

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

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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- κ B 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.¹ 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.^{2,3} 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.⁴ 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.^{5–8}

Anti-inflammatory, antioxidant, and antiatherogenic properties of 4F have been reported under in vivo and in vitro conditions.^{6,7,9} 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.¹⁰ 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.⁸ Both apoA-I and 4F mediate cholesterol efflux from macrophages and alter the composition and function of lipid rafts.⁸ 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

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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.⁸ Previous studies show that M2 activation involves the induction of a new gene program in macrophages.¹¹ 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 protozoans¹² 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.¹² 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- κ B (NF- κ B) 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 in 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-DWFKAFYDKVAEKFKAEF-NH₂, was synthesized by the solid-phase peptide synthesis method.⁵

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.⁸ 4F (50 μ g/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 (2×10^6) 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.¹³ 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 Hefflin 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.¹⁴ A fold change cutoff of $\geq \pm 2$ was used to generate downstream data sets.¹⁵ 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- κ B Activation

I κ B α and p-I κ B α were quantified by immunoblotting. NF- κ B activation was measured as described previously.¹³ Phosphorylation of the NF- κ Bp65 subunit was measured by flow cytometry, whereas translocation of the NF- κ Bp50 subunit was quantified by ELISA using the TransAM NF- κ B 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.⁸

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 TLR4.⁸ 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.¹⁶ 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.^{16,17} 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.¹⁸ 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

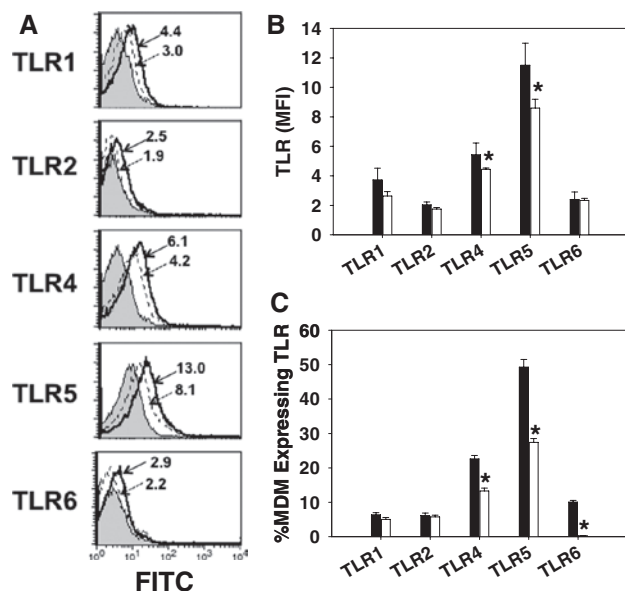


Figure 1. 4F treatment downregulates cell-surface Toll-like receptors (TLRs) in monocyte-derived macrophages (MDMs). Expression of TLRs 1, 2, 4, 5, and 6 was monitored in 4F- and vehicle-treated MDMs by flow cytometry. **A**, Histograms showing surface expression of TLRs in MDMs treated with vehicle (black line) or 4F (dotted line). An isotype control (gray shaded) was run for each sample. The numbers indicate the mean fluorescence intensity (MFI) of each histogram. **B**, 4F-treated cells (white bars) expressed reduced levels of TLRs 4 and 5 per cell as determined by MFI compared with vehicle-treated cells (black bars). **C**, 4F-treated cells (white bars) expressed significantly reduced levels of TLRs 4 to 6 as determined by percentage of cells expressing TLR compared with vehicle-treated cells (black bars). Values are means \pm SEM ($n=3$; $*P < 0.05$ compared with vehicle treatment). FITC indicates fluorescein isothiocyanate.

