Objective—The apolipoprotein A-I (apoA-I) mimetic peptide 4F favors the differentiation of human monocytes to an anti-inflammatory phenotype and attenuates lipopolysaccharide (LPS)-induced inflammatory responses. We investigated the effects of LPS on the Toll-like receptor (TLR) signaling pathway in 4F-differentiated monocyte-derived macrophages.

Methods and Results—Monocyte-derived macrophages were pretreated with 4F or vehicle for 7 days. 4F downregulated cell-surface TLRs (4, 5, and 6) as determined by flow cytometry. 4F attenuated the LPS-dependent upregulation of genes encoding TLR1, 2, and 6 and genes of the MyD88-dependent (CD14, MyD88, TRAF6, interleukin-1 receptor–associated kinase 4, and inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta) and MyD88-independent (interferon regulatory factor 3, TANK-binding kinase 1, and Toll-interleukin 1 receptor domain–containing adaptor-inducing interferon-β) pathways as determined by microarray analysis and quantitative reverse transcriptase polymerase chain reaction. Functional analyses of monocyte-derived macrophages showed that 4F reduced LPS-dependent TLR4 recycling, phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, activation and translocation of nuclear factor-κB and inhibited the secretion of tumor necrosis factor-α and interleukin-6 induced by LPS or lipoteichoic acid. These changes were associated with depletion of cellular cholesterol and caveolin, components of membrane lipid rafts.

Conclusion—These data suggest that disruption of rafts by 4F alters the assembly of TLR–ligand complexes in cell membranes and inhibits proinflammatory gene expression in monocyte-derived macrophages, thus attenuating the responsiveness of macrophages to LPS. (Arterioscler Thromb Vasc Biol. 2012;32:2631–2639.)

Key Words: apolipoprotein mimetic peptide • inflammation • macrophage • microarray analysis • Toll-like receptor signaling

Reduced plasma levels of high-density lipoprotein (HDL) cholesterol are associated with cardiovascular disease and serve as an independent predictor of coronary risk. HDL possesses anti-inflammatory and antioxidant properties that have been ascribed to apolipoprotein A-I (apoA-I), the major protein component of HDL. The ability of apoA-I to protect against atherogenic lesion formation has been studied extensively in animal models and humans. Strategies to manipulate HDL levels and its functional properties have recently included the use of apoA-I mimetic peptides. A family of these 18-residue, amphipathic peptides has been developed in our laboratory. These peptides bear no sequence homology to apoA-I but mimic many of its physiological effects. Among this family of apoA-I mimetics, the peptide 4F has been extensively studied.

Anti-inflammatory, antioxidant, and antiatherogenic properties of 4F have been reported under in vivo and in vitro conditions. Beneficial effects of 4F have been ascribed to its ability to improve HDL function, as reflected by the conversion of HDL from a proinflammatory to an anti-inflammatory particle. Although both apoA-I and 4F have been shown to inhibit atherosclerosis, a major difference in the ability of each molecule to associate with oxidized lipids has been demonstrated. The higher affinity binding of 4F to oxidized lipids could thus explain its efficacy in reducing vascular injury associated with the accumulation of reactive lipid metabolites. Our previous data suggest that the peptide also exerts direct protective effects at the cellular level, thus altering the response to inflammatory stimuli. Both apoA-I and 4F mediate cholesterol efflux from macrophages and alter the composition and function of lipid rafts. Cell-surface receptors and associated signaling molecules reside in rafts and require the scaffolding provided by these structures for their function. By virtue of their ability to mediate cholesterol efflux, apoA-I and 4F may thus regulate cellular activation processes and downstream signaling cascades.

Macrophages are a versatile and heterogeneous group of cells that vary in phenotype and function. They play a key role in inflammation, immunity, and lipid metabolism via the...
production of inflammatory mediators and cytokines. We previously reported that apoA-I and 4F alter macrophage phenotype by promoting the differentiation of monocytes to an alternatively activated M2 macrophage phenotype. Previous studies show that M2 activation involves the induction of a new gene program in macrophages. The present study was, therefore, undertaken to determine whether 4F induces genotypic changes that alter the responsiveness of these cells to proinflammatory stimuli. Our data show that 4F reduces the expression of genes that encode Toll-like receptor (TLR) family members and associated adaptor proteins. TLRs are a class of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), highly conserved motifs present in bacteria, viruses, fungi, and protozoans and initiate signals that activate innate immune responses. Ten TLR isoforms have been identified in human monocytes/macrophages. TLR1, 2, 4, 5, and 6 are present on the cell surface and recognize bacterial cell-surface PAMPs such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS). TLR3, 8, and 9 are localized intracellularly and respond to nuclear components (DNA and RNA) of bacteria and viruses. Herein, we show that the stimulation of TLR signaling genes by LPS is significantly attenuated in monocyte-derived macrophages (MDMs) previously treated with 4F. These changes were associated with a reduction in TLR4 recycling, nuclear factor-κB (NF-κB) activation/translocation, cytokine synthesis/secretion, and cellular cholesterol content. It is proposed that 4F-mediated depletion of membrane cholesterol alters the TLR signaling pathway and confers cytoprotective responses of the peptide in a wide spectrum of inflammatory diseases.

Materials and Methods

Additional details are given in the online-only Data Supplement.

Cell Culture Reagents

Cell culture medium RPMI1640 was obtained from American Tissue Culture Collection (USA). All other cell culture materials were obtained from Cellgro. Fetal bovine serum and Ficoll (Fico/Lite LymphoH) were purchased from Sigma Chemical Co and Atlanta Biologicals (USA), respectively. Conjugated antibodies were obtained from BD Biochemicals (CA) and eBiosciences (CA). Details are mentioned in appropriate sections. LPS (Salmonella abortus equi) was from Alexis, USA.

Peptide Synthesis

The apoA-I mimetic 4F, an 18-residue class A amphipathic helical peptide with the sequence Ac-DWFKAFYDKVAΕKFKEAF-NH₂, was synthesized by the solid-phase peptide synthesis method.

