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Atorvastatin affects TLR4 clustering via lipid raft modulation

Praveen Chansrichavala [a], Udom Chantharaksri [a], Piyamitr Sritara [b], Nathamon Ngaosuwankul [c], Sansanee C. Chaiyaroj [c,d,*]

[a] Department of Pharmacology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand
[b] Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand
[c] Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand
[d] Laboratory of Pharmacology, Chulabhorn Research Institute, Bangkok, Thailand

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ABSTRACT

Statins, HMG-CoA reductase inhibitors, are used widely in the treatment of hypercholesterolemia. Apart from lowering lipid levels, statins have been shown to have anti-inflammatory effects. Previously we showed that atorvastatin inhibits NF-κB activation, dose and time dependently, in LPS-TLR4 signaling pathway. In this study, we investigated the anti-inflammatory mechanism of atorvastatin via Toll-like receptor 4 (TLR4) in murine pro-B cell lines transfected with TLR4. Co-treatment of LPS-stimulated cells with both atorvastatin and mevalonate rescued NF-κB activation and TLR4 blockade demonstrated that atorvastatin does not exert its inhibitory effect via TLR4 receptor–ligand binding mechanism. Further investigation into the anti-inflammatory mechanism has shown that atorvastatin causes an impairment of TLR4 recruitment into the lipid raft thereby affecting anti-inflammatory responses. In contrast, mevalonate repaired lipid raft function leading to TLR4 clustering in the lipid raft. Together, these data suggest that atorvastatin exerts its anti-inflammatory effect via lipid raft modification. This novel finding offers another insight into the pleiotropic effects of atorvastatin and may be applicable to other pattern recognition receptors that utilize membrane lipid raft as a platform for signal transduction.

1. Introduction

Cardiovascular disease (CVD) is one of the leading causes of death in the world, including the United States and most European countries, in spite of substantial widespread therapeutic use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins in the treatment of CVD. Large scale clinical trials have shown the beneficial effect of statins in both primary and secondary prevention, and the subsequent marked reduction of coronary events [1,2], largely owing to the reduction of plasma levels of low density lipoprotein (LDL).

Statins inhibit the biosynthesis of cholesterol and its downstream precursors at the conversion of HMG-CoA to mevalonate, the rate limiting step of the pathway, where these precursors are isoprenoid products of mevalonate. Presently, statins are used extensively in clinical practice for prevention and treatment of hypercholesterolemia to reduce cardiovascular-related morbidity and mortality. This effect is partially mediated through the modulation of lipid profiles, but it has recently been demonstrated that statins have pleiotropic effects, which include anti-inflammatory effects in atherosclerotic plaques [3–5]. Although statins have no specific action in immunosuppression, inhibition of lymphocyte function by statin therapy has been observed both *in vitro* and *in vivo* [6,7]. Weitz–Schmidt et al. have shown that statins can bind to a novel allosteric site within the β2 integrin function-associated antigen-1 (LFA-1), independent of mevalonate pathway production [6]. Additionally, statins were found to inhibit the induction of MHC Class II expression by IFN-γ and hence act as repressors of MHC-II mediated T cell activation [7].

Accumulated evidence suggests that one of the pleiotropic effects of statins is mediated through NF-κB activation and production of pro-inflammatory cytokines. Atorvastatin, a third generation of statin, has been shown to reduce proinflammatory markers (tumor necrosis factor [TNF], IL-1, and IL-6) as well as soluble intercellular adhesion molecule-1 (sICAM-1), and C-reactive protein (CRP) in hypercholesterolemic patients [5]. Ortego has demonstrated that atorvastatin reduces NF-κB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells, leading to plaque stabilization [8]. Apart from this, a second generation statin, simvastatin reduced the expression of cytokines IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) in circulating monocytes [4]. Additionally, Hernandez-Presa has also demonstrated that simvastatin reduces NF-κB activity in peripheral mononuclear and in plaque cells of rabbit atheroma [9]. Wang et al. has also shown that fluvastatin, another synthetic compound of statin family, inhibits the expression of tumor necrosis factor-α (TNF-α) and activation of NF-κB activity in C-reactive protein (CRP) stimulated endothelial cells [10]. Taken together these findings clearly indicated an anti-inflammatory effect of statins which will probably be beneficial in atherosclerosis.

* Corresponding author. Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand. Tel.: +66 81 637 0666; fax: +66 2 644 5411.
E-mail addresses: scscy@mahidol.ac.th, Praveen@tropmedres.ac (S.C. Chaiyaroj).

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There is growing evidence that Toll-like receptor 4 (TLR4) signaling is implicated in atherogenesis. Activation of TLR4 by lipopolysaccharide (LPS), its ligand, results in the downstream activation of NF-κB and the production of inflammatory cytokines. Oxidized LDL (oxLDL) have been suggested to upregulate TLR4 [11] in murine macrophages and it has been proposed that oxLDL may also act as a ligand for TLR4 [12]. In addition, upregulation of TLR4 in circulating monocytes has also been observed [13,14]. TLR4 signaling requires the adaptor protein MyD88: MyD88-deficient mice show a marked reduction in early atherosclerosis [15]. Previously, the study done by our group [16] has shown that indeed when LPS-stimulated cells were treated with atorvastatin, the activation of NF-κB was reduced and that the attenuation was in a MyD88 dependent mannerism. Moreover, the attenuation of NF-κB was observed only in LPS-stimulated cells expressing TLR4 implying that TLR4 is important in atorvastatin-mediated NF-κB inhibition. We also revealed that the effects of atorvastatin on LPS-mediated inhibition of NF-κB occur in a concentration- and time-dependent fashion. However, at lower doses of atorvastatin (0.1 μM and 1 μM), treatment time had to be prolonged for a significant inhibitory effect to be seen [16].

