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SR-BI mediates high density lipoprotein (HDL)-induced anti-inflammatory effect in macrophages

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ABSTRACT

High density lipoprotein (HDL) receptor, scavenger receptor class B, type I (SR-BI), mediates selective cholesteryl ester uptake from lipoproteins into the liver as well as cholesterol efflux from macrophages to HDL. Recently, strong evidence has demonstrated the anti-inflammatory effect of HDL, although the mechanism of action is not fully understood. In this study, we showed that the anti-inflammatory effects of HDL are dependent on SR-BI expression in THP-1 macrophages. Consistent with earlier findings, pretreatment of macrophages with HDL abolished LPS-induced TNFα production. HDL also inhibited LPS-induced NF-κB activation. In addition, knockdown of SR-BI or inhibition of SR-BI ligand binding abolished the anti-inflammatory effect of HDL. SR-BI is a multi-ligand receptor that binds to modified lipoproteins as well as native HDL. Since modified lipoproteins have pro-inflammatory properties, it is unclear whether SR-BI activated by modified HDL has an anti- or pro-inflammatory effect. Glycated HDL induced NF-κB activation and cytokine production in macrophages *in vitro*, suggesting a pro-inflammatory effect for modified HDL. Moreover, inhibition of SR-BI function or expression potentiated glycated HDL-induced TNF-α production, suggesting an anti-inflammatory effect for SR-BI. In conclusion, SR-BI plays an important function in regulating HDL-mediated anti-inflammatory response in macrophages.

1. Introduction

High density lipoprotein (HDL) cholesterol is well known as beneficial cholesterol and inversely correlates with risk of coronary artery disease (CAD) in humans [1]. HDL has been shown to protect against atherosclerosis and other vascular diseases based on anti-atherosclerotic effects such as reverse cholesterol transport and lipid homeostasis [2,3]. HDL promotes reverse cholesterol transport, mediating selective cholesteryl ester uptake from lipoproteins into the liver and steroidogenic tissues, as well as cholesterol efflux from macrophages. Besides its role in regulating cholesterol metabolism, HDL has been shown to exhibit antioxidant and anti-inflammatory effects in the vasculature [4]. HDL has potent anti-inflammatory properties in various types of cells. Many previous reports have shown that reconstituted HDL (rHDL), ApoA-I, as well as HDL suppress NF-κB activation and cytokine expression in various cell types (endothelial cells and monocytes) *in vitro* and in animals [5]. Injections of human apolipoprotein A-I (major HDL protein) into rabbits inhibits vascular inflammation [6]. However, the molecular mechanism behind the anti-inflammatory effect of HDL is not fully understood.

HDL modifications in plasma such as glycation have been observed in diabetes patients and reverse the protective effects of HDL [7], which could explain why patients with Type II diabetes show increased incidence of CAD [8]. Our previous study showed that HDL from the elderly (>75 years) and heavy smokers is highly modified and has no protective properties [9,10]. In addition, modified HDL has pro-atherogenic effects based on lipid phagocytosis and foam cell formation. Macrophages treated with modified HDL from the elderly show enhanced cholesterol influx as well as increased oil red O staining compared to native HDL-treated cells [10]. However, the SR-BI-mediated mechanism and inflammatory effects of glycated HDL in macrophages are not understood.

Scavenger receptor class B type I (SR-BI) is a multi-ligand receptor [11] that binds to a variety of ligands, including modified and native lipoproteins, apoptotic cells, advanced glycation end-products (AGE), oxidized phospholipids, and HDL [12–14]. SR-BI plays an important role in cholesterol homeostasis by interacting with HDL. In the liver, SR-BI mediates the selective uptake of cholesteryl esters (CEs) from HDL and potentiates the reverse cholesterol transport pathway by increasing hepatic excretion of cholesterol.

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Besides its role in cholesterol uptake, SR-BI mediates cholesterol efflux from macrophages to HDL [15]. Thus, SR-BI acts as a bidirectional cholesterol transporter [16]. SR-BI also mediates HDL-induced eNOS activation and NO release in endothelial cells, which is one of the anti-atherogenic effects of HDL [17]. Recent data have suggested that the anti-inflammatory effect of HDL is independent of cholesterol homeostasis. However, due to the sheer variety of SR-BI ligands, it is unclear whether SR-BI is anti- or pro-inflammatory in macrophages. We therefore determined whether or not SR-BI is an anti-inflammatory factor in macrophages.

2. Methods

2.1. Preparation of lipoproteins and synthesis of rHDL

LDL ($1.019 < d < 1.063$), HDL_2 ($1.063 < d < 1.125$), and HDL_3 ($1.125 < d < 1.225$) were isolated from a healthy donor via sequential ultracentrifugation, and apoA-I was purified from human plasma following the standard method [18,19]. Glycation of HDL and cell treatment was carried out as described in our previous report [20,21].