Monocyte Cell Culture

Human subject protocols were approved by the Institutional Review Board of the University of Alabama at Birmingham. Monocytes were isolated from buffy coats (Research Blood Components, MA) obtained from healthy blood donors by Ficoll gradient and adherence as described by Smythies et al. 4F (50 μg/mL) or an equivalent volume of saline vehicle was added to the resulting MDMs on days 1 and 3. Cells were harvested on day 7.

Flow Cytometry

MDMs (2 x 10⁶) were stained with conjugated monoclonal antibodies to various TLRs or control monoclonal antibodies of the same isotype and fluorochrome. Protein expression was monitored by flow cytometry using fluorescence minus one and our established techniques. Data were evaluated by CellQuest software (BD Biosciences, CA).

Microarray

RNA was isolated from MDMs, and its purity was assessed by gel electrophoresis (Agilent 2100 Bioanalyzer). Transcriptional profiling was carried out using the Affymetrix Human Gene ST 1.0 Array in the University of Alabama at Birmingham Heflin Center for Genomic Science using standard methods (Affymetrix GeneChip Expression Technical Manual).

Microarray Data Analysis

Data were analyzed in GeneSpring GX using 1-way ANOVA and the multiple testing correction method of Benjamini–Hochberg. A fold change cutoff of ≥ ±2 was used to generate downstream data sets. To control for the occurrence of false discoveries in the data sets, a corrected P value (q-value) ≤0.05 was calculated. Data sets have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus repository (GEO submission No. GSE36933).

Ingenuity Pathway Analysis

The above data set was analyzed using Ingenuity Pathways Analysis software (version 9; Ingenuity Systems, www.ingenuity.com). Right-tailed Fisher exact test was used to calculate a P value determining the probability that each biological function assigned to that data set is attributable to chance alone.

Quantitation of mRNA and Protein

Real-time polymerase chain reaction and Western Blotting were performed using standard techniques described in the online-only Data Supplement.

NF-κB Activation

1xBt and p-1xBt were quantified by immunoblotting. NF-κB activation was measured as described previously. Phosphorylation of the NF-κBp65 subunit was measured by flow cytometry, whereas translocation of the NF-κBp50 subunit was quantified by ELISA using the TransAM NF-κB ELISA kit (Active Motif, CA).

Cytokine Measurements

LPS-mediated secretion of cytokines was measured by ELISA (BD Biosciences).

Cholesterol Efflux

Peptide-mediated cholesterol efflux was measured in MDMs, as previously described.

Lipid Rafts

Cellular cholesterol was measured using an Amplex Red cholesterol assay kit (Molecular Probes, Eugene, OR). Caveolin-1, a protein component of lipid rafts, was measured by immunoblotting using a rabbit monoclonal antibody (Cell Signaling, MA).

Results

We previously reported that apoA-I and 4F favor the differentiation of human monocytes to an anti-inflammatory M2 phenotype that is resistant to activation by LPS. Importantly, the apoA-I–and 4F-induced macrophage differentiation was accompanied by a significant reduction in the expression of CD14 and TLR4. It was proposed that downregulation of these LPS receptors may be the result of the disruption of lipid rafts by 4F because this peptide effluxes cholesterol from membranes and both CD14 and TLR4 are
known to associate with lipid rafts. In addition to TLR4, other TLRs expressed in the plasma membrane (including TLR1, 2, 4, 5, and 6) and CD14 are known to be recruited as monomers or heterodimers to lipid rafts upon activation. To determine whether the disruption of lipid rafts by 4F influences the expression of TLR1, 2, 4, 5, and 6, flow cytometry experiments were performed in MDMs that were pretreated with vehicle or 4F (Figure 1). Mean fluorescence intensity, as well as the percentage of cells expressing these proteins, was measured using fluorescence minus one analysis. A significant decrease in TLR 4 and 5 protein expression was observed in MDMs treated with 4F compared with vehicle alone in terms of both the percentage of cells expressing each marker and the mean fluorescence intensity for individual cells (Figure 1). A decrease in the expression of TLR1 and TLR2 was also noted but did not reach significance. Percentage of cells expressing TLR 6 expression was virtually undetectable in 4F-treated MDMs (Figure 1C). However, the mean fluorescence intensity for TLR 6 was similar in both vehicle- and 4F-treated cells (Figure 1B).

A Global View of the Effects of LPS on Gene Transcription in MDMs Pretreated With 4F

To better understand the anti-inflammatory mechanism(s) of 4F action at the molecular level, we examined the effects of the peptide on LPS-induced gene expression in MDMs using microarray analysis. Primary human monocytes were treated with 4F or vehicle for 7 days. After aspiration of the medium, cells from both treatment groups were treated with LPS (1 µg/mL) or vehicle for an additional 18 hours. RNA was extracted and subjected to microarray analysis. A total of 28 869 genes were examined and analyzed using GeneSpring software. The probe set was filtered on the basis of signal intensity values and by satisfying the upper and lower percentile cutoffs 20% to 100%, which yielded 23 233 genes that underwent further analyses. LPS significantly altered the expression levels of 5480 genes (P<0.05, q<0.05; ±1.5-fold cutoff) compared with vehicle-treated cells. Transcripts were grouped on the basis of their connectivity and from information contained in the Ingenuity Knowledge Base. Analysis of the top 5 networks using Ingenuity Pathways Analysis revealed the differential expression of genes regulating the following biological functions and disease categories: inflammatory response, antimicrobial response, cell morphology, lipid metabolism, and cell-to-cell signaling and interactions.

