-kinase (JNK) 1 and 2, Vav scaffold protein, IRGM, and mitogen-activated kinases, such as p38 and ERK1/2[60]. We are currently investigating some of these downstream signals which can be activated by HMGB1 and mediate the increased oxLDL uptake. CD36 is a multi-functional receptor which engages not only in oxLDL uptake, but also in apoptosis, caspase activation, angiogenesis, cancer metastasis, inflammation, and many others. Thus, binding between HMGB1 and CD36 should be examined further for other astonishing findings outside atherosclerosis context.

Although various functions of HMGB1have been reported previously, our data suggest that its' another novel role might be LDL scavenging during atherosclerosis. Oxidized LDL induces HMGB1 release[58]. Moreover, excessive LDL uptake causes macrophage apoptosis which enables more HMGB1 release. The secreted HMGB1 can then attach to extracellular proteoglycan molecules, such as syndecan [61] and attract macrophages and monocytes, and additionally can retain more LDLs and modified LDLs (needs evidence in further investigation). These events can worsen lipid accumulation and foam cell formation which can result in a vicious cycle of oxLDL uptake and increased HMGB1 secretion. Therefore, HMGB1 is undoubtedly an important target of atherosclerosis and identifying a binding portion of HMGB1 to oxLDL can be useful for future therapeutic application.

V. CONCLUSION

HMGB1 binds to LDL and modified LDL specifically. HMGB1 bound modified LDL is more actively uptaken by macrophages which can progress them to become foam cells. One of the possible mediators of the observed HMGB1 effect is CD36 scavenger receptor (Figure 5).

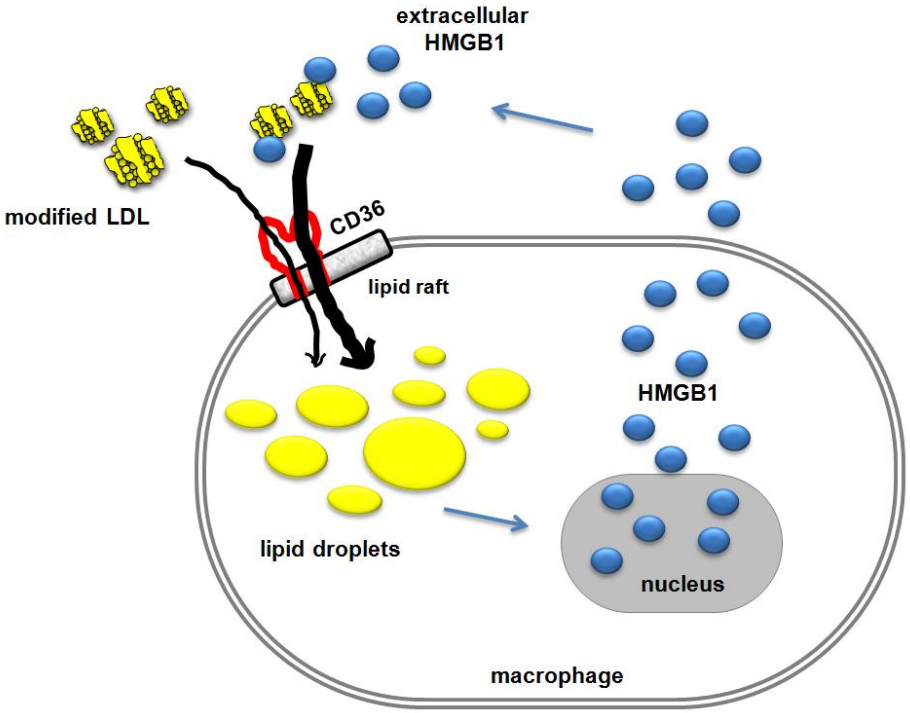


Figure 5. HMGB1 binds to modified LDL and enhances its uptake by macrophages through CD36 scavenger receptor – graphical abstract. Extracellular HMGB1 binds to modified LDL, and the formed HMGB1· modified LDL complex binds to CD36 scavenger receptor in the lipid raft of the macrophage surface and endocytosed more aggressively into the cell, leading to more lipid droplet formation. Excessive LDL ingestion leads to more HMGB1 secretion[58] which can facilitate further increased uptake of oxLDL.