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	IKK α	1147	Conserved helix-loop-helix ubiquitous kinase	–1.824	1.12E-06	0
	IKK β	3551	Inhibitor of κ light polypeptide gene enhancer in B cells, kinase β	–1.505	1.06E-06	0
	IKK γ	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 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 kinase 7	–1.484	1.29E-04	0
Other	I κ B α	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
	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
Trans-membrane receptor	Elk-1	2002	ELK1, member of ETS oncogene family	–1.398	1.92E-05	0
	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
Transporter	TLR10	81793	Toll-like receptor 10	1.081	6.66E-07	0
	LBP	3929	LPS–binding protein	1.069	1.08E-02	0.016

LPS indicates lipopolysaccharide; MDM, monocyte-derived macrophages; IKK, I κ B 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.

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.

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).¹⁹ 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

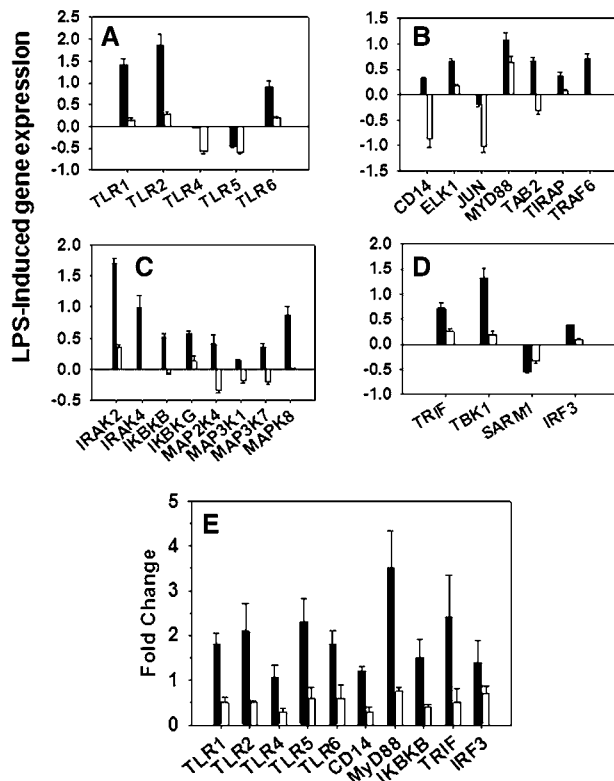


Figure 2. 4F treatment downregulates lipopolysaccharide (LPS)-mediated expression of genes of the MyD88-dependent and MyD88-independent Toll-like receptor (TLR) signaling pathway. Monocyte-derived macrophages (MDMs) were pretreated with 4F (white bars) or vehicle (black bars) for 7 days and then stimulated with LPS (1 μ g/mL) for 18 hours. RNA was extracted, and transcriptional profiling was carried out using the Affymetrix Human Gene ST1.0 array. Absolute mRNA levels obtained were normalized to unstimulated MDMs, pretreated with vehicle. Data are means \pm SEM ($n=4$; $P<0.05$ across all treatments). **A**, Effect of LPS on the expression of surface TLRs; **B**, effect of LPS on adaptor molecules; **C**, effect of LPS on enzymes of the TLR signaling pathway; and **D**, effect of LPS on expression of genes of the MyD88-independent pathway. **E**, mRNA levels of key genes in the TLR pathway in MDMs pretreated with vehicle (black bars) or 4F (white bars) for 7 days and stimulated with LPS (1 μ g/mL) for 18 hours. The data are normalized to β -actin. Values are means \pm SEM ($n=4$). IRAK2 indicates interleukin-1 receptor-associated kinase 2; TRIF, Toll-interleukin 1 receptor domain-containing adaptor-inducing interferon- β ; TBK1, TANK-binding kinase 1; SARM1, sterile α and Toll-interleukin 1 receptor motif-containing 1; IRF3, interferon regulatory factor 3; IKBKG, inhibitor of nuclear factor kappa-B kinase subunit gamma; MAP, mitogen-activated protein.

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 TRIF in 4F-treated MDMs compared with vehicle pretreatment. Furthermore, the reduction in sterile α and Toll-interleukin 1 receptor motif-containing 1 expression elicited by LPS was attenuated in cells pretreated with 4F. These data suggest that 4F may limit inflammatory injury, in part, via inhibition of the TLR signaling pathway by downregulating components of both the MyD88-dependent and MyD88-independent pathways.