4F Modulates Genes of the TLR Pathway

Pretreatment of MDMs with 4F, followed by the addition of LPS, resulted in the upregulation of 2060 and downregulation of 3420 genes. Genes that were significantly (P<0.05, q<0.05) altered in the various treatment groups were analyzed. Prominent effects of the peptide on genes in the inflammatory response and disease category were noted. Specifically, the transcription of genes encoding TLRs, adaptor proteins, and downstream signaling intermediates was significantly reduced (Table). Components of these signaling pathways have been described in the review by Kawai et al and the references therein. Briefly, TLRs recognize and bind their respective ligands that may be present as heterodimers (eg, TLR1 and TLR2) or complexed to other adaptor molecules such as seen in TLR4–MD2–CD14 complex. Ligand binding leads to the recruitment of other adaptor molecules, including Toll-interleukin 1 receptor adaptor protein and myeloid differentiation primary response gene (MyD88). Recruitment of additional adaptor proteins and enzymes, including interleukin-1 receptor–associated kinase and IκB kinase, leads to the activation of transcription factors NF-κB and activator protein-1. These initiate the transcription and synthesis of cytokines and chemokines.

Effects of LPS on the expression of genes in TLR signaling pathways of 4F- and vehicle-treated MDMs are depicted in Figure 2. Expression values for each mRNA were background adjusted and normalized against control (vehicle treatment) using GeneSpring software. Addition of LPS to vehicle-treated MDMs resulted in the upregulation of TLR1, 2, and 6, whereas TLR5 was downregulated (Figure 2A). Gene expression for TLR4 was similar in MDMs treated with LPS and vehicle. 4F treatment of MDMs before the addition of LPS attenuated the effects of LPS on TLR gene expression. Under these conditions, expression levels of TLR1, 2, 4, 5, and 6 were all significantly reduced.

Recognition of PAMPs by TLRs generates a signal that is transmitted via adaptor molecules and kinases to the nucleus, thus initiating an inflammatory response. Effects of 4F on the LPS-mediated transcription of adaptor molecule genes were
assessed. LPS upregulated all the adaptor molecules, except Jun, compared with MDMs pretreated with vehicle alone (Figure 2B). In contrast, a significant downregulation of all adaptor molecules was observed when LPS was added to cells that were pretreated with 4F.

Activation of TLR-dependent signaling pathways results in the phosphorylation and activation of multiple cellular intermediates. Accordingly, we analyzed effects of 4F and LPS on the gene expression for kinases that are known to participate in inflammatory signaling pathways. Addition of LPS to vehicle-treated MDMs resulted in the upregulation of multiple kinases compared with vehicle controls (Figure 2C). In contrast, 4F pretreatment significantly attenuated the LPS-induced expression of these signaling intermediates compared with vehicle pretreatment.

| Table. Effects of 4F on Gene Expression in LPS-Treated MDMs |
|--------------------------|---------------------------------|-----------------|-----------------|
| Type(s)                  | Symbol | Entrez Gene ID | Entrez Gene Name | Fold Change | P Value | q Value |
| Enzyme                   | TRAF6  | 7189           | TNF receptor–associated factor 6 | −1.634   | 1.00E-03 | 0.002   |
| Kinase                   | IKKα   | 1147           | Conserved helix-loop-helix ubiquitous kinase | −1.824 | 1.12E-06 | 0       |
|                         | IKKβ   | 3551           | Inhibitor of κ light polypeptide gene enhancer in B cells, kinase β | −1.505 | 1.06E-06 | 0       |
|                         | IKKγ   | 8517           | Inhibitor of κ light polypeptide gene enhancer in B cells, kinase γ | −1.364 | 1.26E-04 | 0       |
|                         | IRAK2  | 3656           | Interleukin-1 receptor–associated kinase 2 | −2.565 | 9.41E-10 | 0       |
|                         | IRAK4  | 51135          | Interleukin-1 receptor–associated kinase 4 | −1.978 | 2.08E-05 | 0       |
|                         | JNK1   | 5599           | Mitogen-activated protein kinase 8 | −1.808 | 7.50E-05 | 0       |
|                         | MEKK1  | 4214           | Mitogen-activated protein kinase kinase 1 | −1.543 | 2.94E-04 | 0.001   |
|                         | MKK4   | 6416           | Mitogen-activated protein kinase kinase 4 | −1.692 | 1.42E-03 | 0.003   |
|                         | PKR    | 5610           | Eukaryotic translation initiation factor 2-α kinase 2 | −1.905 | 1.54E-13 | 0       |
|                         | TAK1   | 6885           | Mitogen-activated protein kinase kinase 7 | −1.484 | 1.29E-04 | 0       |
| Other                    | IkBα   | 4792           | Nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor, α | −1.716 | 4.63E-07 | 0       |
|                         | MD-2   | 23643          | Lymphocyte antigen 96 | −1.583 | 6.03E-06 | 0       |
|                         | MYD88  | 4615           | Myeloid differentiation primary response gene (88) | −1.359 | 3.74E-06 | 0       |
|                         | TAB2   | 23118          | TGF-β activated kinase 1/MAP3K7 binding protein 2 | −1.998 | 5.14E-06 | 0       |
|                         | TIRAP  | 114609         | Toll-interleukin 1 receptor (TIR) domain containing adaptor protein | −1.240 | 3.51E-04 | 0.001   |
| Transcription regulator  | c-Fos  | 2353           | FBJ murine osteosarcoma viral oncogene homolog | 1.060  | 5.38E-06 | 0       |
|                         | c-Jun  | 3725           | Jun proto-oncogene | −1.758 | 1.67E-03 | 0.003   |
|                         | Elk-1  | 2002           | ELK1, member of ETS oncogene family | −1.398 | 1.92E-05 | 0       |
| Trans-membrane receptor  | CD14   | 929            | CD14 molecule | −2.287 | 4.68E-03 | 0.008   |
|                         | TLR1   | 7096           | Toll-like receptor 1 | −2.385 | 2.69E-04 | 0.001   |
|                         | TLR2   | 7097           | Toll-like receptor 2 | −2.979 | 6.79E-05 | 0       |
|                         | TLR3   | 7098           | Toll-like receptor 3 | −1.643 | 1.89E-08 | 0       |
|                         | TLR4   | 7099           | Toll-like receptor 4 | −1.443 | 5.68E-03 | 0.009   |
|                         | TLR5   | 7100           | Toll-like receptor 5 | 1.208  | 9.13E-05 | 0       |
|                         | TLR6   | 10333          | Toll-like receptor 6 | −1.626 | 1.14E-03 | 0.003   |
|                         | TLR8   | 51311          | Toll-like receptor 8 | −2.036 | 1.39E-08 | 0       |
|                         | TLR9   | 54106          | Toll-like receptor 9 | 1.200  | 6.55E-03 | 0.01    |
|                         | TLR10  | 81793          | Toll-like receptor 10 | 1.081 | 6.66E-07 | 0       |
| Transporter             | LBP    | 3929           | LPS-binding protein | 1.069  | 1.08E-02 | 0.016   |

