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Atorvastatin Attenuates TLR4-mediated NF-κB Activation in a MyD88-Dependent Pathway

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SUMMARY Antigen presenting cells such as dendritic cells and macrophages have recently been detected in atherosclerotic plaques. Toll-like receptors expressed on the surface of these cells, have been implicated in ongoing inflammatory responses in the plaques. In this study, we investigated the anti-inflammatory effect of atorvastatin, a lipid lowering drug, *via* Toll-like receptor 4 (TLR4) *in vitro*, employing murine pro-B cell lines transfected with hTLR4/MD2 and MyD88/hTLR4/MD2 systems. The results showed that atorvastatin at 10 μM significantly attenuated NF-κB activation within 24 hours while at lower doses of 0.1 and 1 μM, treatment time had to be prolonged up to 48 hours for a significant inhibition to occur. The inhibition of NF-κB was also observed in a cell line co-transfected with MyD88 and TLR4 suggesting that the attenuation of NF-κB by atorvastatin occurred in a MyD88 dependent fashion.

Atherosclerotic plaques contain many blood-borne inflammatory and immune cells, including macrophages and dendritic cells (DCs) and the presence of these antigen presenting cells (APCs) in atherosclerotic plaques has been described and indicated in the progression of atherosclerosis.[1] Recently, DCs were detected in atherosclerotic plaques of vascular specimens[2,3] and among foam cells in atherosclerotic lesions. Moreover, macrophages, which express various receptors such as scavenger receptors, can recognize oxidized low density lipoprotein (oxLDL). Fully oxLDL contributes to atherogenesis in part by virtue of being ingested into macrophages via these specific scavenger receptors resulting in the differentiation of the macrophages into foam cells.[4] These APCs amplify the inflammatory responses in atherosclerotic lesions as follows: DCs, by the induction of T-cell proliferation and secretion of pro-inflammatory cytokines and macrophages by secreting pro-inflammatory cytokines and oxidative stress induction which causes further injury to vessels. These processes all contribute to the progression of atherosclerosis eventually leading to plaque disruption.

APCs express pattern recognition receptors (PRRs) on their surfaces, including Toll-like receptors (TLRs). Past studies have found an increase in the expressions of TLR1, TLR2, and TLR4 in human

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atherosclerotic lesions[5] and the expression of TLR4 but not TLR2 is upregulated by oxLDL.[6] It is possible that oxLDL has a role as a ligand of TLR4.[6,7] The binding of oxLDL to TLR4 leads the activation of transcription factor NF-κB, ultimately leading to the synthesis of inflammatory cytokines and chemokines. The best studied TLR4 polymorphism is an amino acid substitution from aspartic acid to glycine at position 299 (D299G). The D299G polymorphism is associated with a reduced risk for carotid artery atherosclerosis and acute coronary events.[7] People with this polymorphism also have lower concentrations of circulating pro-inflammatory cytokines such as IL-6, fibrinogen and soluble cell adhesion molecule 1. An association between TLR4 function and atherosclerosis is consistent with findings showing that mRNA and proteins are more abundant in plaques of atherosclerotic lesions than in unaffected vessels.[6] Variants of TLR4 have also been reported to modify the efficacy of statin therapy.[8] In addition, activation of TLR4 requires an adaptor protein, MyD88, for signal transduction. Bjorkbacka *et al.*[9] confirmed that MyD88-deficient mice showed a marked reduction in early atherosclerosis. Inactivation of the MyD88 pathway led to a reduction in atherosclerosis through a decrease in macrophage recruitment to the artery wall which resulted in reduced chemokine levels.