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- κ Bp65

Binding of LPS to TLR4 induces a signaling pathway that involves interleukin 1 receptor adaptor protein, MyD88, interleukin-1 receptor-associated kinase, TRAF6, and I κ B kinase. A series of phosphorylation reactions leads to the activation and translocation of NF- κ B, which plays a key role in inducing inflammatory genes. NF- κ B is a heterodimer of p65 and p50 subunits and is sequestered in the cytoplasm by inhibitor I κ B proteins.²⁰ Phosphorylation of I κ B α leads to its degradation through ubiquitination and the concomitant release of NF- κ B, which translocates to the nucleus and induces the expression of inflammatory genes. Because our data suggested that 4F downregulated genes encoding adaptor molecules and kinases that promote the activation of NF- κ B, we monitored effects of the peptide on the LPS-dependent phosphorylation of I κ B α . MDMs were pretreated with 4F or vehicle, followed by exposure to LPS (1 μ g/mL) for 30 minutes. Cell lysates were subjected to electrophoresis and immunoblotted for I κ B α and p-I κ B α . A representative blot is shown in Figure 3A. LPS treatment of control MDMs reduced I κ B α with a concomitant increase in p-I κ B α (p-I κ B α /I κ B α ratio increased from 0.17 to 0.9). In contrast, I κ B α expression was reduced in 4F-treated MDMs compared with vehicle-treated cells (65% reduction), which was not altered by LPS. Furthermore, expression of p-I κ B α was significantly reduced in 4F-treated cells (p-I κ B α /I κ B α ratio increased from 0.07 to 0.1), reflecting stabilization of the NF- κ B/I κ B α complex.

Several studies have shown that phosphorylation of the p65 subunit of NF- κ B enhances the transactivation potential of NF- κ B and is required for the optimal induction of NF- κ B target genes.^{20,21} Therefore, we also studied the phosphorylation and translocation of NF- κ B using flow cytometry and ELISA methods. MDMs that were pretreated with 4F or vehicle were exposed to LPS (1 μ g/mL) for 5 minutes, an exposure period associated with optimal phosphorylation of NF- κ Bp65 in blood monocytes.¹³ 4F treatment reduced LPS-dependent p-NF- κ Bp65 formation by 80% compared with MDMs that were pretreated with vehicle (Figure 3B–3D). The reduction in p-NF- κ Bp65 was seen both as percentage of cells expressing p-NF- κ Bp65 (Figure 3C) and as mean fluorescence intensity (Figure 3D).

In subsequent studies, we tested effects of 4F on the nuclear translocation of activated NF- κ B by measuring nuclear