LPS indicates lipopolysaccharide; MDM, monocyte-derived macrophages; IKK, IkB kinase; TNF, tumor necrosis factor; MAP, mitogen-activated protein. Data represent fold changes in gene expression compared with LPS treatment in the absence of 4F.

TLR signaling pathways are activated by both a MyD88-dependent pathway (common to all TLRs, except TLR3) and a MyD88-independent pathway (specific to TLR3 and TLR4). Because LPS is a TLR4 ligand, it activates TLR signaling via both pathways. To determine whether the inhibition of TLR signaling in 4F-treated MDMs was related to changes in the MyD88-independent pathway, we examined the effect of 4F on the expression of several components of this pathway. Specifically, the MyD88-independent genes interferon regulatory factor 3, sterile α and Toll-interleukin 1 receptor motif–containing 1, TANK-binding kinase 1, and Toll-interleukin 1 receptor domain–containing adaptor-inducing interferon-β (TRIF) were analyzed. Expression of interferon regulatory factor 3, TANK-binding kinase 1, and TRIF was...
upregulated by LPS in MDMs compared with vehicle controls (Figure 2D), whereas sterile α and Toll-interleukin 1 receptor motif–containing 1, a negative regulator of TRIF-dependent TLR signaling, was downregulated. On the other hand, LPS treatment downregulated interferon regulatory factor 3, TANK-binding kinase 1, and interferon regulatory factor 3, interferon regulatory factor 3, IKKBKG, inhibitor of nuclear factor kappa-B kinase subunit gamma, MAP, mitogen-activated protein.

The microarray data for key genes in the TLR pathway were validated by quantitative reverse transcriptase polymerase chain reaction (Figure 2E). Fold changes in the mRNA expression of TLR1, TLR2, TLR4, TLR5, TLR6, CD14, MyD88, IKBKB, TRIF, and interferon regulatory factor 3 (normalized to β-actin) were measured in MDMs that were pretreated with 4F or vehicle, followed by addition of LPS (Figure 2). In each case, the decrease observed in the microarray data (Figure 2) was supported by quantitative reverse transcriptase polymerase chain reaction experiments. Western blots for some key gene products confirmed that the decrease in mRNA was associated with corresponding changes in protein levels (Figure I in the online-only Data Supplement).

**4F Impairs the Phosphorylation and Nuclear Translocation of NF-κBp65**

Binding of LPS to TLR4 induces a signaling pathway that involves interleukin 1 receptor adaptor protein, MyD88, interleukin-1 receptor–associated kinase, TRAF6, and IκB kinase. A series of phosphorylation reactions leads to the activation and translocation of NF-κB, which plays a key role in inducing inflammatory genes. NF-κB is a heterodimer of p65 and p50 subunits and is sequestered in the cytoplasm by inhibitor IκB proteins. Phosphorylation of IκBα leads to its degradation through ubiquitination and the concomitant release of NF-κB, which translocates to the nucleus and induces the expression of inflammatory genes. Because our data suggested that 4F downregulated genes encoding adapter molecules and kinases that promote the activation of NF-κB, we monitored effects of the peptide on the LPS-dependent phosphorylation of IκBα. MDMs were pretreated with 4F or vehicle, followed by exposure to LPS (1 μg/mL) for 30 minutes. Cell lysates were subjected to electrophoresis and immunoblotted for IκBα and p-IκBα. A representative blot is shown in Figure 3A. LPS treatment of control MDMs reduced IκBα with a concomitant increase in p-IκBα (p-IκBα/IκBα ratio increased from 0.17 to 0.9). In contrast, IκBα expression was reduced in 4F-treated MDMs compared with vehicle-treated cells (65% reduction), which was not altered by LPS. Furthermore, expression of p-IκBα was significantly reduced in 4F-treated cells (p-IκBα/IκBα ratio increased from 0.07 to 0.1), reflecting stabilization of the NF-κB/IκBα complex.

Several studies have shown that phosphorylation of the p65 subunit of NF-κB enhances the transactivation potential of NF-κB and is required for the optimal induction of NF-κB target genes. Therefore, we also studied the phosphorylation and translocation of NF-κB using flow cytometry and ELISA methods. MDMs that were pretreated with 4F or vehicle were exposed to LPS (1 μg/mL) for 5 minutes, an exposure period associated with optimal phosphorylation of NF-κBp65 in blood monocytes. 13 4F treatment reduced LPS-dependent p-NF-κBp65 formation by 80% compared with MDMs that were pretreated with vehicle (Figure 3B–3D). The reduction in p-NF-κBp65 was seen both as percentage of cells expressing p-NF-κBp65 (Figure 3C) and as mean fluorescence intensity (Figure 3D).

In subsequent studies, we tested effects of 4F on the nuclear translocation of activated NF-κB by measuring nuclear
NF-κBp50 content. Previous studies in blood monocytes showed that this is a time-dependent phenomenon, with maximum translocation observed at 60 minutes. Accordingly, we tested effects of LPS on activated NF-κBp50 translocation in vehicle- and 4F-treated MDMs over this time period. The LPS-induced translocation of NF-κBp50 was significantly reduced in cells that were pretreated with 4F compared with vehicle controls (Figure 3E). These data further confirm the protective effect of 4F in attenuating inflammatory responses through reduced activation of NF-κB.