Statins, potent inhibitors of the cholesterol biosynthesis, have been reported to have other pleiotropic effects that are independent of lowering lipids. These beneficial effects include anti-inflammatory effects in atherosclerotic plaques.[10-12] Although statins have no specific immunosuppressive action, an inhibition of lymphocyte functions by statins has been observed both *in vitro* and *in vivo*.[13,14] There is also compelling evidence towards the role of statins in the suppression of dendritic cell maturation. A report from Yilmaz *et al.*[15] has confirmed that statins indeed do suppress the maturation of dendritic cells dose-dependently, and that the maturation markers are decreased in comparison to unstimulated DCs. A later work by the same group has also supported the presence of DCs in the rupture-prone regions of vulnerable plaques.[16] Additionally, in a more recent study, it was found that statins suppress endocytosis, secretion of pro-inflammatory cytokines and DC-induced T cell proliferation.[17]

Accumulated evidence suggests that one of the pleiotropic effects of statins is mediated through the activation of NF-κB and the production of pro-inflammatory cytokines. Atorvastatin, a third generation statin, has been shown to reduce pro-inflammatory 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.[12] Ortego[18] has demonstrated that atorvastatin reduces NF-κB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells, leading to plaque stabilization. Therefore, in the present study we investigated the ability of atorvastatin to reduce pro-inflammatory responses via TLR4 using a murine pro-B cell line transfection system.

MATERIALS AND METHODS

Drugs and chemicals

Atorvastatin was kindly provided by Berlin Pharmaceutical Industry Co., Ltd., Bangkok, Thailand. The atorvastatin used in this study has undergone a quality control analysis by Dr. Reddy's Laboratories Ltd. (Hyderabad, India). A certificate of analysis has been issued indicating that the results for the tested compound matched the specifications. When tested for impurities by HPLC, only 0.39% total impurities were reported, which was less than the specified threshold of 1.0%. Bacterial lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Culture medium and its supplements were purchased from GIBCO, Invitrogen Corporation, Carlsbad, CA, USA.

Cells, cell cultures and stimulation

The 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, Institute of Medical Science, University of Tokyo, Tokyo, Japan). 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 the lipofectamine (Promega, Madison, WI, USA) method. Briefly, Ba cell lines were cultured in

RPMI-1640 medium 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).

To study the effect of atorvastatin on LPS-stimulated cells, approximately 10^5 cells were seeded into a 96-well plate. The cells were stimulated with 1 μg/ml LPS for 24 hours, and then treated with atorvastatin at various concentrations (0.1, 1, 10 μM) and incubated for different periods of time (0, 12, 24, or 48 hours). The cells were not washed between the first and second cultures (after LPS stimulation the cells were treated with atorvastatin without a change of media).

Flow cytometric analysis

Approximately 10^6 cells (Ba/κB, Ba/hTLR4/MD2, Ba/MyD88 and Ba/MyD88/hTLR4/MD2) were stained with antibody (Ab) against human Toll-like receptor 4 (hTLR4) (Alexis Biochemical, Switzerland) or irrelevant mouse IgG (Caltag Laboratories, Burlingame, CA, USA) as a negative control. Cells were stained with primary antibodies, 5 μg/ml anti-hTLR4 Ab and an isotype-specific control for each cell line and incubated at 4°C for 30 minutes in the dark followed by incubation with fluorescein isothiocyanate isomer 1 (FITC) conjugated rabbit anti-mouse immunoglobulin (DakoCytomation, Denmark) at a dilution of 1:40 at 4°C for 30 minutes in the dark. The surface expressions of hTLR4 were then analyzed using FACSCanto (Becton & Dickenson, USA).

Confirmation of MyD88 and TLR4 expression

Total RNA was extracted from 1×10^6 cells of each cell line 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). Thirty cycles of PCR with *Taq* polymerase (Promega, WI, USA) were performed at 95°C for 50 seconds (denaturing), 50°C for 50 seconds (annealing) and 72°C for 90 seconds (extension). After the last cycle, the reaction was left at 72°C for 7 minutes. The primers used for the RT-PCR were: TLR4, TGG ATA CGT TTC CTT ATA AG and GAA ATG GAG GCA CCC CTT C; MyD88, TGC TGG AGC TGG GAC CCA GCA TTG AGG AGG A and TCA GAC ACA CAC AAC TTC AGT CGA TAG; and β-actin, TCA TGA AGT GTG ACG TTG ACA TCC GT and CCT AGA AGC ATT TGC GGT GCA CGA TG.