Lipid rafts are microdomains enriched with sphingolipids and cholesterol that float within the liquid-disordered bilayer of cellular membranes. These rafts also contain many lipid-modified signaling proteins such as tyrosine kinases of the Src family, GPI (glycosylphosphatidylinositol)-linked proteins among other adaptor proteins. It is believed that these rafts serve as platforms for the formation of multicomponent transduction complexes [17]. Many immune receptors are recruited into lipid raft upon stimulation and dissociate from the raft in the absence of stimulation [18,19]. TLR signaling has also been suggested to occur in lipid raft. Upon stimulation with specific ligand, the membrane-associated TLRs are recruited into the raft domain where signaling complexes are formed to transduce signals [20]. To date it remains unclear how statin administration affects the role of TLR clustering in lipid rafts. In the present study we investigated the role of raft-dependent TLR4 clustering in LPS-stimulated pro-B cells in response to atorvastatin.

2. Materials and methods

2.1. Drugs, chemicals and antibodies

Atorvastatin was kindly provided by Berlin Pharmaceutical Industry Co., Ltd., Bangkok, Thailand. Atorvastatin has undergone quality control analysis by Dr. Reddy's laboratories Ltd. (Hyderabad, India). A certificate of analysis indicates that the result for the tested compound matches the specifications. 0.39% of total impurities were reported [less than the specified threshold (1.0%)] when tested for impurities by HPLC. Mevalonate, bacterial lipopolysaccharide (LPS), and protease inhibitors were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Company, St. Louis, MO, USA). Culture media and its supplements were purchased from GIBCO®, Invitrogen Corporation, Carlsbad, CA, USA.

All primary antibodies used in immunoblotting were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA) and secondary antibodies were from Amersham Biosciences (GE Healthcare, Buckinghamshire, UK). Flotillin-1 was used as a marker for raft fractions while transferrin receptor, a non-raft resident, was used to verify the non-raft fractions.

2.2. Transfection and cell culture

Murine pro-B cell lines (Ba), Ba/κB and Ba/hTLR4/MD2, were kind gifts from Prof. Kensuke Miyake (Department of Microbiology and Immunology, Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan) [16,21–23]. Cell lines Ba/MyD88 and Ba/MyD88/hTLR4/MD2 were constructed by transfecting the two original lines mentioned above with hMyD88 plasmid (InvivoGen, San Diego, CA, USA) in our laboratory using lipofectamine™ (Promega, Madison, WI, USA) method.

Ba cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, penicillin-streptomycin, 50 μM 2-mercaptoethanol (2-ME), non-essential amino acids (NEAA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 2 ng/ml mouse interleukin-3 (R&D Systems, Minneapolis, MN, USA).

2.3. Cell stimulation and luciferase assay

For the blocking assay, approximately 10^5 cells were stimulated with 1 μg/ml LPS for 24 h, and then pre-blocked for 30 min with various concentrations (1, 5, 10 μg/ml) of either anti-TLR4 (HTA 125) monoclonal antibodies or control mIgG2A antibodies (Alexis Biochemicals, San Diego, CA, USA). Cells were then treated further with atorvastatin (10 μM) for 24 h.

To study the effect of atorvastatin and mevalonate on LPS-stimulated cells, approximately 10^5 cells were seeded into a 96-well plate. Cells were stimulated with 1 μg/ml LPS for 24 h. Cells were then further treated with atorvastatin at 10 μM alone or co-treated with 100 μM mevalonate and incubated for different periods of time (0, 12, 24, or 48 h). Cells were not washed between first and second cultures (after LPS stimulation, cells were treated with atorvastatin and mevalonate without change of media).

At the indicated time point, luciferase activity was measured by using the Steady Glo® Luciferase Assay System (Promega, Madison, WI, USA) according the manufacturer's instructions. Briefly, cells were flushed and 100 μl of cells were removed and transferred to a new 96-well white plate. 100 μl of the Steady Glo® substrate was then added and incubated in the dark for 5 min. Luciferase activity was measured using the Wallac Victor 1420 automated microplate reader machine (Perkins Elmer, Waltham, MA).

2.4. RNA isolation and RT-PCR

Total RNA was extracted from 1×10^6 Ba/hTLR4/MD2 cells using the MasterPure™ RNA Purification Kit (EPICENTRE®, Madison, WI, USA) according to the manufacturer's instructions. cDNA was synthesized from 50 ng/ml RNA using SuperScript® III Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). PCR was performed with *Taq* polymerase (Promega, WI, USA) for 30 cycles at 95 °C for 50 s (denaturing), 50 °C for 50 s (annealing) and 72 °C for 90 s (extension). After the last cycle, the reaction was left at 72 °C for 7 min. PCR primer sequences were as follows: TLR4, 5′-TGG ATA CGT TTC CTT ATA AG-3′ (forward) and 5′-GAA ATG GAG GCA CCC CTT C-3′ (reverse); MyD88, 5′-TGC TGG AGC TGG GAC CCA GCA TTG AGG AGG A-3′ (forward) and 5′-TCA GAC ACA CAC AAC TTC AGT CGA TAG-3′ (reverse); and β-actin, 5′-TCA TGA AGT GTG ACG TTG ACA TCC GT-3′ (forward), and 5'-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′ (reverse).

2.5. Flow cytometric analysis

Flow cytometry was utilized to determine TLR4 surface expression under various conditions. 10^6 cells of Ba/hTLR4/MD2 were stimulated with 1 μg/ml LPS for 24 h and further treated with 10 μM atorvastatin for 24 h. Unstimulated cells and cells stimulated with LPS alone were used as negative and positive controls, respectively. Briefly, Ba/hTLR4/MD2 cells were stained with 5 μg/ml primary antibodies (anti-hTLR4 Ab and isotype-specific control) and incubated at 4 °C for 30 min in dark followed by fluorescein isothiocyanate isomer 1 (FITC) conjugated rabbit anti-mouse Immunoglobulins (DakoCytomation, Denmark) at the dilution 1:40 at 4 °C for 30 min in dark. TLR4 surface expressions were then analyzed using FACSCanto (Becton & Dickenson, USA).