REFERENCES

1. Moore JK, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: a dynamic balance. Nature Rev 2013;13:709-21.
2. Miller YI, Choi SH, Wiesner P, Fang L, Harkewicz R, Hartvigsen K, et al. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. Circ Res 2011108:235–48.
3. Dobrian A, Mora R, Simionescu M, Simionescu N. In vitro formation of oxidatively-modified and reassembled human low-density lipoproteins: antioxidant effect of albumin. Biochim Biophys Acta 1993;1169(1):12-24.
4. Areschoug T, Gordon S, Egesten A, Schmidt A, Herwald H. Contributions to microbiology. Switzerland: Karger; 2008. P.45–60.
5. Plüddemann A, Mukhopadhyay S, Gordon S. The interaction of macrophage receptors with bacterial ligands. Expert Rev Mol Med 2006;8:1–25.
6. Plüddemann A, Mukhopadhyay S, Gordon S. Innate immunity to intracellular pathogens: macrophage receptors and responses to microbial entry. Immunol Rev 2011; 240:11–24.
7. Palani S, Maksimow M, Miiluniemi M, Auvinen K, Jalkanen S, Salmi M. Stabilin-1/CLEVER-1, a type 2 macrophage marker, is an adhesion and scavenging molecule on human placental macrophages. Eur J Immunol 2011; 41:2052–63.
8. Shimaoka T, Nakayama T, Fukumoto N, Kume N, Takahashi S, Yamaguchi J, et al. Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. J Leukoc Biol 2004;75:267–74.
9. Santiago-Garcia J, Kodama T, Pitas R. The class A scavenger receptor binds to proteoglycans and mediates adhesion of macrophages to the extracellular matrix. J Biol Chem 2003;278:6942–6.

10. Murshid A, Gong J, Calderwood SK, Henderson B, Pockley AG. Cellular trafficking of cell stress proteins in health and disease. Netherlands: Springer; 2012. p.215–27.
11. Yang Z, Ming XF. CD36: the common soil for inflammation in obesity and atherosclerosis? Cardiovasc Res 2011;89:485–6.
12. Xu S, Ogura S, Chen J, Little PJ, Moss J, Liu P. LOX-1 in atherosclerosis: biological functions and pharmacological modifiers. Cell Mol Life Sci 2013;70:2859–72.
13. Silverstein RL, Li W, Park YM, Rahaman SO. Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. Trans Am Clin Climatol Assoc 2010;121:206–20.
14. Mitra S, Goyal T, Mehta JL. Oxidized LDL, LOX-1 and Atherosclerosis Cardiovasc Drugs Ther 2011;25:419–29.
15. Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, et al. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 2002;277:49982–8.
16. Kuchibhotla S, Vanegas D, Kennedy DJ, Guy E, Nimako G, Morton RE, et al. Absence of CD36 protects against atherosclerosis in ApoE knock-out mice with no additional protection provided by absence of scavenger receptor A I/II. Cardiovasc Res 2008;78:185–196.
17. Greaves DR, Gordon S. The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. J Lipid Res 2008;50:S282–6.
18. Kunjathoor VV, Febbraio M, PodrezAE, Moore JK, Andersson L, Koehn S, et al. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 2002;277:49982-8.
19. Endeman G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. J BiolChem 1993;268:11811–6.

20. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR-γ. Cell 1998;93:229–40.
21. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPAR-γ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 1998;93:241–52.
22. Carr AC, McCall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive oxygen species; reaction pathways and antioxidant protection. Arterioscler Thromb Vasc Biol 2000;20: 1716–23.
23. Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, et al. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nature Immunol 2010;11:155–61.
24. Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhelawon B, Carpenter SB, et al. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. Nature Immunol 2013;14:812–20.
25. Febbraio M, Podrez EA, Smith JD, Hajjar DP, Hazen SL, Hoff HF, et al. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. J Clin Invest 2000;105:1049–56.
26. Guy E, Kuchibhotla S, Silverstein R, Febbraio M. Continued inhibition of atherosclerotic lesion development in long term western diet fed CD360/ apoE0 mice. Atherosclerosis 2007;192:123–30.
27. Kuchibhotla S, Vanegas D, Kennedy DJ, Guy E, Nimako G, Morton RE, et al. Absence of CD36 protects against atherosclerosis in ApoE knock-out mice with no additional protection provided by absence of scavenger receptor A I/II. Cardiovasc Res 2008;78:185–196.
28. Park YM, Febbraio M, Silverstein RL. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. J Clin Invest 2009;119:136–45.
29. Podrez EA, Byzova TV, Febbraio M, Salomon RG, Ma Y, Valiyaveettil M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a pro-thrombotic phenotype. Nat Med 2007;13:1086–95.