Reconstituted HDL (rHDL) was prepared via sodium cholate dialysis at a molar ratio of 95:5:1:150 (palmitoyloleoyl phosphatidylcholine:cholesterol:apoA-I:sodium cholate) as previously described by our research group [20].

2.2. Silencing SR-BI gene transcription in macrophages

THP-1 macrophages were cultured in 6-well plates to 30% confluency. Cells were then incubated with 80 μM siRNA (SR-BI siRNA; GGACAAGUUCGGAUUAUUUdTdT, Dharmacon, Thermo Scientific) and Lipofectamine 2000 (Invitrogen) in RPMI with 0.1% FBS for 5 h. Cells were incubated in culture medium (RPMI with 10% FBS) for an additional 48 h. Cells were then incubated with HDL (50 μg/ml) or PBS for 16 h, washed, and stimulated with LPS (10 ng/ml) for 4 h. We used a scrambled siRNA (Qiagen) sequence as a control.

2.3. Gene expression analysis

Total RNA was extracted from cultured macrophages using a commercial miniprep kit (Qiagen) and reverse-transcribed to cDNA using MultiScribe reverse transcriptase and random primers (High capacity cDNA reverse transcription kit, AB Applied Biosystems). Specific primers were used to measure mRNA expression by real-time PCR (TNFα; Forward 5′-CCCCAGGGACCTCTCTCTAA-3′; Reverse 5′-TGAGGTACAGGCCCTCTGAT-3′; IL6 Forward 5′-GCCTTC GGTCCAGTTGCCTT-3′; Reverse 5′-GCA GAATGAGATGAGTTGTC-3′; ICAM Forward 5′-GGCTGGAGCTGTTTGAGAAC-3′; Reverse 5′-ACTG TGGGGTTCAACCTCTG-3′; SR-BI Forward 5′-ATGATCGTGATGGT GCCGTC-3′; Reverse 5′-TGTTGCTTTTGTGCCTGAAC-3′; GAPDH Forward 5′-CTCATGACCACAGTCCATGC-3′; Reverse 5′-ATGTAGGCCAT GAGGTCCAC-3′; ABCA1 Forward 5′-CCTTGGGTTCAGGGGATT AT-3′; Reverse 5′-TTCATGCTGGTGTCTTTCTGG-3′; ABCG1 Forward 5′-AACATGGAGGCCACTGAGAC-3′; Reverse 5′-GGCCACCAACTCACC ACTAT-3′). Samples were amplified in triplicate for TNFα, SR-BI, IL6, ICAM, and GAPDH (as a housekeeping gene) using a ABI Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Relative quantification was achieved using the delta CT method based on detection with SYBR-Green I (Invitrogen, Carlsbad, CA, USA).

2.4. Western blot analysis

Cells were lysed in urea lysis buffer (4 M urea, 62.5 mM Tris-HCl, 2% SDS, 1 mM EDTA) containing protease and phosphatase inhibitor cocktail (Sigma), and equal amounts of protein from cell lysates were subjected to Western blot analysis. Specific primary antibodies for human SR-BI (1:2000 dilution, Novus Biologicals), apolipoprotein A1 (1:1000, AbChem), GAPDH (1:2000, Antibody verify), phospho-IκB (1:1000, Santa Cruz Biotech), and human β-actin (1:5000, Bethyl) were used. HRP-conjugated goat secondary anti-rabbit IgG and a chemiluminescent substrate (SuperSignal, Thermo Scientific) were used to visualize proteins.

2.5. Immunostaining for surface SR-BI

Cells grown on glass coverslips were fixed with 4% paraformaldehyde and incubated with blocking buffer containing 1% FCS and 1% BSA in PBS. Primary rabbit anti-SR-BI (Novous, 1:500) was applied to the same buffer overnight at 4 °C. Coverslips were washed with PBS, incubated with Alexa 594-conjugated anti-rabbit secondary antibody (1:1000, Molecular Probes) at room temperature for 2 h, and washed again. Coverslips were mounted for immunofluorescence microscopy and analyzed with a Nikon A1 confocal laser-scanning microscope equipped with a ×63 oil immersion objective.

2.6. Statistical analysis

Statistical analysis was performed using PRISM5.0 for Windows. Statistical evaluation was done by independent two-tailed *t*-test and ANOVA, followed by Turkey's test. A p value $\leqslant 0.05$ was considered statistically significant. Data were expressed as mean ± SEM.