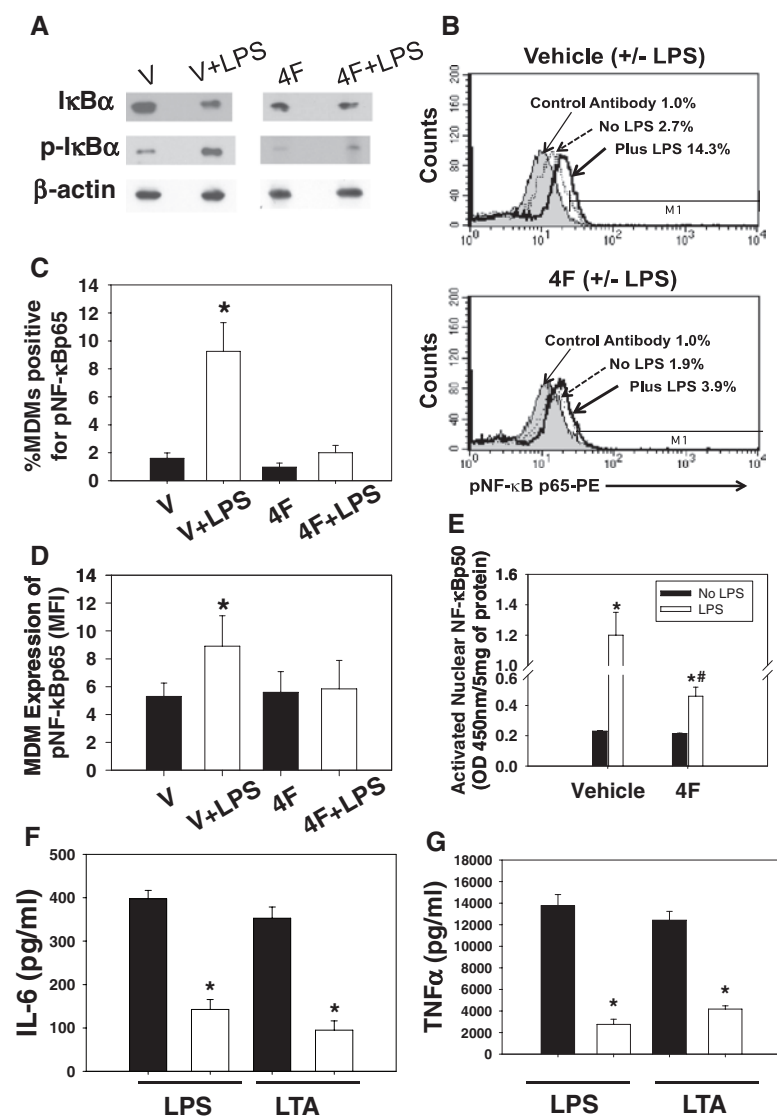


Figure 3. 4F pretreatment decreases the activation of nuclear factor (NF)-κBp65 and nuclear translocation of activated NF-κBp50 in lipopolysaccharide (LPS)-treated monocyte-derived macrophages (MDMs). **A**, Western blots for IκBα and p-IκBα. 4F treatment decreased IκBα and inhibited LPS-stimulated p-IκBα. **B**, Phosphorylation of NF-κBp65 in MDMs treated with LPS (1 μg/mL) for 5 minutes was determined by flow cytometry. Representative histograms show an increase in p-NF-κBp65 on LPS stimulation (black line) compared with resting cells (dotted line) in vehicle-treated (V) MDMs (upper panel). Such an increase was not seen in 4F-treated cells (lower panel). Gray histograms represent cells treated with control antibody. **C**, In the absence of LPS, p-NF-κBp65 was similar in MDMs pretreated with V and 4F. Addition of LPS to V-pretreated cells (V+LPS) revealed a significant (**P*<0.05) increase in p-NF-κBp65 compared with V alone. In contrast, p-NF-κBp65 was not increased by LPS in 4F-pretreated cells (4F+LPS). Results from 5 independent donors are presented. **D**, Data from (B) expressed as mean fluorescence intensity (MFI) of pNF-κBp65. 4F-treated MDMs show significant decrease in MFI compared with V-treated MDMs (*n*=5; **P*<0.05). **E**, Nuclear transport of activated NF-κBp50 was quantified by ELISA in MDMs treated with LPS (1 μg/mL) for 60 minutes. 4F pretreatment significantly decreased nuclear translocation of activated NF-κBp50. Data are means±SEM (*n*=5; **P*<0.05 compared with unstimulated V-pretreated cells, ***P*<0.05 compared with LPS-stimulated V-pretreated cells). **F** and **G**, MDMs pretreated with 4F (white bars) or V (black bars) were treated with LPS (1 μg/mL) or lipoteichoic acid (LTA) (1 μg/mL) for 6 hours. Interleukin (IL)-6 (F) and tumor necrosis factor-α (TNF-α) (G) levels in conditioned media were determined by ELISA. Data are means±SEM (*n*=6; **P*<0.05 compared with respective V controls).