30. Chen K, Febbraio M, Li W, Silverstein RL. A specific CD36-dependent signaling pathway is required for platelet activation by oxidized low-density lipoprotein. Circ Res 2008;102:1512–9.
31. Chen K, Li W, Major J, Rahaman SO, Febbraio M, Silverstein RL. Vav guanine nucleotide exchange factors link hyperlipidemia and a prothrombotic state. Blood 2011;117:5744–50.
32. Fuentes QE, Fuentes QF, Andrés V, Pello OM, de Mora JF, Palomo GI. Role of platelets as mediators that link inflammation and thrombosis in atherosclerosis. Platelets 2013;24:255–62.
33. Wong HS, Jaumouillé V, Freeman SA, Doodnauth SA, Schlam D, Canton J, Mukovozov IM, Saric A, Grinstein S, Robinson LA.Chemokine Signaling Enhances CD36 Responsiveness toward Oxidized Low-Density Lipoproteins and Accelerates Foam Cell Formation. Cell Rep 2016;14(12):2859-71.
34. Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. HMGB1 as a DNA-binding cytokine. J Leukoc Biol 2002;72:1084 –91.
35. Urbonavicute V, Furnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, Bianchi ME, Kirschning C, Wagner H, Manfredi AA, Kalden JR, Schett G, Rovere-Querini P, Herrman M, Voll RE. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. J Exp Med 2008;205:3007–18
36. Zimmermann K, Volkel D, Pable S, Lindner T, Kramberger F, Bahrami S, Scheiflinger F. Native versus recombinant high-mobility group B1 proteins: functional activity in vitro. Inflammation 2004;28:221–9.
37. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191-5.
38. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248–51.
39. Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, et al. Endogenous HMGB1 regulates autophagy. J Cell Biol 2010;190:881-92.

40. Qin S, Wang H, Yuan R, Li H, Ochani M, Ochani K, et al. Role of HMGB1 in apoptosis-mediated sepsis lethality. J Exp Med 2006;203:1637-42.
41. Bell CW, Jiang W, Reich CF 3rd, Pisetsky DS. The extracellular release of HMGB1 during apoptotic cell death. Am J Physiol Cell Physiol 2006;291:C1318-25
42. Porto A, Palumbo R, Pieroni M, Aprigliano G, Chiesa R, Sanvito F, Maseri A, Bianchi ME. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. FASEB J 2006;20:1955–63.
43. Degryse B, Bonaldi T, Scaffidi P, Muller S, Resnati M, Sanvito F, et al. The high mobility group (HMG) boxes of the nuclear protein HMGB1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J Cell Biol 2001;152:1197–1206.
44. Fiuza C, Bustin M, Talwar S, Tropea M, Gerstebberger E, Shelhamer JH, et al. Inflammation-promoting activity of HMGB1 on microvascular endothelial cells. Blood 2003;101:2652–60.
45. Penzo M, Molteni R, Suda T, Samaniego S, Raucci A, Habiel DM, et al. Inhibitor of NF-ƙB kinases α and β are both essential for high mobility group box 1-mediated chemotaxis. J Immunol 2010;184: 4497–4509.
46. Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. J Leuk Biol 2007;81:59–66.
47. Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB1 is required for the migration of maturing dendritic cells. J Leuk Biol 2007;81:84 –91.
48. Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, et al. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. Mol Med 2003;9:37–45.
49. Kalinina N, Agrotis A, Antropova G, DiVitto G, Kanellakis P, Kostolias G, et al. Increased expression of the DNA binding cytokine HMGB1 in human atherosclerotic lesions: role of activated macrophages and cytokines. Arterioscler Thromb Vasc Biol 2004;24:2320 –5.