2.6. Isolation of lipid rafts

Approximately 1×10^8 Ba/hTLR4/MD2 cells were either stimulated with 1 μg/ml LPS for 24 h, or treated further with 10 μM atorvastatin or simultaneously with 100 μM mevalonate. Cells were then lysed with 1% Brij 58 lysis buffer (1% (v/v) Brij 58, 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA) containing protease inhibitors (5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A) for 30 min on ice. The lysates were mixed (1:1) with 80% sucrose. The lysate/sucrose mixture was carefully overlaid onto 60% sucrose. Then 800 μl of each sucrose concentration (i.e. 20%, 15%, 10%, 5%) were carefully overlaid on top of the 40% lysate/sucrose mixture creating a sucrose gradient. The gradients were then subjected to centrifugation at 287,500 × *g* for 18 h in a SW55 ti rotor (Beckman Instruments) at 4 °C. After sucrose gradient fractionation, twelve 400 μl (F1-F12) were collected from the top of the tube. Flotillin-1 and transferrin receptor were subsequently used as markers to verify the lipid raft and non-raft fractions in cells, respectively, by using immunoblotting technique.

2.7. Immunoblotting

To verify lipid raft isolation, fractions were resolved by SDS-PAGE and transferred to nitrocellulose membrane (PROTRAN, Whatman, Schleicher & Schuell, Germany). Membranes were then blocked with blocking buffer (phosphate buffered saline containing 1% Tween 20 and 5% skim milk) overnight at 4 °C and incubated with raft and non-raft specific primary antibodies, namely anti-Flotillin-1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and anti-transferrin receptor (CD 71) polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA), at 1:200 for 4 h at room temperature. Membranes were then incubated with horseradish peroxidase (HRP) conjugated isotype-specific secondary antibodies (DakoCytomation, Glostrup, Denmark) for 2 h and developed using a colorimetric system.

In order to detect and quantitate the presence of TLR4 in lipid raft disruption by atorvastatin, each fraction of the treated cell lysate was slot-blotted onto the nitrocellulose membrane. After incubating with blocking buffer (1% Tween 20 PBS containing 5% fetal bovine serum) at 4 °C overnight, the membrane was then incubated with anti-TLR4 monoclonal antibodies diluted at 1:200 for 2 h followed by ECL Plex Cy5 conjugated goat-anti-rabbit IgG (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) for 1 h in the dark at room temperature. A Typhoon scanner (Typhoon TRIO Variable Mode Imager, GE Healthcare Lifesciences) with Image Quant TL V2005 software was used to detect the fluorochrome emission signal.

2.8. Statistical analysis

Statistical analysis was performed with software SPSS version 14.0 using an independent paired *T*-test and *P*-value < 0.05 were considered to be statistically significant. All values are represented as mean ± SD.

3. Results

3.1. Atorvastatin does not exert its inhibitory effect on NF-κB activation via TLR4 receptor-ligand interaction

Previously the study done by our group [16] has shown that atorvastatin inhibits NF-κB in dose and time dependent fashion, with the most prominent effect seen at 10 μM and treatment time at 24 h. Additionally, because the inhibitory effect of atorvastatin requires expression of TLR4, it is necessary to verify that if cells were co-incubated with LPS and atorvastatin, would LPS still be able to activate NF-κB or not. Despite co-stimulation of cells with both LPS and atorvastatin, LPS was still able to activate NF-κB activities as time increased from 12 h to 24 h (data not shown).

Next, a blocking assay was performed to further rule out the possibility that atorvastatin does not directly interact through TLR4 receptor-ligand binding mechanism, LPS-stimulated cells were pre-blocked with either control mouse IgG2A monoclonal antibody or anti-hTLR4 antibody (1, 5, and 10 μg/ml) and then incubated with or without 10 μM atorvastatin. In LPS-stimulated cells expressing TLR4 (Ba/hTLR4/MD2), pre-blocking with isotype control antibodies (mouse IgG) provoked some reduction of NF-κB activation (although not statistically significant) in comparison to atorvastatin treated LPS-activated cells (Fig. 1).

However, when cells expressing TLR4 were pre-blocked with anti-hTLR4 MAb prior to atorvastatin treatment, a significant reduction (*P*-value < 0.05) in NF-κB activation was observed, but not as drastic as that observed in cells treated with atorvastatin alone (*P*-value < 0.01). In addition, cells treated with monoclonal antibodies and atorvastatin, showed further reduction of NF-κB activation as compared to cells treated with monoclonal antibodies alone. This reduction was

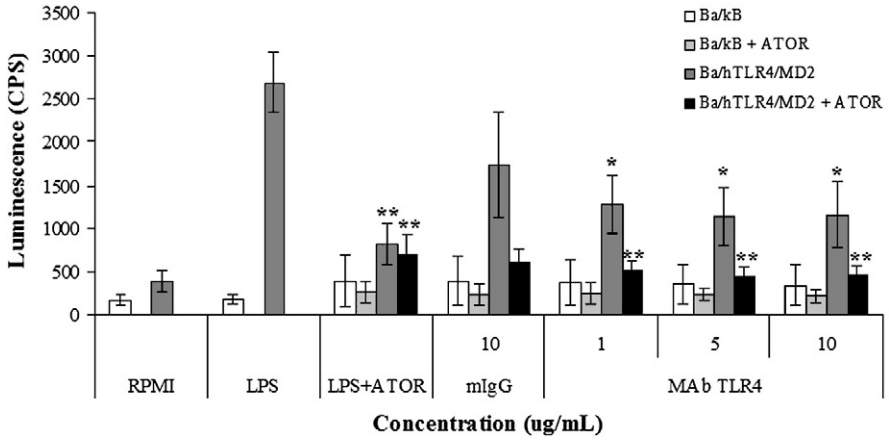

Fig. 1. Attenuation of NF-κB activation in response to TLR4 blockade at LPS-binding site. Ba/κB and Ba/hTLR4/MD2 cells were stimulated with 1 μg/ml LPS for 24 h, and then pre-blocked for 30 min with various concentrations (1, 5, 10 μg/ml) of either anti-TLR4 (HTA 125) monoclonal antibodies or control mIgG2A antibodies (10 μg/ml). Cells were then treated further with atorvastatin (10 μM) for 24 h. LPS-stimulated cells treated with atorvastatin alone (LPS + ATOR) were used as a positive control. The data are expressed as mean ± SD of four data sets from independent experiments and $^*P < 0.05$, $^{**}P < 0.01$ are considered to be statistically significant.

statistically significant (P-value<0.01) in comparison to cells treated with only atorvastatin (Fig. 1). Ba/κB cells were used as negative control. Collectively, these data suggest that although blocking TLR4 at LPS-binding site with MAb produced a significant inhibitory effect on NF-κB activation, the addition of atorvastatin helped further to reduce NF-κB activation.