Luciferase assay

Cells were co-transfected with a Luc-NF-κB reporter construct. The luciferase activity was measured by using the Steady Glo Luciferase Assay System (Promega, Madison, WI, USA). The assay was performed according the manufacturer's instructions. Briefly, the cells were flushed and 100 μl of cells were removed and transferred to a new 96-well white plate. One hundred microliters of the Steady Glo substrate were added and incubated in the dark for 5 minutes. The luciferase activity was measured using the Wallac Victor 1420 automated microplate reader machine (Perkins Elmer, Waltham, MA).

Statistical analysis

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

RESULTS

Confirmation of MyD88 and TLR4 expression in Ba cell lines

Prior to experimentation, the expression of both TLR4 and MyD88 was confirmed in all cell lines. Since TLR4 is expressed on the surface of cells, flow cytometric analysis was used to detect TLR4 surface expression in all cell lines. Figs. 1A and 1B show that in the cell lines Ba/κB and Ba/MyD88, the intensity peak resembled that of the isotype specific control, *i.e.* no shift of the peak was observed, suggesting that TLR4 was not expressed in these two cell lines. But TLR4 surface expression was found in the cell lines Ba/hTLR4/MD2 and Ba/MyD88/hTLR4/MD2 (Figs. 1C and 1D), as indi-