NF-κBp50 content. Previous studies in blood monocytes showed that this is a time-dependent phenomenon, with maximum translocation observed at 60 minutes.¹³ 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 results⁸ 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^{8,22} 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.⁸ 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,²³ 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

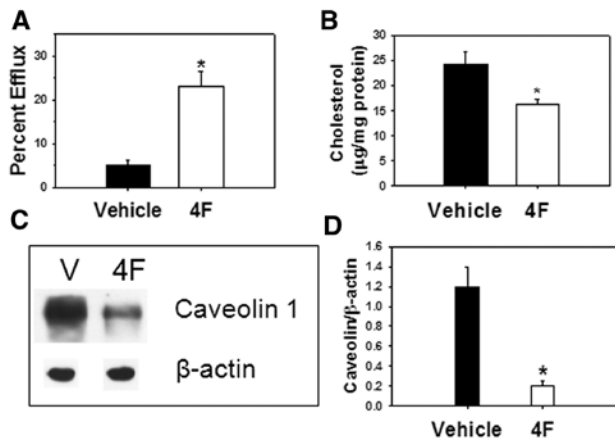


Figure 4. 4F decreases cell-associated cholesterol and caveolin-1, markers of lipid rafts, in monocyte-derived macrophages (MDMs). **A**, 4F increases cholesterol efflux from MDMs. **B**, 4F decreases cell-associated cholesterol. **C**, Immunoblot for caveolin-1 shows decreased caveolin-1 expression in 4F-pretreated MDMs compared with vehicle-treated (V) cells. **D**, Analysis of band intensities revealed a significant reduction in caveolin-1 in 4F-treated MDMs. Data are means \pm SEM (n=4; *P<0.05 compared with V treatment).

to disrupt lipid rafts. MDMs were treated with M β CD (10 mmol/L) for 1 hour. Preliminary studies showed that higher concentrations of M β CD and prolonged exposure times significantly reduced MDM viability (Figure II in the online-only Data Supplement). M β CD 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 M β CD inhibits LPS-mediated cytokine secretion by mouse macrophages.^{24,25} Although the cytotoxicity of M β CD 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,⁸ 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 lose 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),

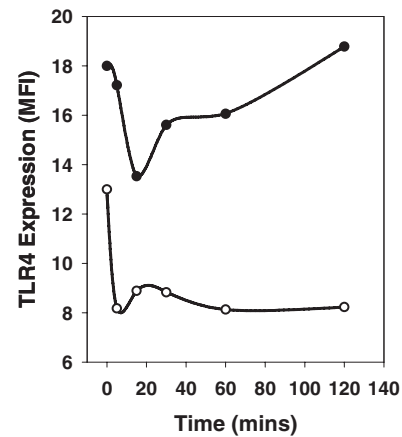


Figure 5. 4F pretreatment impairs lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) 4 recycling. Vehicle (black circles) or 4F-treated (open circles) monocyte-derived macrophages (MDMs) were stimulated with LPS (1 μ g/mL) for 0, 5, 15, 30, 60, and 120 minutes. The mean fluorescence intensity (MFI) for cell-surface TLR4 was determined at each time point. MDMs from 3 different donors were examined. A representative graph of MFI for TLR4 as a function of time is shown. LPS stimulation of vehicle-treated MDMs shows the well-established decrease of surface TLR4 (MFI) at early time points and an increase at later time points indicating TLR4 recycling. 4F treatment, however, not only decreases overall TLR4 (MFI) but also abolishes the cell's capacity to recycle TLR4.

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 mimetic 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- κ B.¹⁹

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.¹⁸ 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.¹⁹ 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- κ B 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.²⁸ Because different TLRs are activated by different ligands,¹⁹ 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.⁸ 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.⁸ Depletion of cholesterol disrupts lipid rafts, membrane microdomains rich in cholesterol, and sphingolipids that serve as platforms for cell-surface signaling complexes.³⁰ 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.⁸ 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- κ B signaling prevents intestinal macrophage release of proinflammatory cytokines but does not inhibit host bactericidal function.¹³ Similar to our observation with 4F-treated MDMs, a decrease in the expression of TLR2 and TLR4, downregulation of MyD88, and impairment of NF- κ B phosphorylation/translocation were observed in intestinal macrophages compared with blood monocytes.¹³

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.^{17,31} Zhu et al¹⁷ showed that macrophages from macrophage-specific ATP-binding cassette transporter A1 null (Abca1^{-M/-M}) 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.¹⁷ 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.³² 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.¹² 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.³³ The formation of this functional complex permits the activation of downstream signaling intermediates that culminate in NF- κ B 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- κ B signaling. These events are summarized in Figure 6.