3.2. Mevalonate rescues the inhibitory effect seen in atorvastatin treated cells

Because statins reversibly inhibit HMG CoA reductase and mevalonate is the product of HMG CoA reductase, we investigated whether mevalonate could rescue the inhibitory effect of atorvastatin on NF-κB activation. LPS-stimulated cells were treated with atorvastatin (10 μM) simultaneously with mevalonate (100 μM) further for 0, 12, and 24 h. Results in Fig. 2A indicate that mevalonate can significantly reverse the inhibitory effect of atorvastatin on NF-κB activation (P-value<0.05) when compared to cells treated with atorvastatin alone. In Ba/hTLR4/MD2 cells, the increase of NF-κB induction went from 21.23 ± 9.27 folds to 38.90 ± 23.39 folds, while in Ba/MyD88/hTLR4/MD2 cells, the increase was from 15.05 ± 6.65 folds to 26.58 ± 14.99 folds. However, as seen with earlier experiments, the effect is only observed in cells expressing TLR4, while in Ba/MyD88 (a negative control), there was no change. In addition, the result also showed that mevalonate could reverse this effect most potently after the 24 h treatment. When the treatment time was prolonged to 48 h, there was no change in fold induction observed (Fig. 2B). The data also indicate that mevalonate can restore NF-κB activation to near normal levels by comparison with cells stimulated with LPS alone, suggesting that the inhibitory effect of atorvastatin was not of an irreversible nature.

The interaction of mevalonate with TLR4-induced NF-κB activation was further investigated. Cells were stimulated with different concentrations of mevalonate (1, 10 and 100 μM) for various times (0, 12, and 24 h) and activation of NF-κB was measured. Our data show that mevalonate could not stimulate NF-κB activation in any cell lines, particularly in cell lines expressing TLR4 (data not shown). Interestingly, when LPS-stimulated cells were treated with mevalonate alone, NF-κB activation was still observed (Fig. 2A), implying that mevalonate did not interact with TLR4-induced NF-κB activation. Taken together, this suggests that TLR4 does not play a role in assisting mevalonate to exert action: mevalonate likely functions through other unknown mechanisms.

3.3. Atorvastatin does not alter TLR4 expression at both mRNA and protein levels in the transfected system

The next investigation was to elucidate the inhibitory effect of atorvastatin on the expression of TLR4 in our transfected system. As shown in Fig. 3A, no significant difference change in TLR4 expression at mRNA level in LPS-stimulated cells treated with atorvastatin when compared to cells stimulated with LPS was observed. Further analysis by flow cytometry also revealed that the surface expression of TLR4 remains the same in both LPS-stimulated cells alone and LPS-stimulated cells treated with atorvastatin (Fig. 3B).

3.4. Verification of lipid raft isolation

Before performing further experiments with lipid raft, fractions retrieved from sucrose gradient were first determined for the presence of Flotillin-1 (a raft marker), transferrin receptor (a non raft marker), and TLR4 (Fig. 4). Fractions 2–4 were designated as lipid