3. Results

3.1. SR-BI up-regulation by HDL in THP-1 macrophages

Macrophage activation leads to cytokine release and recruitment of monocytes, which is an early event in the development of atherosclerosis. Therefore, we used macrophages to study the anti-inflammatory effect of SR-BI. To study the function of SR-BI in macrophages, we examined SR-BI expression in THP-1 macrophages, which were differentiated from monocytes by PMA treatment for 48 h. We first examined the mRNA expression of SR-BI as well as other known HDL-binding receptors, specifically ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), in THP-1 cells by RT-PCR. As shown in Fig. 1, endogenous SR-BI expression was observed in THP-1 macrophages. Interestingly, SR-BI expression was significantly elevated by HDL treatment at both mRNA and protein levels (Fig. 1B and C), which supports the notion that HDL is internalized via SR-BI. The apoA-I (28 kDa) band appeared only after HDL treatment (Fig. 1B), suggesting internalization of apoA-I through endogenous HDL receptors in THP-1 macrophages. Next, localization of SR-BI in macrophages was examined by immunostaining. SR-BI localized mainly to the cytoplasm as well as the cell surface. We also observed increased surface expression of SR-BI upon HDL treatment for 15 min (Fig. 1D). We next examined whether or not SR-BI expression is up-regulated by lipid-free apoA-I, the major apolipoprotein of HDL, and reconstituted HDL (rHDL). As we shown in Fig. 1E, both apoA-I and rHDL elevated SR-BI expression, whereas LDL had no effect.

3.2. Anti-inflammatory effect of HDL requires SR-BI in macrophages

In prior studies, we tested the hypothesis that HDL has anti-inflammatory effects in macrophages mediated by SR-BI. In this study, inflammatory function was assessed by measuring production of pro-inflammatory cytokines or adhesion molecules such as TNFα, IL6, and ICAM in macrophages. For this, we used

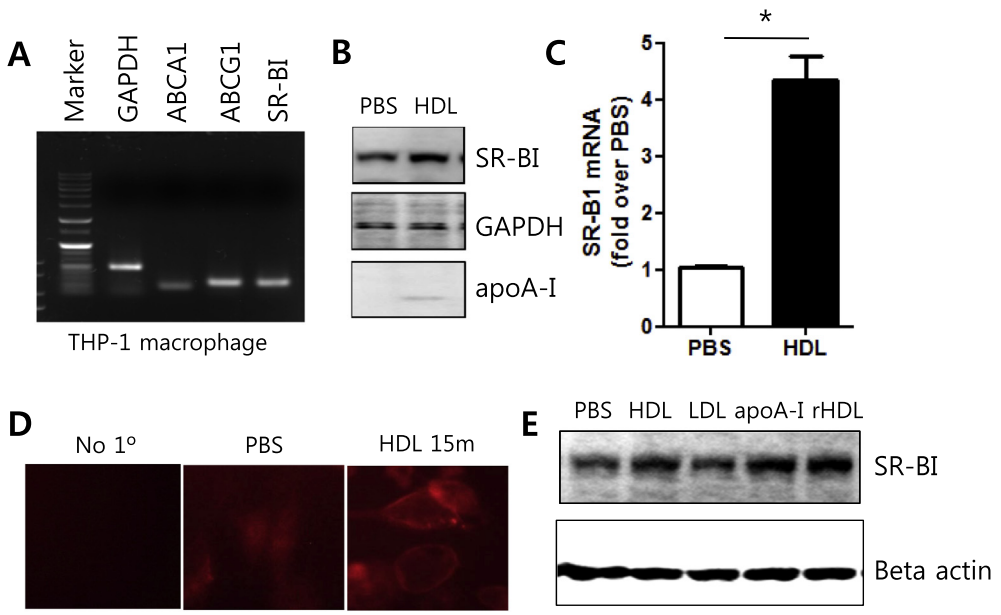

Fig. 1. Expression of SR-BI in THP-1 macrophages. (A) mRNA expression of HDL receptors ABCA1, ABCG1, and SR-BI as detected by RT-PCR. (B) Western blotting for SR-BI protein expression. Membrane was re-blotted with anti-GAPDH (loading control) and anti-apoA-I antibodies. (C) SR-BI mRNA was quantified by real-time RT-PCR after treatment with HDL (50 μg/ml) for 4 h. Values are means ± SEM of three determinations. *$p < 0.05$ vs PBS control. (D) Immunostaining for SR-BI in macrophages after HDL treatment for 15 min. Cell surface expression of SR-BI is clearly shown after HDL treatment (arrow). (E) Western blotting for SR-BI. Cells were lysed after treatment with HDL (50 μg/ml), LDL (100 μg/ml), apoA-I (50 μg/ml), and reconstituted HDL (rHDL, 50 μg/ml) overnight.

lipopolysaccharide (LPS) as a stimulator of classical activation in macrophages. LPS dramatically increased production of TNF-α, IL-6, and ICAM (Fig. 2). The effect of HDL (50 μg/ml) on the response to LPS stimulation was assessed by comparing cytokine production levels in cells treated with LPS prepared in the presence of carrier (PBS). Pretreatment with HDL significantly reduced LPS-induced production of TNFα, IL-6, and ICAM. Further, HDL pretreatment reduced production of TNFα by 51% ($p < 0.05$). Similarly, HDL treatment significantly reduced LPS-induced IL6 production by 70% and ICAM by 56% ($p < 0.05$). To exclude the possibility of HDL-mediated non-specific blocking of LPS binding, BSA was used as vehicle instead of PBS. As shown in Supplementary Fig. S1, the anti-inflammatory effect of HDL was observed.