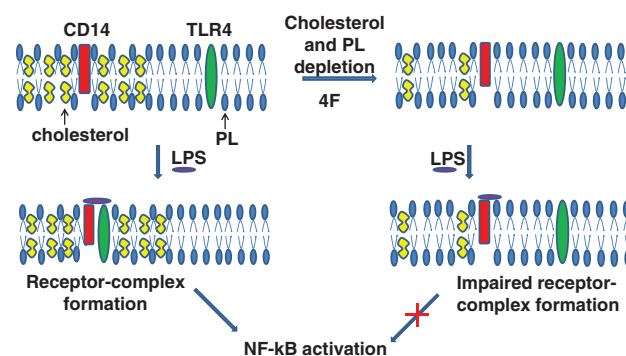


Figure 6. Proposed mechanism for 4F-mediated inhibition of the Toll-like receptor (TLR) signaling pathway. Lipopolysaccharide (LPS) induces the recruitment of TLR4 to lipid rafts, which forms a functional complex with CD14 leading to the activation of nuclear factor (NF)- κ B. 4F treatment of cells disrupts 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.

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Disclosures

G.M.A. is a principal in Bruin Pharma, Inc and holds stock in Lipimetix, LLC. The other authors have no conflicts to report.

References

- Castelli WP. Cholesterol and lipids in the risk of coronary artery disease—the Framingham Heart Study. *Can J Cardiol*. 1988;5A:10A.
- Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM, Lauer MA, Sheldon WS, Grines CL, Halpern S, Crowe T, Blankenship JC, Kerensky R. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA*. 2003;290:2292–2300.
- Moore RE, Navab M, Millar JS, Zimetti F, Hama S, Rothblat GH, Rader DJ. Increased atherosclerosis in mice lacking apolipoprotein A-I attributable to both impaired reverse cholesterol transport and increased inflammation. *Circ Res*. 2005;97:763–771.
- Anantharamaiah GM. Synthetic peptide analogs of apolipoproteins. *Meth Enzymol*. 1986;128:627–647.
- Datta G, Chaddha M, Hama S, Navab M, Fogelman AM, Garber DW, Mishra VK, Epand RM, Epand RF, Lund-Katz S, Phillips MC, Segrest JP, Anantharamaiah GM. Effects of increasing hydrophobicity on the physical-chemical and biological properties of a class A amphipathic helical peptide. *J Lipid Res*. 2001;42:1096–1104.
- White CR, Datta G, Mochon P, Zhang Z, Kelly O, Curcio C, Parks D, Palgunachari M, Handattu S, Gupta H, Garber DW, Anantharamaiah GM. Vasculoprotective effects of apolipoprotein mimetic peptides: an evolving paradigm in hdl therapy. *Vasc Dis Prev*. 2009;6:122–130.
- Navab M, Anantharamaiah GM, Reddy ST, Fogelman AM. Apolipoprotein A-I mimetic peptides and their role in atherosclerosis prevention. *Nat Clin Pract Cardiovasc Med*. 2006;3:540–547.
- Smythies LE, White CR, Maheshwari A, Palgunachari MN, Anantharamaiah GM, Chaddha M, Kurundkar AR, Datta G. Apolipoprotein A-I mimetic 4F alters the function of human monocyte-derived macrophages. *Am J Physiol Cell Physiol*. 2010;298:C1538–C1548.
- Navab M, Yu R, Gharavi N, Huang W, Ezra N, Lotfizadeh A, Anantharamaiah GM, Alipour N, Van Lenten BJ, Reddy ST, Marelli D. High-density lipoprotein: antioxidant and anti-inflammatory properties. *Curr Atheroscler Rep*. 2007;9:244–248.
- Van Lenten BJ, Wagner AC, Jung CL, Ruchala P, Waring AJ, Lehr RI, Watson AD, Hama S, Navab M, Anantharamaiah GM, Fogelman AM. Anti-inflammatory apoA-I-mimetic peptides bind oxidized lipids with much higher affinity than human apoA-I. *J Lipid Res*. 2008;49:2302–2311.
- Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol*. 2006;177:7303–7311.
- Chang ZL. Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflamm Res*. 2010;59:791–808.
- Smythies LE, Shen R, Bimczok D, Novak L, Clements RH, Eckhoff DE, Bouchard P, George MD, Hu WK, Dandekar S, Smith PD. Inflammation energy in human intestinal macrophages is due to Smad-induced IkappaBalpha expression and NF-kappaB inactivation. *J Biol Chem*. 2010;285:19593–19604.