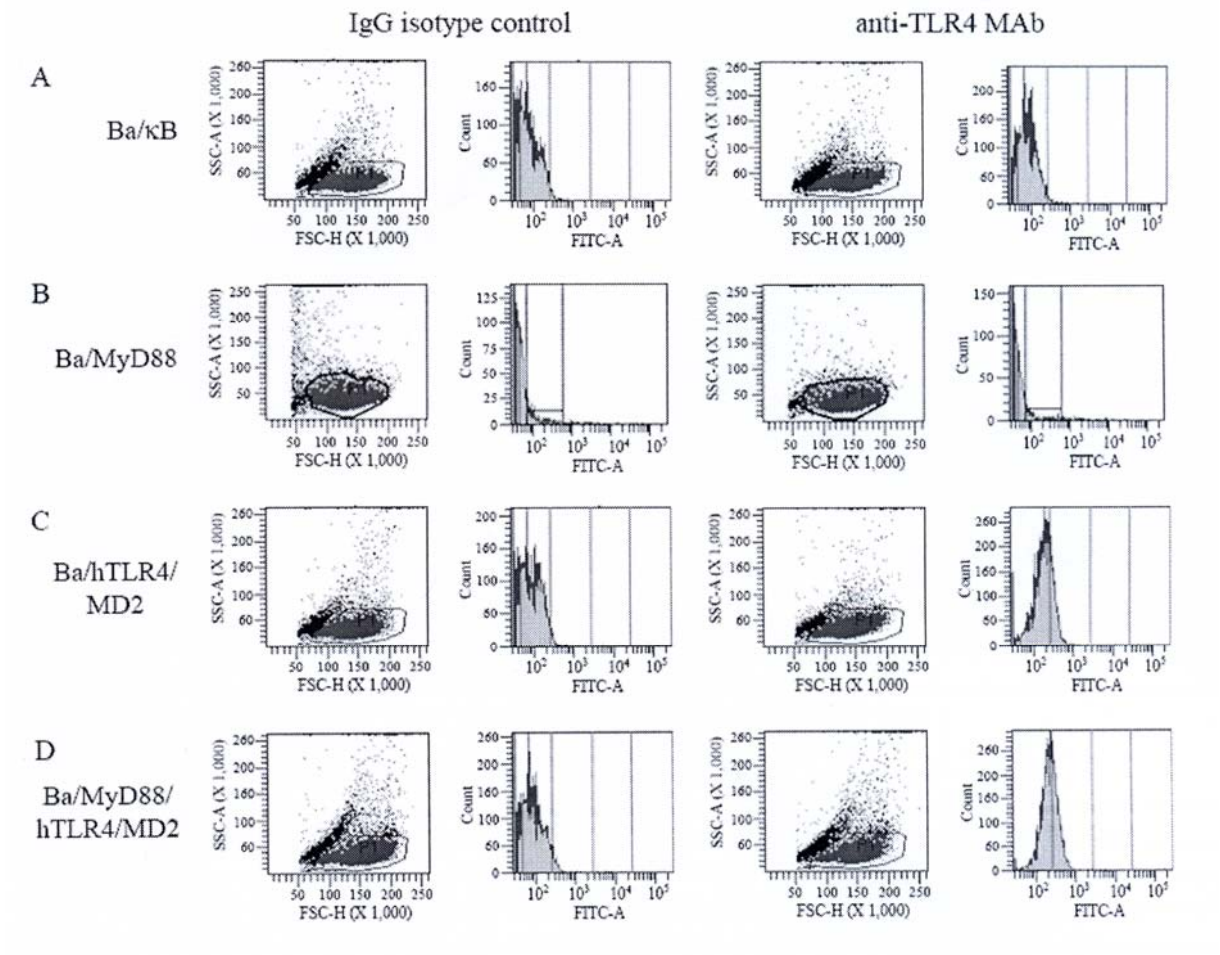

Fig. 1 Flow cytometric analysis of the surface expression of TLR4 in each cell line. TLR4 expression was confirmed in the cell lines Ba/hTLR4/MD2 and Ba/MyD88/hTLR4/MD2 while no TLR4 expression was found in the untransfected cell lines Ba/κB and Ba/MyD88.

cated by an increase in the intensity of fluorescence when compared to each cell line's individual isotype specific control.

In order to check for MyD88 expression, the total RNA was extracted and cDNA was synthesized from the RNA and amplified with PCR using specific primers. Beta-actin was used as internal control and amplicon bands were present in all cell lines as shown in Fig. 2. MyD88 was found expressed in the cell lines Ba/MyD88 and Ba/MyD88/hTLR4/MD2, as illustrated by the presence of a band in the designated MyD88 lane shown in Figs. 2B and 2D. Regarding the cell lines Ba/κB and Ba/hTLR4/MD2, the absence of the band in the MyD88 lane (Figs. 2A and 2C, respectively) demonstrated the lack of MyD88 expression in these cell lines. These findings were consistent with the nature of each cell line. In addition to this, TLR4 expression was also detected in both Ba/hTLR4/MD2 and Ba/MyD88/hTLR4/MD2 cells, while no such expression was found in the untransfected cell lines Ba/κB and Ba/MyD88, depicted by the presence and absence of the relevant bands in the TLR4 lane, respectively. Taken together, these results showed conclusively that there was TLR4 expression in the lines concerned, namely, Ba/hTLR4/MD2 and Ba/MyD88/hTLR4/MD2, while MyD88 expression was found in the lines Ba/MyD88 and Ba/MyD88/hTLR4/MD2. Neither TLR4 nor MyD88 were found expressed in Ba/κB which is consistent with the nature of this untransfected cell line.

Atorvastatin-induced attenuation of NF-κB activation in a concentration-dependent manner