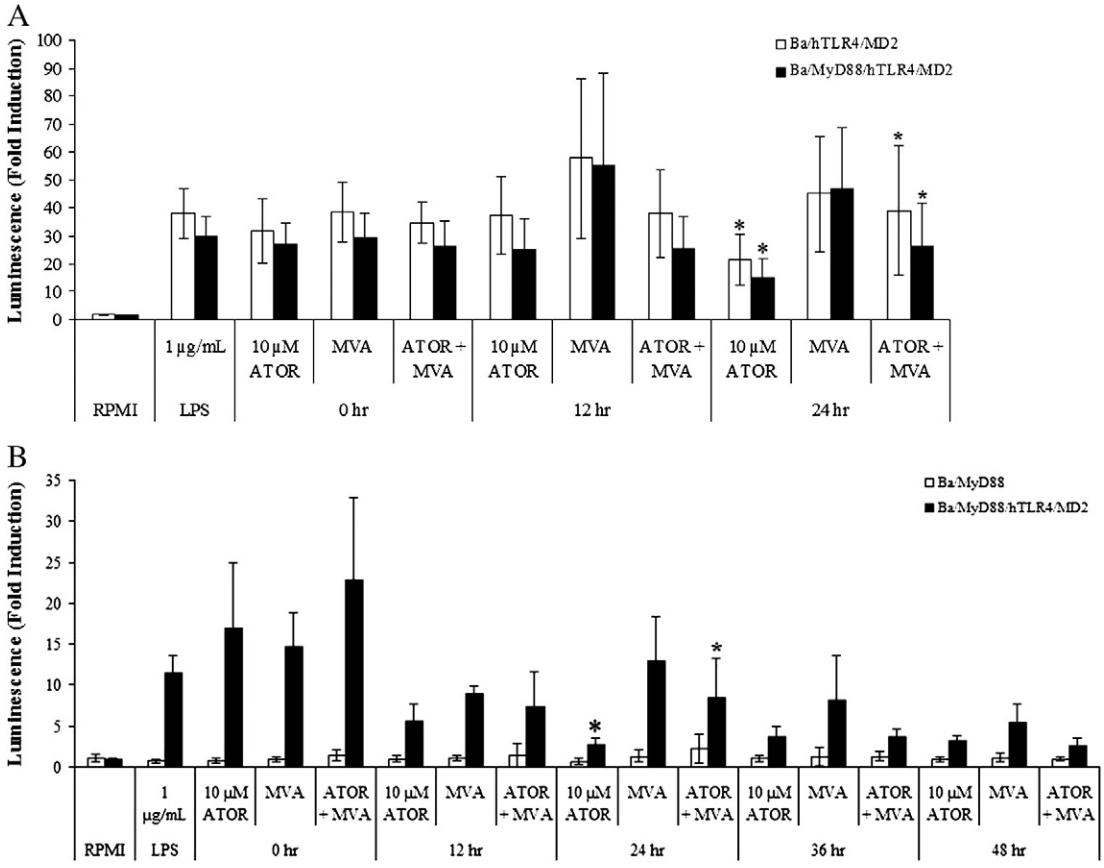

Fig. 2. Mevalonate rescue effect on atorvastatin treated cells. LPS-stimulated cells (Ba/MyD88, Ba/hTLR4/MD2 and Ba/MyD88/hTLR4/MD2) were further treated with either atorvastatin (ATOR) at 10 μM alone or simultaneously with mevalonate (MVA) for (A) 24 h and (B) 48 h. Ba/MyD88 cells were used as negative control. The data are expressed as fold induction and expressed as mean ± SD of four data sets from independent experiments and $^*P<0.05$ is considered to be statistically significant.

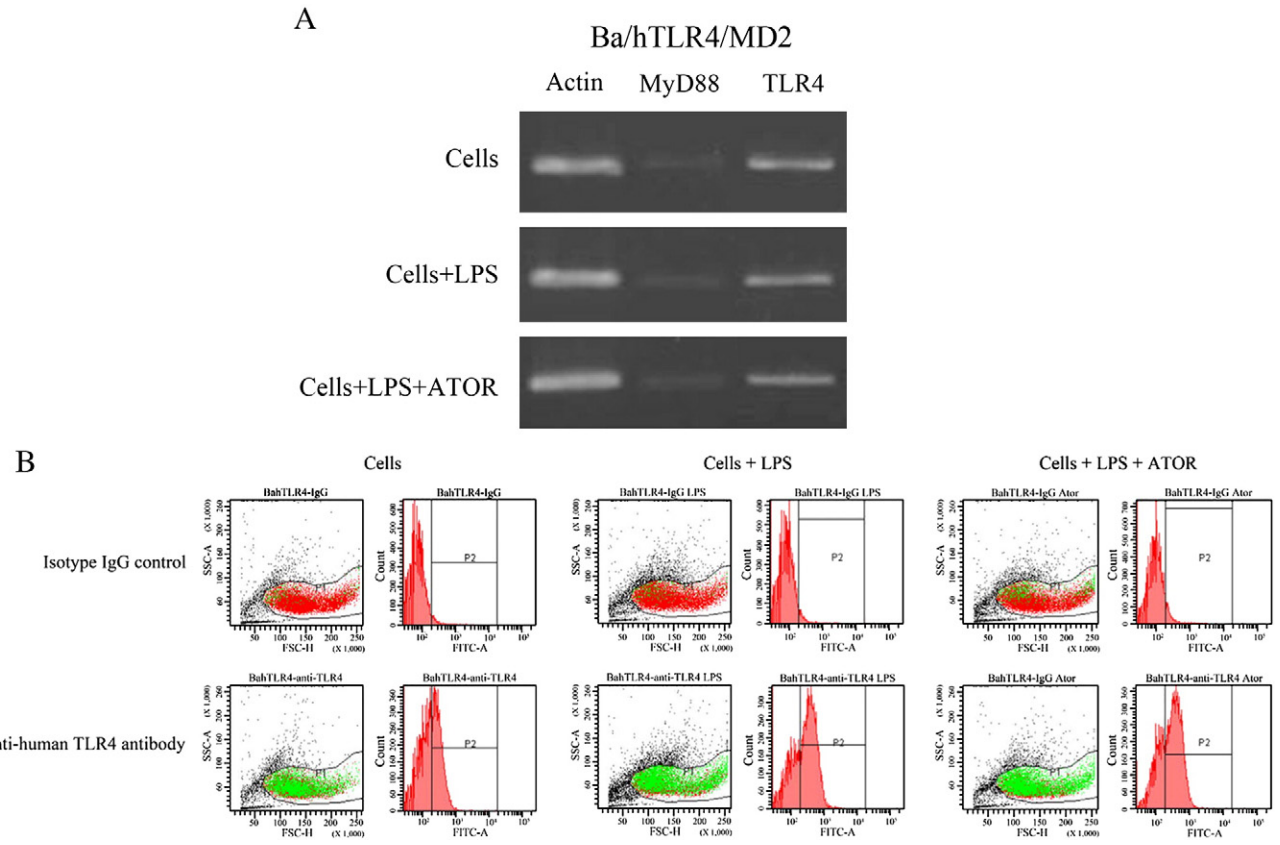

Fig. 3. Effect of atorvastatin on TLR4 expression. (A). Ba/hTLR4/MD2 were stimulated with 1 μg/ml LPS for 24 h and further treated with 10 μM for 24 h. RNA was extracted and RT-PCR was performed and TLR4 mRNA expression was determined. β-actin was used an internal control. All RT-PCR bands are representative data of three independent experiments. (B). Ba/hTLR4/MD2 were stimulated with 1 μg/ml LPS for 24 h alone or further treated with 10 μM atorvastatin for 24 h. These cells were then stained with primary antibodies (anti-hTLR4 Ab and isotype-specific control) and followed by fluorescein isothiocyanate isomer 1 (FITC) conjugated rabbit anti-mouse Immunoglobulins. TLR4 surface expressions were then analyzed. The top row of FACS plots represented the isotype control while the bottom row represents anti-hTLR4 staining.

rafts (or detergent resistant membranes, DRMs) as the interface band of isolated raft appeared around these fractions and Flotillin-1 also reside in these fractions. These fractions, which have been identified as lipid raft fractions, were then used to carry on with the next slot-blot experiments to study the impact of atorvastatin and mevalonate on TLR4 recruitment into lipid rafts.