4F Attenuates Inflammatory Effects of LPS
Because 4F inhibited the activation and nuclear translocation of NF-κB, we next confirmed that this treatment prevented cytokine release in response to LPS (TLR4 ligand) and LTA (TLR2 ligand). Both LPS and LTA induced the secretion of interleukin-6 and tumor necrosis factor-α in conditioned medium. In contrast, 4F pretreatment attenuated the release of cytokines in response to these TLR ligands (Figure 3F and 3G). These data indicate that pretreatment of cells with 4F attenuates their responsiveness to LPS and LTA.

4F Treatment Depletes MDMs of Cholesterol and Caveolin 1
Our earlier results suggested that 4F-mediated effects were related to disruption of lipid rafts. We further substantiated this observation by assessing cellular cholesterol content and rafts by alternate methods. Lipid rafts serve as platforms for many cell-surface receptor complexes and signaling molecules. We and others have shown that 4F efficiently mediates cholesterol efflux from macrophages. Incubation of MDMs with 4F (50 µg/mL) increases the efflux of cholesterol in the medium (Figure 4A) and significantly reduces expression of rafts on MDMs. To extend these findings, changes in cell-associated cholesterol were monitored in MDMs pretreated with 4F or vehicle. As expected, cells pretreated with 4F showed a dramatic reduction in cholesterol content (Figure 4B). Furthermore, cellular content of caveolin-1, a raft protein that plays a key role in cell signaling and cholesterol transport, was significantly reduced in 4F-treated cells (Figure 4C).

In related experiments, we monitored effects of methyl-β-cyclodextrin (MβCD) on LPS-mediated cytokine secretion. MβCD is a cholesterol-depleting agent that is known...
to disrupt lipid rafts. MDMs were treated with MβCD (10 mmol/L) for 1 hour. Preliminary studies showed that higher concentrations of MβCD and prolonged exposure times significantly reduced MDM viability (Figure II in the online-only Data Supplement). MβCD treatment reduced cellular cholesterol content by 40%, consistent with changes in cholesterol obtained with 4F pretreatment (Figure III in the online-only Data Supplement). This response was associated with a reduction in LPS-induced tumor necrosis factor-α and interleukin-6 release from MDMs (Figure IV in the online-only Data Supplement). These data confirm previous reports showing that MβCD inhibits LPS-mediated cytokine secretion by mouse macrophages.24,25 Although the cytotoxicity of MβCD necessitated a shorter exposure period than that for experiments with 4F (1 hour versus 7 days, respectively), these results support our hypothesis that cholesterol depletion and disruption of lipid rafts attenuate proinflammatory responses to LPS. It is proposed that this mechanism is an important component underlying the anti-inflammatory effects of 4F.

4F Impairs TLR4 Recycling in MDMs

After stimulation by LPS, the LPS–CD14–TLR4–MD2 complex is rapidly internalized, along with the cholesterol-rich lipid raft domain to the Golgi, and is recycled to the plasma membrane.26 Because we reported that surface TLR4 and CD14 in MDMs are reduced by 4F,14 we next investigated the effect of 4F on TLR4 recycling. A representative graph showing the effect of LPS on cell-surface TLR4 expression as a function of time is depicted in Figure 5. MDMs (n=3) were treated with LPS for the times indicated in Figure 5, and the cell-surface expression of TLR4 was monitored by flow cytometry. Freshly isolated monocytes rapidly lost surface TLR4 (5–15 minutes) but recover within 1 to 2 hours after LPS stimulation (data not shown). Adherent MDMs exhibit a similar response to LPS (Figure 5). In contrast, MDMs treated with 4F had significantly lower basal levels of TLR4 (Figure 5), consistent with Figure 1 and, importantly, did not regain their surface TLR4 expression within 120 minutes after LPS stimulation. Because CD14, MD2, and lipid rafts play a role in the recycling phenomenon, it is possible that the 4F-mediated decrease in CD14 and MD2 (Figure 2) may prevent the reassembly of the TLR4–ligand complex.

Discussion

The ability of the apoA-I mimic peptide 4F to reduce the expression of proinflammatory mediators is thought to provide the basis for its protective effects in a wide array of disease states.27 We previously reported that 4F reduces inflammation and improves survival in animals with experimental sepsis.28 Sepsis complications arise in response to the release of components of Gram-negative and Gram-positive bacteria in circulation. LPS is a component of the outer membrane of Gram-negative bacteria that induces gene expression29 and activates an inflammatory signaling cascade via binding to TLR4.19 Similarly, LTA is released from the membrane of Gram-positive bacteria and engages TLR2 receptors. TLRs are pattern recognition receptors that induce immune responses by recognizing PAMPs. TLR binding induces signaling cascades that transmit the PAMP recognition signal from the cell membrane to the nucleus. Although TLR2 and TLR4 are activated by different ligands, their signaling pathways converge, leading to the activation of NF-κB.29

In the present study, we show that 4F not only attenuates the expression of TLR1, 2, 4, 5, and 6 in LPS-treated MDMs but also inhibits the expression of important adaptor molecules in the MyD88-dependent and MyD88-independent pathways. MyD88 is one of the well-studied adaptor molecules that plays a crucial role in TLR signaling for all TLRs except TLR3. Studies by Kawai et al showed that responses to TLR2, TLR4, TLR7, and TLR9 ligands were abolished in MyD88 knockout
mice. However, TLR3- and TLR4-mediated signaling via an MyD88-independent pathway has also been observed. Ligand binding to TRIF mediates TLR3-induced regulated and normal T cell expressed and secreted production as well as TLR4-mediated MyD88-independent tumor necrosis factor-α synthesis. Our studies show that pretreatment with 4F significantly downregulated MyD88 in LPS-treated MDMs.