NF-κB is a key regulator of TNF-α, IL-6, and ICAM gene expression [22]. Therefore, we measured NF-κB activity after LPS and HDL treatments. As shown in Fig. 2D, LPS elevated phospho-IκB levels by 30 ± 3% ($p < 0.05$) relative to PBS-treated macrophages. Treatment with HDL reduced LPS-induced phospho-IκB expression back to basal levels.

To determine whether or not scavenger receptor SR-BI mediates the inhibitory effects of HDL against chemokine expression, siRNAs were used to knockdown SR-BI in THP-1 macrophages, and Western blotting was used to confirm siRNA knockdown efficiency (70% knockdown, Fig. 2E insert). Control cells (scrambled siRNA) incubated with LPS for 4 h showed significant elevation of TNFα mRNA levels. Pre-incubation of control cells (scrambled siRNA) with HDL inhibited TNFα levels (32% reduction of LPS, $p < 0.05$), as shown in Fig. 2E. To confirm the anti-inflammatory function of SR-BI, we used anti-SR-BI antibody (Novus, 1:500) and observed the effects. LPS significantly increased TNFα levels in control cells incubated with control antibody (anti-HA), whereas HDL suppressed LPS-induced TNFα levels (53% reduction, $p < 0.05$). However, inhibition of SR-BI with anti-SR-BI abolished HDL-induced reduction of TNFα levels (only 10% reduction, not significant).

3.3. Glycated HDL induces inflammation in macrophages

Non-enzymatic glycation has been implicated in inflammation in many cells [23], whereas HDL has anti-inflammatory effects (Fig. 3). Therefore, we tested whether or not glycated HDL has a pro- or anti-inflammatory effect in macrophages. For this, we synthesized glycated HDL and measured advanced glycated end product formation in HDL. After 3 days of incubation with fructose (final 250 mM) and HDL (1 mg/ml) under 5% CO_2 at 37 °C, non-enzymatic glycation was observed (Fig. 3A). Fluorospectroscopic observation revealed around 9-fold greater production of glycated end products using glycated (gHDL) compared with normal HDL.

Macrophages were treated with gHDL to observe its effect on TNFα production, a marker of inflammation. THP-1 macrophages were treated with native HDL and gHDL (50 μg/ml) for 4 h after serum starvation. The level of TNFα mRNA was quantitated by real-time PCR. gHDL treatment induced significant production of TNFα as shown in Fig. 3B (80% of LPS-induced TNFα production, $p < 0.05$) in macrophages, whereas treatment of cells with native HDL had no such effect on TNFα production (Fig. 3C).

To examine whether or not gHDL stimulates TNFα production via the NF-κB pathway, NF-κB activity was measured using phospho-IκB antibody. As a positive control, we treated cells with oxLDL (100 μg/ml) for 15 min after serum starvation in THP-1 macrophages. Treatment with oxLDL increased phospho-IκB levels, whereas PBS and HDL had no effect on phosphorylation of IκB. Interestingly, gHDL by itself induced NF-κB activation based on phosphor-IκB (70% of oxLDL-induced response, Fig. 3D).

3.4. Blocking SR-BI increases glycated HDL-induced inflammation

As shown in Fig. 4A, gHDL slightly increased SR-BI expression, but the increase was significantly smaller than that observed in HDL-treated cells (Supplementary Fig. S2). Immunostaining for SR-BI also showed that gHDL did not stimulate expression of