3.5. Atorvastatin results in a decreased recruitment of TLR4 into lipid raft

Since atorvastatin inhibitory effect on NF-κB was not through modulation of TLR4 expression, lipid raft on cell membrane, where signaling complexes are formed, were studied. To elucidate how atorvastatin influences NF-κB activation, the effect of atorvastatin on lipid raft integrity by monitoring the recruitment of TLR4 to the lipid raft domain were investigated. A profound increase of TLR4 in detergent resistant membranes (DRMs) or lipid raft domain (F2–F4) was observed in LPS-stimulated cells, while there was a prominent reduction of TLR4 found within DRMs in atorvastatin treated cells. In contrast, in untreated cells, less TLR4 was found within DRMs when compared to LPS-stimulated cells (Fig. 5). A line graph was also plotted to show the profile of cells treated with LPS alone or with atorvastatin in comparison to untreated cells (a negative control). It was demonstrated that, in LPS-stimulated cells there was a significant increase in fluorescence intensity (212.33 ± 1.10%) while a significant reduction (102.33 ± 0.65%) in the fluorescence intensity was observed in atorvastatin-treated cells ($P<0.01$) (Fig. 5). Taken together, these data suggest that atorvastatin causes defect in TLR4 recruitment into lipid raft, which is important for LPS-induced TLR4 signaling, and results in the decreased NF-κB activation observed in this study.

3.6. Mevalonate restores TLR4 recruitment into lipid raft through modulation of lipid raft

Finally, the mechanism underlying the mevalonate rescue effect observed on NF-κB activation was also investigated. Upon treating LPS-stimulated cells simultaneously with atorvastatin and mevalonate, the level of TLR4 found in DRMs was restored, though not to 100% (Fig. 6A). As seen from the individual fractions (F2, F3, and F4), the relative fluorescence in cells treated simultaneously with mevalonate and atorvastatin was higher than in cells treated with atorvastatin alone in all DRM fractions (F2, F3, and F4). However, the relative fluorescence was not as high as that of LPS-stimulated cells.

Fig. 6B demonstrates that when compared to a positive control (100%), atorvastatin-treated cells gave significant lower fluorescence intensity, 43.95 ± 11.95% (P-value<0.01), while a significant increase in intensity was observed in cells treated with both atorvastatin and mevalonate (93.02 ± 7.98%) (P-value<0.01). Altogether, these individual DRM fractions demonstrated a reduction trend in TLR4 recruitment to lipid rafts in atorvastatin treated cells, while there was an increase in TLR4 recruitment in mevalonate co-treated cells. In corroboration with the NF-κB luciferase assays suggesting that mevalonate can reverse the inhibitory effect of atorvastatin, these findings indicate that the rescue effect of mevalonate is through lipid raft modulation.

4. Discussion

Accumulating evidence has pointed out that statin, a commonly prescribed drug for the treatment of hypercholesterolemia, has pleiotropic effects that are believed to be lipid-lowering independent.

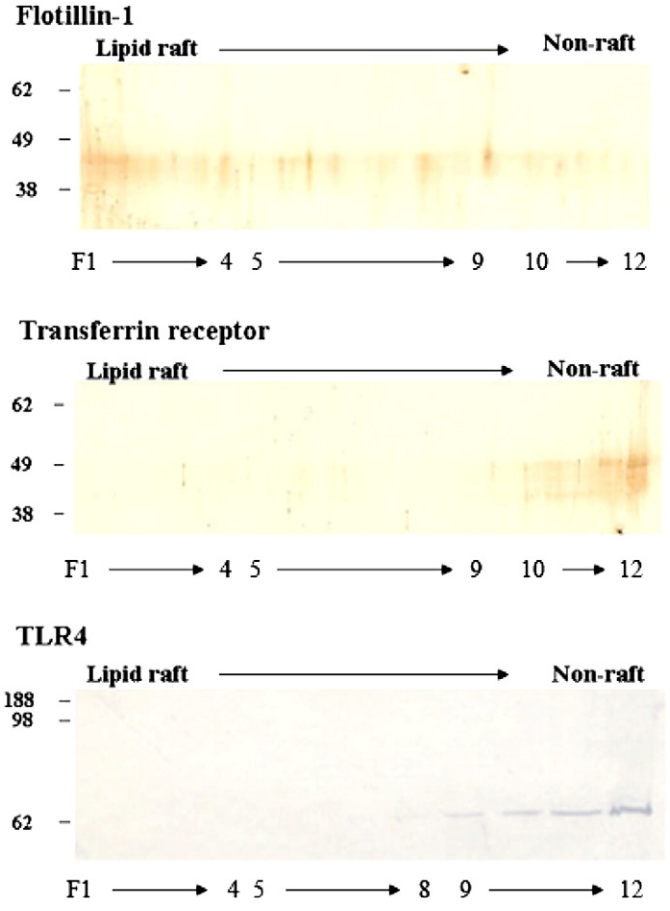

Fig. 4. Verification of lipid raft. Ba/hTLR4/MD2 cells lysed by 1% Brij 58 lysis buffer were subjected to sucrose gradient centrifugation. Twelve 400 μL fractions were collected and Western blotted for flotillin-1 (F2-F4), transferrin receptor (F10-F12), and TLR4, which resides in the non raft fractions (F9-F12).

Possible mechanisms by which statins reduce CVD risk independent of their lipid regulation include anti-thrombotic and anti-inflammatory effects [4,5,24], plaque stabilization [25–28], and endothelial wall relaxation resulting in lower intra-arterial pressure and increased blood flow [29–31]. Recently, numerous studies have pointed out that statins have immunomodulating effects which can influence various immunological disorders including atherosclerosis [3,32–34]. It is only recently that inflammation has been suggested to play a pivotal role in atherosclerosis [35–38]. Past studies have indicated an ongoing process of inflammatory responses in amplifying the progression of atherosclerosis resulting in plaque disruption, thrombosis and eventually myocardial infarction.