The downregulation of TLR2 and TLR4 and their downstream genes, MyD88, interleukin-1 receptor–associated kinase 4, TRAF6, TRAF3, and mucosa-associated lymphoid tissue lymphoma translocation protein 1, by 4F would be expected to decrease the responsiveness of MDMs to LPS and LTA. Indeed, we found that 4F inhibited the LPS-induced phosphorylation and nuclear translocation of NF-κB subunits and the secretion of interleukin-6 and tumor necrosis factor-α in these cells. This is consistent with our previous observation that 4F administration reduces inflammatory injury in rodent models of sepsis. Because different TLRs are activated by different ligands, our data further suggest that 4F may attenuate the response of macrophages to a variety of PAMPs.

In the present study, monocytes were isolated from blood of healthy donors and cultured under conditions that yield MDMs, as described under Methods section. No information was provided by the vendor regarding age, sex, ethnicity, and potential risk factors of donors, and a phenotypic characterization of freshly isolated monocytes was not performed. The possibility that a given blood sample may favor a monocyte subpopulation represents a potential limitation of the study. To overcome this limitation, experiments were repeated multiple times with cells from different donors. We previously reported that these culture conditions yield MDMs that display characteristics of M1 macrophages. Furthermore, 4F treatment induced an anti-inflammatory phenotype in these monocyte-derived cells. By virtue of its high affinity for lipids, 4F mediates cholesterol and phospholipid efflux from plasma membranes. Depletion of cholesterol disrupts lipid rafts, microdomain microdomains rich in cholesterol, and sphingolipids that serve as platforms for cell-surface signaling complexes. Thus, modulation of lipid rafts may directly alter both cell responsiveness and underlying patterns of gene expression. This is supported by our observation that the 4F-mediated disruption of lipid rafts in MDMs was associated with the differentiation of these cells to an anti-inflammatory M2 phenotype. To elucidate the mechanisms underlying the anti-inflammatory effects of 4F, we now show that 4F-treated MDMs are less responsive to LPS than vehicle-treated cells and that this change was associated with major differences in gene expression. Downregulation of the TLR signaling pathway by 4F may permit a functional host defense response, while attenuating inflammatory injury at the cellular level, as has been recently shown in human intestinal macrophages where inhibition of NF-κB signaling prevents intestinal macrophage release of proinflammatory cytokines but does not inhibit host bactericidal function. Similar to our observation with 4F-treated MDMs, a decrease in the expression of TLR2 and TLR4, downregulation of MyD88, and impairment of NF-κB phosphorylation/translocation were observed in intestinal macrophages compared with blood monocytes.

The role of lipid rafts in cell signaling has been studied by manipulating cellular cholesterol levels by either depletion or loading of cholesterol. A direct association between raft cholesterol levels and cell responsiveness to TLR2 and TLR4 ligands has been demonstrated in primary mouse macrophages. Zhu et al showed that macrophages from macrophage-specific ATP-binding cassette transporter A1 null (Abca1 null) mice accumulate significantly more free cholesterol than those from wild-type mice and were more responsive to TLR2, TLR4, TLR7, and TLR9 ligands. The hyperresponsiveness to LPS noted in this study was ascribed to an increase in the cholesterol content of rafts and the concentration of TLR4 receptors in these structures. In the present study, we show an association among cholesterol efflux, lipid raft disruption, and decreased expression of TLR4, CD14, and MD2. Our data further suggest that raft cholesterol influences TLR4 recycling, because cholesterol depletion by 4F attenuated this response. This is consistent with previous studies demonstrating that raft-disrupting drugs inhibit TLR recycling. Cholesterol depletion thus favors an anti-inflammatory phenotype in macrophages by reducing the assembly of TLR4 in lipid rafts and downregulating TLR4–MyD88–dependent and TLR4–MyD88–independent signaling in these cells.

Inflammation plays a key role in preventing microbial injury. However, unregulated activation of TLR-induced responses may lead to sepsis, atherosclerosis, Alzheimer disease, and certain cancers. Results of the present study show that 4F regulates the recognition of PAMPs by downregulating TLRs and downstream adaptor proteins, thus attenuating inflammatory signaling via the MyD88-dependent and MyD88-independent pathways. Activation of the TLR pathway by LPS is dependent on the colocalization of TLR4 and CD14 in lipid rafts. The formation of this functional complex permits the activation of downstream signaling intermediates that culminate in NF-κB activation and the synthesis of proinflammatory cytokines. Data presented in the present study show that 4F disrupts lipid rafts by effluxing cholesterol and phospholipids. It is proposed that this inhibits the formation of a functional TLR4–CD14 complex, thus preventing the upregulation of LPS-responsive genes and activation of NF-κB signaling. These events are summarized in Figure 6.

Figure 6. Proposed mechanism for 4F-mediated inhibition of the Toll-like receptor (TLR) signaling pathway. Lipopolysaccharide (LPS) induces the recruitment of TLR4 to lipid rafts, which forms a functional complex with CD14 leading to the activation of nuclear factor (NF)-κB. 4F treatment of cells disrupts lipid rafts, thus preventing the formation of TLR4–CD14 complex and activation of cells by LPS. LPS may still bind to phospholipids (PLs) in the membrane, but because of the disruption of the lipid rafts, a functional ligand–receptor complex formation is impaired.
Results of the present study may explain, in part, the protective effect of 4F in multiple disease states associated with acute and chronic inflammation.

Acknowledgements
We thank Hailin Lu for excellent technical assistance.

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Disclosures
G.M.A. is a principal in Bruin Pharma, Inc and holds stock in Colitis Foundation of America (L.E.S.). The other authors have no conflicts to report.

Sources of Funding
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SUPPLEMENT MATERIAL

Regulation of Pattern Recognition Receptors by the Apolipoprotein A-I Mimetic Peptide 4F

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From the Departments of Medicine¹, Physiology and Biophysics², Genetics³, The Heflin Center for Genomic Science⁴, and Biochemistry and Molecular Genetics⁵, University of Alabama at Birmingham, Birmingham, AL
Supplementary Data

Materials and Methods

Cell culture reagents. Cell culture medium RPMI1640 was obtained from American Tissue Culture Collection (ATCC, USA). All other cell culture materials were obtained from Cellgro. Fetal bovine serum (FBS) and Ficoll (Fico/Lite LymphoH) were purchased from Sigma Chemical Co. and Atlanta Biologicals (USA) respectively. Conjugated antibodies were obtained from BD Biochemicals (CA, USA) or eBiosciences (CA, USA). Details are mentioned in appropriate sections. Lipopolysaccharide (LPS) was from Alexis, USA.