To study the effect of various concentrations of atorvastatin on the NF-κB activation, cells were stimulated with 1 μg/ml LPS for 24 hours and then treated with various concentrations (0.1, 1, 10 μM) of atorvastatin. As shown in Fig. 3A, when Ba/hTLR4/MD2 cells (grey bars) were treated with 0.1 μM, 1 μM, and 10 μM atorvastatin, there was a 29.24%, 31.33%, and 55.57% inhibition of NF-κB, respectively. In the Ba/MyD88/hTLR4/MD2 cell line (black bars), 0.1 μM, 1 μM, and 10 μM atorvastatin treatments resulted in a 10.88%, 15.97%, and 59.86% inhibition of NF-κB, respectively. No NF-κB activation was observed in the control cell line Ba/κB (white bars) under any condition. Statistical analysis was done to compare the LPS-stimulated cells. Notably, no significant change in the NF-κB activation was observed in the Ba/MyD88 line, even when stimulated with LPS. The NF-κB activation was comparable to that of the untransfected line, Ba/κB (Fig. 3B). These results suggest that atorvastatin can reduce NF-κB activation concentration-dependently, with a significant decrease in luciferase activity seen at 10 μM (p-value < 0.05). However, this was only observed in cell lines expressing TLR4 (Ba/hTLR4/MD2 and Ba/MyD88/hTLR4/MD2), in cells lacking TLR4 expression on its surface (Ba/κB and Ba/MyD88), there was no significant difference between atorvastatin treated and untreated cells when compared to a positive control. Moreover, our results also revealed that in addition to the inhibition of the TLR4 mediated NF-κB activation, atorvastatin significantly inhibited the NF-κB activation in a MyD88 dependent manner (Fig. 3A). Collectively, these results suggest that atorvastatin can attenuate NF-κB activation through TLR4 in a concentration-dependent fashion.

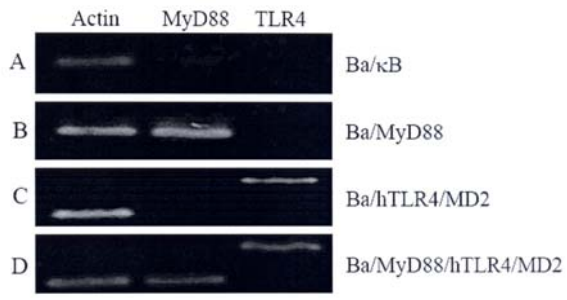

Fig. 2 RT-PCR confirmation of MyD88 and TLR4 expression. MyD88 expression was confirmed in lines Ba/MyD88 and Ba/MyD88/hTLR4/MD2 while none was found in lines Ba/κB and Ba/hTLR4/MD2. Moreover, TLR4 expression was also confirmed in lines Ba/hTLR4/MD2 and Ba/Myd88/hTLR4/MD2. Neither MyD88 nor TLR4 expression were found in the untransfected line Ba/κB. β-actin was used as internal control.

Atorvastatin-induced attenuation of NF-κB activation in a time-dependent manner

Next, an experiment was performed to investigate whether the inhibitory effect of atorvastatin would be more prominent at lower doses, *i.e*. 0.1 and 1 μM, if the treatment time was prolonged. Cell lines were stimulated under the same conditions as mentioned above, but the atorvastatin incubation time was prolonged up to 48 hours. Fig. 4 demonstrates that when Ba/MyD88/hTLR4/MD2 cells (black bars) were treated with 0.1 and 1 μM atorvastatin up to 48 hours, the degrees of inhibition, compared to LPS-stimulated cells, were 28.59% (p-value < 0.05) and 35.07% (p-value < 0.05), respectively. However, under prolonged treatment with 10 μM, the inhibition of NF-κB was only slightly increased (from 42.18% to 51.67%). These data show that atorvastatin can attenuate NF-κB activation in LPS-stimulated pro-B cells expressing TLR4 in a time dependent manner.

DISCUSSION

The beneficial effects of statins for cardiovascular diseases particularly atherosclerosis as well as the underlying mechanism of these effects, *i.e*. the reduction of blood cholesterol, are well documented.[19,20] Apart from the reduction of cholesterol levels, statins have also been shown to have other independent pleiotropic effects.[10-12] Possible mechanisms by which statins reduce the risk of CVD independent of their lipid regulation include antithrombotic and anti-inflammatory effects[11,12,21] plaque stabilization,[16,22] and endothelial wall relaxation