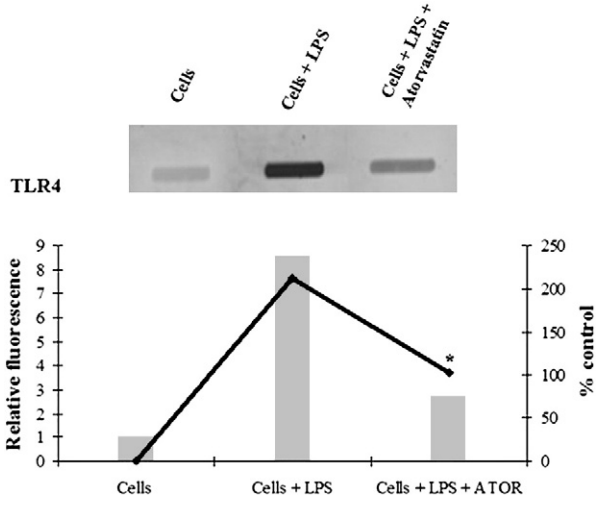

Fig. 5. Effect of atorvastatin on TLR4 recruitment into lipid raft. Pooled raft fractions (F2-F4) of untreated, LPS-treated, and LPS and atorvastatin treated Ba/hTLR4/MD2 cells were slot-blotted for TLR4 and plotted. All blots are representative of at least three independent experiments and $^{*}P$-value<0.01 is considered to be statistically significant.

Although many TLRs related studies employ macrophages, monocytes or endothelial cells as the models in their studies, we select IL-3 dependent murine pro-B cells which have been transfected with hTLR4/MD2, MyD88/hTLR4/MD2 and NF-κB reporter construct for this study because of the exclusivity of TLR4 expression on pro-B cells. This transfection system has been previously used in many studies [16,21–23] regarding TLR4 signaling pathway as it offers the advantage of excluding other PRRs and TLRs out in the regards to NF-κB activation.

Numerous studies suggest that statin can lead to a reduction in pro-inflammatory cytokines in monocytes, dendritic cells and macrophages, a result of NF-κB activation [5,24,39,40]. In concordance with these studies, previous work done by our group also showed that atorvastatin attenuates TLR4-mediated NF-κB activation in murine pro-B cells transfected with TLR4 [16]. The observation that, when cells are co-treated with atorvastatin and LPS simultaneously, activation of NF-κB by LPS was still seen as exposure time increased suggests that there is no competition between LPS and atorvastatin in binding with TLR4 (data not shown). This could be explained by the nature of TLR4 in recognizing a lipoprotein, (LPS bound to LBP), while atorvastatin is a completely synthetic compound and was not recognized by TLR4. Evidence from our blocking assay further suggests that the attenuation of NF-κB activities is unlikely to occur through direct receptor–ligand interaction (Fig. 1).

Accumulating evidence has shown that the addition of mevalonate, a precursor of cholesterol, simultaneously with statins could reverse the inhibitory effect of statins. Here, we reported that when LPS-stimulated cells expression both MyD88/hTLR4/MD2 and hTLR4/MD2 were treated simultaneously with atorvastatin and mevalonate, the inhibitory effect could be reversed best at 24 h (Fig. 2). Possible explanations for this observation are that the time that takes atorvastatin to exert its maximal inhibitory effect is at 24 h and mevalonate concentrations may decline over time due to natural degradation in this *in vitro* system. This mechanism was further explored. By using LPS-activated cells as positive control, we found that when cells were treated with mevalonate alone, activation of NF-κB was not observed (data not shown). Varying doses of mevalonate did not invoke any responses. Furthermore, when LPS-stimulated cells were treated with mevalonate, NF-κB activation was still observed. Overall, these results suggest that mevalonate does not exert its rescue effect via the LPS-TLR4-mediated NF-κB signaling pathway but another unknown mechanism is likely responsible. Although it is postulated that mevalonate replenishes cells with cholesterol to elicit rescue, this is yet to be confirmed.

Moreover, the surface expressions of TLR4 and TLR2 on monocytes have been reported to decrease upon statins administration *in vivo* by Niessner et al. and Methe et al. [41,42]. However, here we reported that upon atorvastatin treatment, TLR4 expression remains the same at both mRNA and protein levels (Fig. 3). Although our result is different from the previous groups, it is important to note that the system used in our study was the transfected system where there is an overexpression of TLR4 while Niessner and Methe used *in vivo* system. This finding is of particular importance because the variations of overall TLR4 expression in the raft study where TLR4 recruitment into the lipid raft domain upon atorvastatin treatment was investigated can be eliminated.

In lymphocytes, the most important receptors expressed are members of multichain immune recognition receptor (MIRR). These