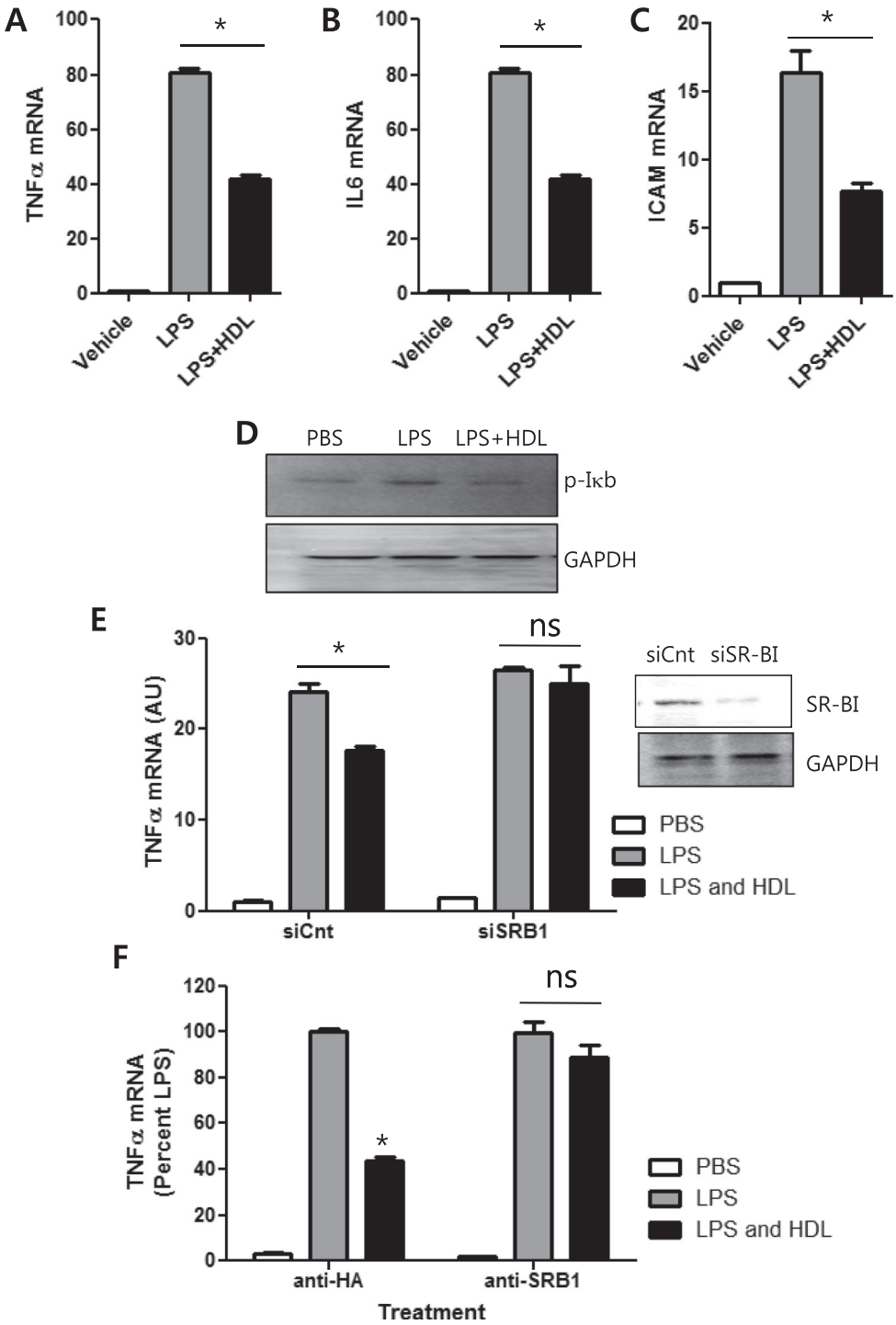

Fig. 2. Anti-inflammatory effect of HDL is mediated by SR-BI. (A) TNFα production in LPS-stimulated THP-1 macrophages with HDL (50 μg/ml) pretreatment. (B and C) Real-time PCR for IL6 (B) and ICAM (C) mRNA in THP-1 macrophages under the same conditions as A. (D) NF-κB activation. Phosphorylation of IκB was analyzed in cells with LPS (10 ng/ml) for 15 min after pretreatment with HDL or PBS by Western blotting. (E) Anti-inflammatory effect of HDL after knockdown of SR-BI. Knockdown rate of SR-BI was measured by Western blotting, shown in insert panel. (F) TNFα production in LPS-stimulated THP-1 macrophages with HDL (50 μg/ml) pretreatment after blocking SR-BI function. Values are means ± SEM of three determinations from at least two independent experiments. $^{*}p < 0.05$, ns, not significant. $^{*}p < 0.05$ from ANOVA, Tukey's multiple comparison test.

SR-BI after 4 h of incubation (data not shown). However, gHDL increased surface SR-BI localization as shown in cells treated with HDL for 15 min, similar to native HDL treatment for 15 min (Figs. 4C and 1D). gHDL treatment also induced apoA-I internalization as shown by Western blotting (Supplementary Fig. S3), suggesting gHDL can interact with SR-BI.