Peptide synthesis. The apoA-I mimetic 4F, an 18-residue class A amphipathic helical peptide with the sequence Ac-DWFKAHYDKVAEKFKEAF-NH2, was synthesized by the solid phase peptide synthesis method1. Peptide purity was assessed by mass spectral analysis and analytical high-performance liquid chromatography (HPLC). Peptide concentration was determined using ε280=7,300 M⁻¹cm⁻¹. The peptide was found to be endotoxin free as determined by the Limulus amebocyte lysate assay QCL-1000 kit (Lonza, Inc).

Monocyte cell culture. Human subject protocols were approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB). Blood monocytes used in these studies were isolated from buffy coats obtained from Research Blood Components, Inc (Boston, MA). The collection of blood samples by this vendor conforms with guidelines of the American Association of Blood Banks. Blood was obtained upon informed consent from healthy male and female donors between the ages of 18 and 65. Monocytes were isolated from buffy coats obtained from normal, healthy, donors by Ficoll gradient and adherence2. Adherent MDMs were washed with fresh medium and incubated with complete RPMI 1640 medium containing saline vehicle (V) or 4F (50µg/10⁶ cells) for 7 days. Our previous studies showed that this concentration of the peptide had a maximal effect on monocyte differentiation over the 7 day treatment period2. Medium (with and without 4F) was replenished on day 3. On day 7, cells that
had been pre-treated with vehicle or 4F were washed and analyzed by flow cytometry, as
described below. In other studies, MDMs that were pre-treated with vehicle or 4F were
challenged with LPS (1µg/ml) for an additional 18hrs. In this manner, we tested the resultant
effects of 4F on LPS-induced gene expression.

**Flow Cytometry.** MDMs (2 x 10^6) were stained with phycoerythrin-, allophycocyanin or FITC-
conjugated monoclonal antibodies to TLR1, 2, 4, 5 and 6 (eBiosciences), pNF-κBp65, CD13,
HLA-DR (BD Biosciences) or control monoclonal antibodies of the same isotype and
fluorochrome. Protein expression was established by flow cytometry, using fluorescence minus
one (FMO) and our established techniques^3^. Data were evaluated by CellQuest software (BD
Biosciences) and are expressed as mean fluorescence intensity (MFI) and percentage of cells
expressing each marker.

**MicroArray.** RNA was isolated from MDMs, and its purity was assessed by gel electrophoresis
(Agilent 2100 Bioanalyzer). Transcriptional profiling was carried out using the Affymetrix Human
Gene ST 1.0 Array in the UAB Heflin Center for Genomic Science using standard methods
(Affymetrix GeneChip Expression Technical Manual). The Human Gene ST 1.0 chip consists of
28,869 well-annotated genes with 764,885 distinct probes. Briefly, 300ng of total RNA from
each sample was used to generate double strand cDNA by linear amplification using T7-linked
random primers and reverse transcriptase. Subsequently, cRNA was generated by standard
methods (Affymetrix) followed by ssDNA fragmentation, end label biotinylation and preparation
of hybridization cocktail. The arrays were hybridized overnight at 45°C, and then washed,
stained, and scanned the next day.

**Microarray Data Analysis.** Data acquisition software (Affymetrix GeneChip Command Console
Software) was used to generate a cell intensity (CEL) file from the stored images containing a
single intensity value for each probe cell on the array. The CEL files were imported into
GeneSpring GX 11.5.1 (Agilent Technologies, Santa Clara, CA). Using the RMA16
summarization algorithm, GeneSpring GX summarized the CEL data files. Briefly, expression values for each mRNA were obtained by the Robust Multi-array Analysis (RMA) method of Irizarry. This protocol adjusts for the background on the raw intensity scale, carries out a non-linear quantile normalization of the perfect match values, log transforms the background-adjusted perfect match values, and carries out a robust multi-chip analysis of the quantile normalized log transformed values. Each sample underwent baseline transformation to the mean of the control samples. Entities were filtered based on their signal intensity values by satisfying the upper and lower percentile cutoffs 20-100%. Filtered data was further processed in GeneSpring GX by using a one-way ANOVA and the multiple testing correction method of Benjamini Hochberg. A fold change cut-off greater than ±2 was used to generate downstream datasets. To control for the occurrence of false discoveries in the datasets, a corrected p-value (q-value) d 0.05 was used. The data have been submitted to the NCBI’s Gene Expression Omnibus repository. The GEO submission number is GSE36933.

**Ingenuity Pathway Analysis.** The above data set was analyzed using Ingenuity Pathways Analysis (IPA) software (ver 9: Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)). The data set contained gene identifiers and corresponding expression values and was uploaded into the application. Each identifier was mapped to its corresponding object in Ingenuity’s Knowledge Base. A fold-change cutoff of ±2 was set to identify target molecules whose expression was significantly up- or down-regulated. These Network Eligible molecules were overlaid onto a global molecular network developed from information contained in Ingenuity’s Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. The Functional Analysis identified the biological functions that were most significant to the dataset. Molecules from the dataset that met the ±2 fold-change cutoff and were associated with biological functions in Ingenuity’s Knowledge Base were considered for
analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.