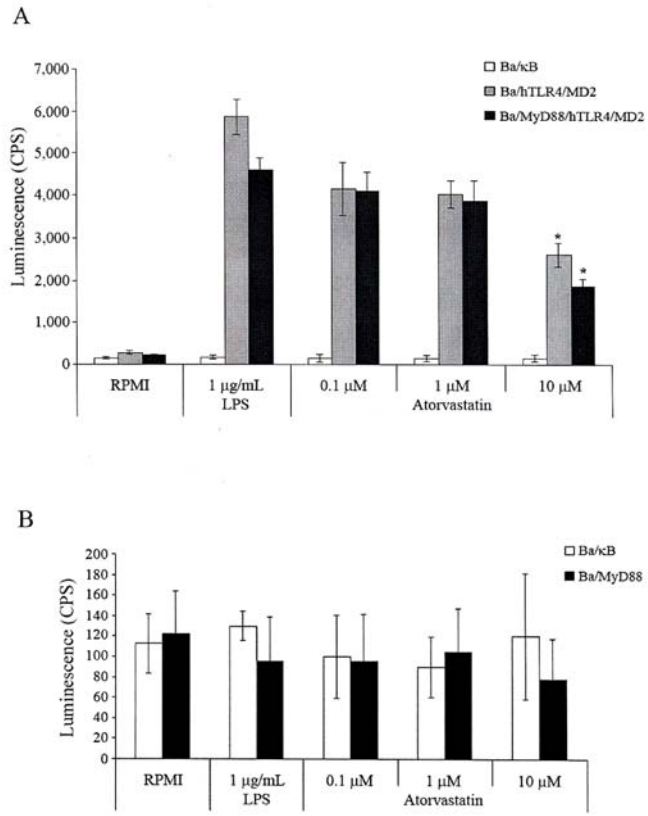

Fig. 3 Concentration- dependent inhibitory effect of NF-κB activation by atorvastatin. LPS-stimulated cells were treated with various concentrations of atorvastatin (ATOR) and incubated for 24 hours. At the indicated time points, the NF-κB luciferase activities were measured. (A) At 24 hours, there was a significant reduction (p-value < 0.05) in the NF-κB activation by atorvastatin (10 μM), however this only occurred in lines that expressed TLR4. (B) In line Ba/MyD88, no activation of NF-κB was observed under any conditions. The activation of luciferase activities was comparable to that of the untransfected cell line Ba/κB. The data are expressed as mean ± SD of four data sets from independent experiments.

resulting in lower intra-arterial pressure and increased blood flow.[23-25]

Both innate and adaptive immunity have been implicated in the role of atherogenesis. TLR4, a member of the PRRs, was of particular interest here in this study. Lipopolysaccharide (LPS)[26] is an exogenous ligand for TLR4 and it has been found that TLR4 deficient mice are hyporesponsive to LPS.[27] Upon activation of TLR4 by LPS, the signaling can be either MyD88 independent or dependent and both pathways can result in the activation of the downstream transcriptional factor, NF-κB.[26] The importance of TLR4 in the pathogenesis of atherosclerosis has been stated by Xu and his colleagues[6] who found that TLR4 was expressed in human atherosclerotic plaques and up-regulated by oxLDL whereas TLR2 was not up-regulated by oxLDL. Moreover, a functional association between TLR4 and atherosclerosis has been shown in apolipoprotein E knockout mice and in humans.[28,29] Taken together, this evidence suggests an important role of TLR4 in atherosclerosis. To date no studies have pointed out significant roles for other TLRs.

Because of earlier reports suggesting that statins can lead to a reduction in pro-inflammatory cytokines which is a subsequent result of NF-κB activation,[12,15-17, 21,30] *in vitro* effects of atorvastatin on LPS-stimulated cells were investigated. We found that only in LPS-stimulated cells expressing TLR4, inhibition of NF-κB activities by atorvastatin could