- Benjamini B, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B*. 1995;57:289–300.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4:249–264.
- Gaus K, Rodriguez M, Ruberu KR, Gelissen I, Sloane TM, Kritharides L, Jessup W. Domain-specific lipid distribution in macrophage plasma membranes. *J Lipid Res*. 2005;46:1526–1538.
- Zhu X, Owen JS, Wilson MD, Li H, Griffiths GL, Thomas MJ, Hiltbold EM, Fessler MB, Parks JS. Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol. *J Lipid Res*. 2010;51:3196–3206.
- Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373–384.
- Takeda K, Akira S. TLR signaling pathways. *Semin Immunol*. 2004;16:3–9.
- Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J Biol Chem*. 1999;274:30353–30356.
- Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem Sci*. 2005;30:43–52.
- Sherman CB, Peterson SJ, Frishman WH. Apolipoprotein A-I mimetic peptides: a potential new therapy for the prevention of atherosclerosis. *Cardiol Rev*. 2010;18:141–147.
- Razani B, Woodman SE, Lisanti MP. Caveolae: from cell biology to animal physiology. *Pharmacol Rev*. 2002;54:431–467.
- Yvan-Charvet L, Welch C, Pagler TA, Ranalletta M, Lamkanfi M, Han S, Ishibashi M, Li R, Wang N, Tall AR. Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. *Circulation*. 2008;118:1837–1847.
- Koseki M, Hirano K, Masuda D, Ikegami C, Tanaka M, Ota A, Sandoval JC, Nakagawa-Toyama Y, Sato SB, Kobayashi T, Shimada Y, Ohno-Iwashita Y, Matsuura F, Shimomura I, Yamashita S. Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-alpha secretion in Abca1-deficient macrophages. *J Lipid Res*. 2007;48:299–306.
- Latz E, Visintin A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA, Golenbock DT, Espevik T. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J Biol Chem*. 2002;277:47834–47843.
- White CR, Anantharamaiah GM, Datta G. HDL mimetic peptides: novel therapeutic strategies for the treatment of inflammatory vascular disease. In: Tsugikazu Komoda, ed. *The HDL Handbook*. London, UK: Academic Press; 2010.
- Zhang Z, Datta G, Zhang Y, Miller AP, Mochon P, Chen YF, Chatham J, Anantharamaiah GM, White CR. Apolipoprotein A-I mimetic peptide treatment inhibits inflammatory responses and improves survival in septic rats. *Am J Physiol Heart Circ Physiol*. 2009;297:H866–H873.
- Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal*. 2001;13:85–94.
- Fessler MB, Parks JS. Intracellular lipid flux and membrane microdomains as organizing principles in inflammatory cell signaling. *J Immunol*. 2011;187:1529–1535.
- Zhu X, Lee JY, Timmins JM, Brown JM, Boudyguina E, Mulya A, Gebre AK, Willingham MC, Hiltbold EM, Mishra N, Maeda N, Parks JS. Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages. *J Biol Chem*. 2008;283:22930–22941.
- Triantafilou M, Manukyan M, Mackie A, Morath S, Hartung T, Heine H, Triantafilou K. Lipoteichoic acid and toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. *J Biol Chem*. 2004;279:40882–40889.
- Triantafilou M, Gamper FG, Haston RM, Mouratis MA, Morath S, Hartung T, Triantafilou K. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *J Biol Chem*. 2006;281:31002–31011.