**NF-κB Activation.** NF-κB activation was measured as described previously. Phosphorylation of NF-κB p65: MDMs treated with vehicle or 4F for 7 days, were cultured in the absence or presence of LPS (1µg/ml) for 5 min at 37°C. Cells were then washed with cold PBS, and permeabilized with Perm Buffer III (BD Biosciences), washed and stained with anti-p-NFκBp65-PE or control antibodies. Labeled MDMs were analyzed by flow cytometry using FMO analysis to establish the MFI of p-NFκBp65 expression on MDMs and the percentage of MDMs expressing p-NFκBp65 above the 1% cursor set by isotype controls. Translocation of NF-κB: Nuclear extracts were prepared from 10^7 cells treated with LPS (1µg/ml) for 60 mins at 37°C, using the NE-PER kit (Nuclear and Cytoplasmic Extraction Reagents, Pierce). NF-κB DNA binding was assessed using NF-κB ELISA by measuring the ability of activated NF-κBp50 to bind to an immobilized NF-κB consensus binding site (Active Motif, CA). The bound NF-κBp50 was detected by an antibody specific to activated NF-κBp50 and the signal quantified by addition of a secondary antibody conjugated to horseradish peroxidase.

**Real Time PCR.** Gene array data for the regulation of key genes was validated by qRT-PCR. The primer sequences used are listed in Table I. MDMs pretreated with medium or 4F (50µg/ml) were treated with LPS (1µg/ml) for 18h. Cells were harvested, total RNA was isolated (miRVana kit, Ambion, USA) and cDNA generated from total RNA ( Superscript III First strand kit, Invitrogen, USA). Target genes were amplified using Absolute QPCR SYBR Green Mix (Thermo Scientific, USA). The amplification program consisted of 1 cycle of 95°C for 15 mins (enzyme activation) followed by 40 cycles of [95°C, 15sec (denaturation step); 60°C, 30sec (annealing step); 72°C, 30sec (extension step)]. Data were analyzed by the ∆∆CT method and fold change normalized to β-actin.
**Western Blots.** MDMs were harvested and lysed in lysis buffer. Proteins were separated on a 4-20% SDS gel, transferred to nitrocellulose membranes and incubated with antibodies to TLR1, TLR2, TLR4, TLR5, TLR6 (Imgenex, USA), MyD88, IRF3, IκB-α and p-IκB-α (Santa Cruz Biotechnology, CA) overnight at 4°C. After further washing, the membranes were probed with horse radish peroxidase-conjugated secondary antibodies and bands were detected using chemiluminescence (Immobilon Western, Millipore, USA).

**Cytokine Measurements.** At the end of the 7 day treatment period, cytokine release was measured in MDMs that were pre-treated with 4F or vehicle. Medium was aspirated from MDMs and LPS (1µg/ml) or LTA (1µg/ml) was added for a 6hr treatment period. Conditioned medium was then collected, and IL-6 and TNF-α levels were measured by ELISA (BD Biosciences, CA, USA).

**Cholesterol Efflux.** Peptide-mediated cholesterol efflux was measured in MDMs as described by Kritharides et al. Briefly, MDMs grown in RPMI medium were plated at a density of 1 x 10^6 cells per well in a 6-well plate in RPMI containing 10% FBS. MDMs were loaded with [14C]-cholesterol by incubating with [14C]-cholesterol containing acetylated-LDL (Ac-LDL) for 24h. Equilibrated cholesterol enriched cells were washed with PBS and incubated with 1ml of medium containing 4F (50µg/ml). After 12h, the medium was removed and counted. Cells were lysed, counted and protein content was determined by the method of Lowry. Data are expressed as percent cholesterol effluxed.

**TLR4 Recycling.** MDMs treated with vehicle alone or 4F were stimulated with LPS (1µg/ml) for 0, 5, 15, 30, 60 and 120 min. Surface TLR4 expression was determined by phenotyping with TLR4 antibody (eBiosciences) followed by flow cytometry using FMO analysis.

**Lipid Rafts.** Immunoblotting for caveolin. MDMs, pre-treated with 4F or vehicle for 7days were lysed and proteins separated by SDS electrophoresis and transferred to nitrocellulose membranes. Caveolin-1, a protein component of lipid rafts, was measured by immunoblotting
using a rabbit monoclonal antibody (Cell Signaling, MA, USA) and β-actin (loading control) was measured using a mouse monoclonal antibody (Santa Cruz Biotechnology, USA). The bands were scanned and band intensity analysis was performed using LabWorks software (Lablogics, Inc. V4.6.

**Effect of methyl-β-Cyclodextrin.** The effect of methyl-β-cyclodextrin (MβCD) on cellular cholesterol was measured by treating MDMs obtained by incubating monocytes with RPMI medium containing 10% FBS for 7 days with 10 mM MβCD for 60 mins. This concentration and time of incubation have been shown previously to deplete cells of raft cholesterol.  

**Measurement of Cellular Cholesterol.** Cholesterol content of MDMs exposed to media alone, 4F or MβCD was measured as previously described. Briefly, at the end of the experiment, cell monolayers were washed with PBS and lysed in 0.5 ml of a 1% sodium cholate solution in water supplemented with 10 U/ml DNase. Cholesterol content of the cell lysate was measured fluorimetrically using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) as described by the manufacturer and quantitated using a standard. Cell protein of the cell lysates was measured by Lowry method. Cellular cholesterol content is expressed as micrograms of cholesterol per milligram of protein. Cell viability was measured using Trypan Blue. After addition of Trypan Blue, percent viability was calculated.
### Table I. Primer Sequences Used for Real Time PCR

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<td>TLR1_R</td>
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<td>IKKB_R</td>
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F denotes forward primer and R denotes reverse primer.
**Supplementary Figures**

**Figure I**

<table>
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**Figure I**: Western blots for key proteins of the TLR pathway. A decrease in protein expression in 4F treated cells was observed.
Figure II  Effect of MβCD on MDM viability. MDMs, obtained after treating human monocytes for 7 days with vehicle, were treated with MβCD for 60 mins were 100% viable in concentrations up to 10mM; increasing concentrations of MβCD caused greater cell death.
**Figure III.** Cell-associated cholesterol of MDMs treated with vehicle, 4F or MβCD measured using the Amplex Red kit (Invitrogen).
Figure IV  Treatment of MDMs with MβCD attenuates their response to LPS. Decreased (A) TNF-α and (B) IL-6 secretion by MDMs treated with MβCD on stimulation with LPS (1μg/ml).
References