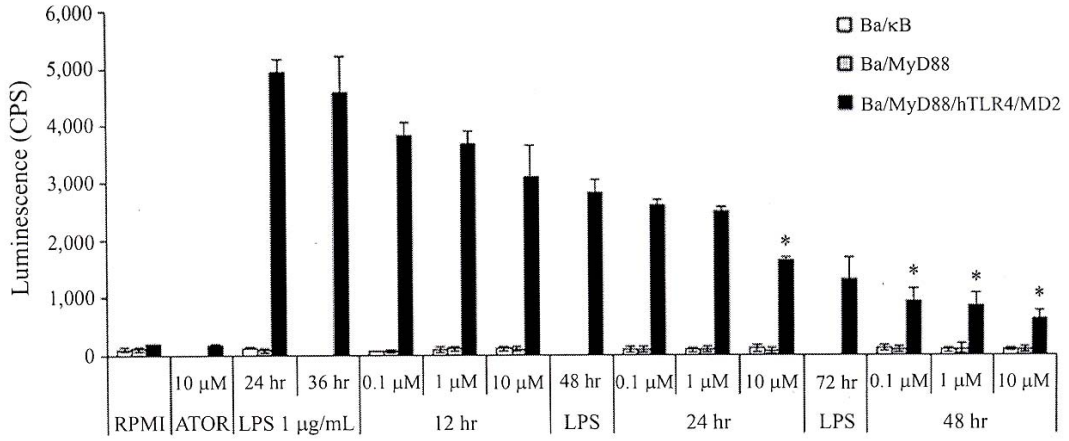

Fig. 4 Time-dependent inhibitory effect of NF-κB activation by atorvastatin. LPS-stimulated cells were treated with various concentrations of atorvastatin (ATOR) and incubated up to 48 hours. At the indicated time points, the NF-κB luciferase activities were measured. At 48 hours, atorvastatin significantly inhibited NF-κB activation at lower doses, 0.1 and 1 μM (p-value < 0.05). The data are expressed as mean ± SD of four data sets from independent experiments.

be observed. This suggests that TLR4 is important in the atorvastatin-mediated NF-κB effect. An examination of the effects of various concentrations of atorvastatin on LPS-stimulated cells as well as of different lengths of treatment revealed that atorvastatin inhibited NF-κB activities in a dose- and time-dependent fashion. However, at lower doses, *i.e.* 0.1 and 1 μM, treatment time had to be prolonged for a significant inhibitory effect to be seen. This is in concordance with studies conducted by Yilmaz and his colleagues [15,17] that showed a significant effect of various concentrations and time of treatment on the outcome of dendritic cell phenotypes. It is important to note that the concentrations used in these *in vitro* conditions are in micromolar (μM) ranges which are not comparable to *in vivo* situations where plasma concentrations are in nanomolar (nM) ranges. One of the possible explanations suggested for this discrepancy is that the hypocholesterolemic activity *in vivo* might not be adequately mimicked enough in *in vitro* situations.[13] Despite this, the observed effects can not be disregarded but a very careful extrapolation of data from *in vitro* to *in vivo* must be considered as well as perhaps a reduction in the concentrations used in *in vitro* experiments in future investigations.

Cellular responses to LPS occur *via* an interaction between LPS and LPS-binding protein (LBP) and CD14, which subsequently leads to TLR4 signaling in both MyD88-dependent and -independent manners.[31,32] Subsequently, in the present study it was also demonstrated that atorvastatin inhibits NF-κB responses in MyD88/hTLR4/MD2 transfectants, indicating that the anti-inflammatory effect of atorvastatin is in a MyD88 dependent fashion.

In summary, the novel findings in this study suggest that atorvastatin has an anti-inflammatory effect *via* TLR4 which is dose and time dependent and in a MyD88 dependent fashion. The present study also differed from other studies in that TLR4 transfectants were used while other studies have used antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages or monocytes and thus a range of PRRs including TLR4 were expressed. The use of transfected cell lines in this study excluded the involvement of other PRRs. During the progression of atherosclerosis and the ongoing inflammatory response, the administration of statins may benefit patients not only by reducing blood cholesterol, but also by providing anti-inflammatory effects resulting in plaque stabilization. This reduces the risk of cardiovascular disease development as a consequence of plaque ruptures. In addition, this novel finding may also be applied to other autoimmune or inflammatory diseases whose pathogenesis stem from inflammation.

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