To determine whether or not SR-BI mediates the pro-inflammatory effects of gHDL against chemokine expression, siRNAs were used to knockdown SR-BI in THP-1 macrophages. There was a significant increase in TNFα mRNA expression (90% of LPS-induced response, $p < 0.05$) in control cells (scrambled siRNA) incubated with gHDL, as shown in Fig. 4D. When SR-BI was knocked down,

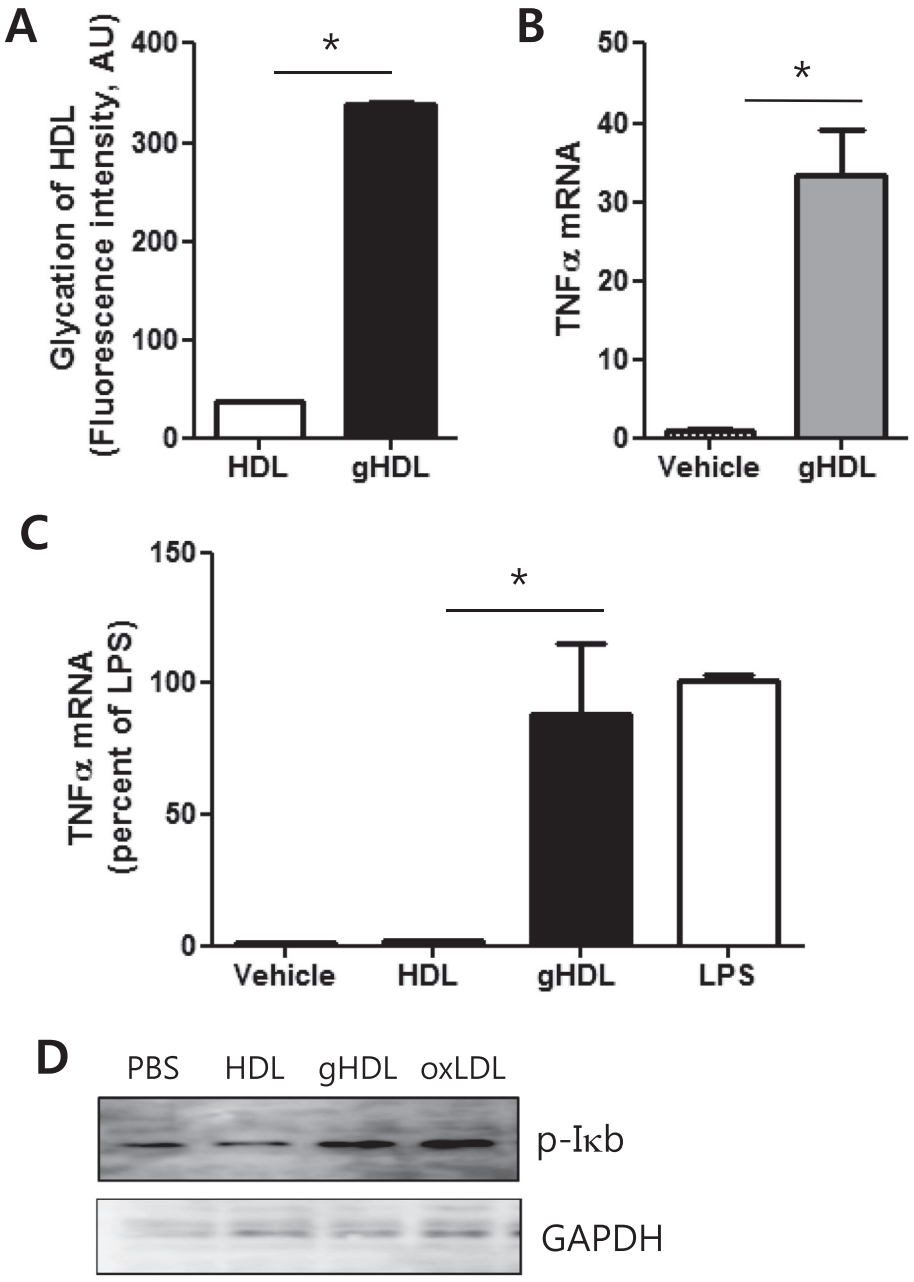

Fig. 3. Glycation of HDL induces inflammation in macrophages. (A) Glycation of HDL. (B) TNFα production in THP-1 macrophages by glycated HDL (gHDL). Values are means ± SEM of three determinations from three independent experiments. (C) TNFα production in THP-1 macrophages stimulated with LPS (10 ng/ml), HDL (50 μg/ml), gHDL (50 μg/ml), or PBS (vehicle). Graph shows percent TNFα mRNA level (mean ± SEM) relative to LPS. $^{*}p < 0.05$. (D) NF-κB activation in THP-1 macrophages.

the pro-inflammatory effect of gHDL on TNFα production was amplified (240% of LPS-induced response with control siRNA). To confirm this observation, we used an SR-BI inhibitor (blocking antibody) to block SR-BI function. Incubation of cells with control antibody (anti-HA) and gHDL significantly increased TNFα levels (25-fold of basal response), as measured by real-time PCR (Fig. 4E). Blocking SR-BI with SR-BI inhibitor, as shown in the knockdown model, potentiated TNFα production 2-fold compared to cells without SR-BI inhibitor.