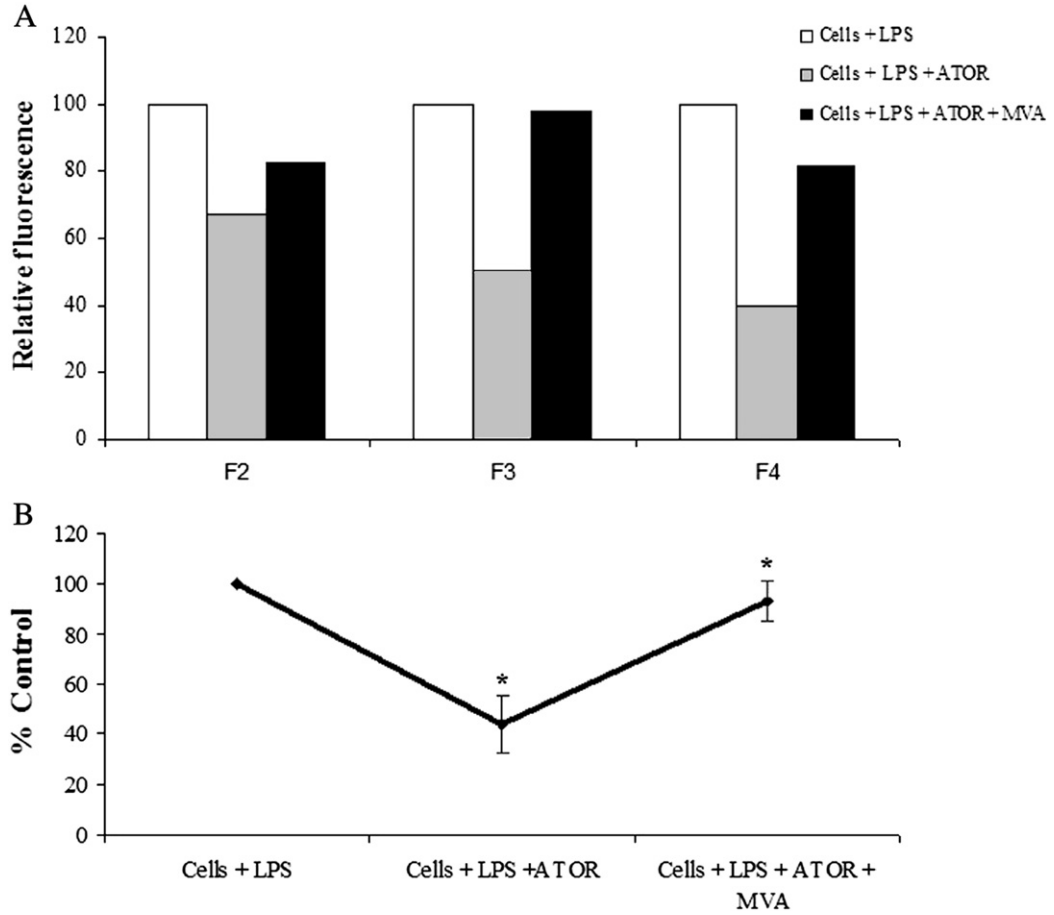

Fig. 6. Effect of mevalonate on TLR4 recruitment into lipid raft. (A). Individual raft fractions (F2, F3, and F4) of LPS-treated, LPS/atorvastatin treated, and LPS/atorvastatin/mevalonate-treated Ba/hTLR4/MD2 cells were slot-blotted for TLR4 and the individual raft fractions band intensities were analyzed and plotted. (B). The differences in the band intensities were plotted against control, LPS-stimulated Ba/hTLR4/MD2 cells. All blots are representative of at least three independent experiments and *P-value < 0.01 is considered to be statistically significant.

include T cell antigen receptor (TCR), B cell antigen receptor (BCR) as well as the receptors for Fc region of antibodies expressed on different leukocyte cells. By themselves, the receptors cannot transduce signals however, the association with other coreceptors or signaling molecules will allow them to do so. Signaling of these immune receptors is thought to occur in microdomains called lipid rafts [17–19], where they serve as platforms for receptor clustering to transduce signals. Moreover, it has been previously shown that cholesterol chelating agents, such as MCβD and statins (HMG-CoA reductase inhibitors), can attenuate TCR signaling [43]. Nevertheless, very little is known in regard to TLR signaling. Although one crucial study relating the role of lipid raft to TLR revealed that ethanol can reduce the recruitment of TLR4 into lipid rafts in LPS-stimulated cells [44]. This insight suggests that as in TCR and BCR signaling, TLR4 moves into the specialized domain (lipid raft) where receptor complexes are formed in order to transduce signals.

The role of statins in lipid raft modulation has been previously demonstrated. Hillyard and her collagues have shown in a series of experiments that statins such as simvastatin and fluvastatin can modulate lipid raft in immune cells [45–47]. It was demonstrated that fluvastatin inhibits a raft-dependent Fcγ receptor signaling in human monocytes by disrupting membrane raft while simvastatin has been shown to reduce NK cell cytotoxicity and T cell proliferation through membrane cholesterol depletion. In particular, Jury et al. have shown that in SLE patients T cells the expression of raft-associated ganglioside, GM1 is increased and CD45, which regulates lymphocyte-specific protein kinase (Lck) activity is found to be more localized in lipid raft domain, atorvastatin can restore levels of Lck by disruption the colocalization of total Lck and CD45 within lipid rafts [43].

In light of this evidence, we further examined a raft-dependent TLR4 signaling in pro-B cells transfected with TLR4 in the present study. We demonstrated that, in untreated cells, more TLR4 was found outside the raft domain while in LPS-stimulated cells, more TLR4 was recruited into the raft domain. However, when LPS-stimulated cells were treated with atorvastatin, less TLR4 was found in the raft domain resulting in attenuation of NF-κB by atorvastatin, hence reduction in the inflammatory responses (Fig. 5). This result suggests that atorvastatin, which inhibits the cholesterol biosynthesis, disrupts lipid raft by depleting cells of cholesterol. Interestingly, when LPS-stimulated cells were also treated simultaneously with atorvastatin and mevalonate, a rescue effect was observed. A possible explanation for this is that there is cell replenishment of cholesterol, a product of mevalonate [46]. These data imply that inhibition of cholesterol synthesis is responsible for membrane raft disruption.

In conclusion, in this study a new proposed mechanism is as follows: In resting cells, TLR4 resides outside the raft and when there is TLR4 activation, TLR4 is recruited into the raft and transduce signals resulting in the activation of NF-κB. However, in the presence of atorvastatin, less TLR4 is recruited into the raft and mevalonate restores the raft integrity by replenish the cells with cholesterol, hence the ability of TLR4 to be recruited into the raft once again.

In summary, the present study has shown an *in vitro* raft-dependent anti-inflammatory effect of atorvastatin and offers a new insight into the anti-inflammatory mechanism of atorvastatin. The novel findings gained from this study and the knowledge of the

emerging concept of the lipid raft may offer a new explanation in regards to the nature of the pleiotropic effects of statins seen both *in vitro* and *in vivo*. In atherosclerosis, where there is an ongoing inflammatory response, statins may benefit patients more than by merely reducing cholesterol level, but also by providing anti-inflammatory responses leading to plaque stabilization. This reduces the risks of cardiovascular diseases development as a consequence of plaque ruptures. In addition, this novel finding may also be applied to other autoimmune or inflammatory diseases where pathogenesis stems from inflammation.

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