50. Inoue K, Kawahara K, Biswas KK, Ando K, Mitsudo K, Nobuyoshi M, et al. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerotic lesions. Cardiovasc Pathol 2007;16:136 –43.
51. Kanellakis P, Agrotis A, Kyaw TS, Koulis C, I Ahrens, S Mori, et al. High-Mobility Group Box Protein 1 Neutralization Reduces Development of Diet-Induced Atherosclerosis in Apolipoprotein E–Deficient Mice. Arterioscler Thromb Vasc Biol 2011;31:313-9.
52. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem 1993;268:11811–6.
53. Nicholson AC, Frieda S, Pearce A, Silverstein RL. Oxidized LDL binds to CD36 on human monocyte-derived macrophages and transfected cell lines: Evidence implicating the lipid moiety of the lipoprotein as the binding site. Arterioscler Thromb Vasc Biol 1995;15:269–75.
54. Acton SL, Scherer PE, Lodish HF, Krieger M. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. J Biol Chem 1994;269:21003–9.
55. Febbraio M, Podrez EA, Smith JD, Hajjar DP, Hazen SL. Hoff HF, et al. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. J Clin Invest 2000;105:1049–56.
56. Chavakis E, Hain A, Vinci M, Carmona G, Bianchi ME, Vajkoczy P, et al. High-Mobility Group Box 1 Activates Integrin-Dependent Homing of Endothelial Progenitor Cells. Circ Res 2007;100:204-12
57. Vogel S, Gawaz M, Neal MD. Platelet-derived HMGB1 is a critical mediator of thrombosis. J Clin Invest 2015;125(12):4638-54.
58. Yu X, Xing C, Pan Y, Ma H, Zhang J, Li W. IGF-1 alleviates oxidized-low density lipoproteins-induced inflammation via reducing HMGB1 release in endothelial cells. Acta Biochim Biophys Sin 2012;44:746–51.

59. Mendis S, Puska P, Norrving B. Global atlas on cardiovascular disease prevention and control. Geneva: World Health Organization. 2011.
60. Park YM. CD36, a scavenger receptor implicated in atherosclerosis. Exp & Mol Med 2014;46:99.
61. Salmivirta M, Rauvala H, Elenius K, Jalkanen M. Neurite growth-promoting protein (amphoterin, p30) binds syndecan. Exp Cell Res 1992;200(2):444-51.

ABSTRACT (IN KOREAN)

HMGB1 의 저밀도 지질단백질과의 결합으로 인한 상호작용 및 대식세포로의 uptake 에 미치는 영향

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동맥경화는 동맥 혈관벽 내막 내 지질의 축적에 의해 발생하는 염증성 심혈관계 질환으로 심근경색 및 뇌졸중을 야기한다. 핵단백질인 HMGB1 은 면역과 염증 반응에 중요한 역할을 하는 것으로 알려져 있다. HMGB1 은 동맥경화증의 진행에 연관 있는 동맥경화증병변 부위에서 증가되어 있다. 본 연구에서는 HMGB1 이 저밀도 지질단백질과 변형된 저밀도 지질단백질에 직접적으로 결합하는 것을 확인하였다. 그리고 HMGB1 을 시간, 농도 의존적으로 처리하였을 때, THP-1 대식세포에서 변형된 저밀도 지질단백질의 uptake 가 증가하였다. 또한 수용성 CD36 청소 수용체 단백질을 추가하였을 경우 변형된 저밀도 지질단백질 uptake 에서의 HMGB1 효과가 감소하였다. 게다가, HMGB1 이 CD36 과 직접 결합을 하는 것을 확인하였고, CD36 을 과발현시키면 변형된 저밀도 지질단백질의 uptake 에 대한 HMGB1 의 효과가 증가되었다. 이를 종합하면 본 연구에서는 HMGB1 이 변형된 저밀도 지질단백질과 결합하여 CD36 청소 수용체를 통한 대식세포로의 uptake 를 증가시켜 동맥경화 형성촉진에 관여함을 제시하고자 한다.

핵심뒤는 말: HMGB1, 저밀도지질단백질, CD36, 동맥경화