4. Discussion

HDL has beneficial effects against inflammatory diseases such as atherosclerosis and diabetes. However, studies on the function of SR-BI, which is the physiological receptor of HDL, have not been reported. Therefore, we studied the role of SR-BI in the inflammatory effect of HDL. Findings of this study: (1) anti-inflammatory effect of HDL is mostly mediated by SR-BI in macrophages, (2) SR-BI expression is up-regulated by HDL but not gHDL, even though both can induce SR-BI trafficking, (3) glycation of HDL alters HDL from anti-inflammatory to pro-inflammatory, and (4) gHDL-mediated TNFα production is amplified by deficiency of SR-BI.

The mechanism involves the anti-inflammatory effect of HDL via inhibition of NF-κB activation, which is similar to that observed in vascular smooth muscle cells, as recently published by van der Vorst et al. [24]. Specifically, rHDL is able to inhibit TNFα-induced production of cytokines such as CCL2, CCL5, and

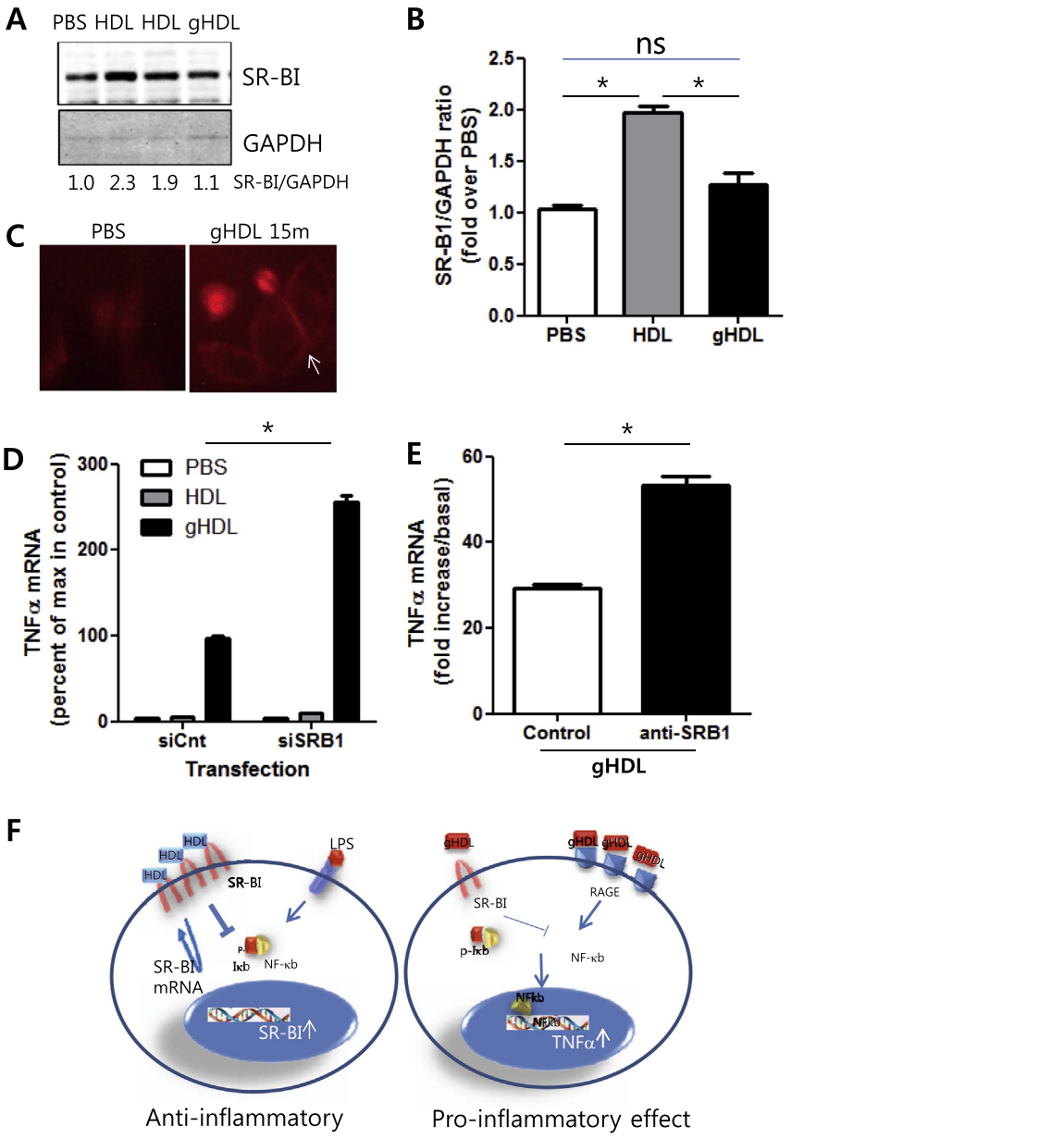

Fig. 4. Blocking SR-BI potentiates gHDL-induced inflammation. (A) SR-BI protein expression in THP-1 treated with HDL or gHDL. (B) Graph shows quantification of SR-BI protein expression from three experiments. (C) Immunostaining for SR-BI in THP-1 macrophages treated with gHDL for indicated times. (D) TNFα production by gHDL after knockdown of SR-BI expression. (E) Values are means ± SEM of three determinations from at least two independent experiments. *$p < 0.05$ from ANOVA, Tukey's multiple comparison test. (F) Schematic diagram of our working hypothesis describing anti-inflammatory effect of SR-BI in macrophages.

CX3CII. Furthermore, SR-BI knockdown can abolish the ability of rHDL to inhibit expression of cytokines. Additionally, our study found that HDL treatment increased SR-BI expression at both mRNA and protein levels. Several mechanisms regulating SR-BI expression have been proposed. Cellular cholesterol level, estrogens, and trophic hormones all regulate SR-BI expression by both transcriptional and post-transcriptional mechanisms [11]. Liver X receptors (LXRs) and retinoic X receptor (RXR) modulate several genes involved in lipid metabolism, including SR-BI, in hepatocytes and macrophages [17]. Incubation of HUVECs with RXR agonist has been shown to induce expression of SR-BI (2.4-fold). High levels of SR-BI have been observed in steroidogenic tissues, and its expression *in vivo* is induced by adrenocorticotropic hormone, suggesting that SR-BI is a physiologically relevant HDL receptor providing cholesterol substrate for steroid hormone synthesis [25]. In our study, SR-BI in macrophages was mostly localized to the cytoplasm, and HDL increased surface SR-BI expression (Fig. 1D). Cytoplasmic expression of SR-BI has also been observed in adipocytes, and insulin and AngII increases membrane SR-BI expression via the AKT-dependent pathway [26]. HDL-induced SR-BI expression might play a role in amplifying the anti-inflammatory effect of HDL in macrophages in a sort of positive feedback loop. It is known that the amount of SR-BI is highly correlated with function of HDL-mediated cholesterol efflux in different types of tissues. The rate of efflux per microgram of HDL/ml is well correlated with the expression level of SR-BI in cellular membranes [27]. Based on our observation, we hypothesize that up-regulation of SR-BI by HDL could have strong anti-inflammatory effects, which can inhibit pro-inflammatory signals in LPS-treated macrophages (Fig. 4F).

SR-BI was originally cloned as a scavenger receptor for modified LDL [12]. Ohgami et al. also reported that SR-BI could serve as an advanced glycation end product (AGE) receptor in CHO cells over-expressing SR-BI [28]. AGE proteins can be ligands for SR-BI, and the ligand-binding site on SR-BI for AGE proteins is different from that for HDL, suggesting different signaling pathways [28]. Our study showed native HDL treatment up-regulated SR-BI expression, whereas gHDL had little effect. Additionally, gHDL activated macrophages by increasing NF-κB activation and pro-cytokine production, whereas native HDL inhibited macrophage activation stimulated by LPS (Figs. 2 and 3). Interactions between proteins with AGEs and Receptor for AGE (RAGE) or CD36 (scavenger receptor type B) in macrophages are known to induce production of several cytokines via NF-κB activation [29], which may explain gHDL-induced TNFα production. However, it remains unknown what kind of receptors and downstream signaling pathways are involved in regulation of gHDL-induced NF-κB activation, release of cytokine production, and macrophage activation, which are important early steps in progression of atherosclerosis. Many other receptors of gHDL, such as RAGE in macrophages, might be involved in the pro-inflammatory response of gHDL. Therefore, we hypothesize that blocking SR-BI does not alter the pro-inflammatory effect of gHDL, although it does potentiate cytokine production, suggesting gHDL-mediated SR-BI signaling is partially anti-inflammatory.

In conclusion, the anti-inflammatory effect of HDL is mediated by SR-BI in macrophages. Glycation of HDL alters the properties of HDL from anti- to pro-inflammatory and gHDL-mediated TNFα production is amplified by deficiency of SR-BI. The current results have important therapeutic implications, wherein activation of SR-BI has the potential to protect against plaque formation or systematic inflammation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.028.

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