Effect of omega-3 fatty acids on toll-like receptor 4-mediated macrophage inflammation and its regulation

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Abstract

Macrophages are a major source of pro-inflammatory factors in the arterial intima and play a central role in the development of atherosclerotic plaque. Macrophages express toll-like receptor 4 (TLR4), a plasma membrane receptor, which when activated triggers the nuclear factor κB (NFκB) and mitogen-activated protein kinase signaling pathways leading to the production of pro-inflammatory cytokines. TLR4 expression and signaling have been positively associated with atherosclerotic lesion formation. Very long-chain polyunsaturated fatty acids, specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have anti-inflammatory effects on macrophages, while saturated fatty acids have pro-inflammatory effects. However, the effect of enriching macrophages with EPA, DHA, or a saturated fatty acid on TLR4 cell surface expression and TLR4-mediated production of pro-inflammatory cytokines is not well characterized. **We hypothesized that the production of pro-inflammatory cytokines would be downregulated in EPA- or DHA-enriched macrophages stimulated with TLR4 ligand, which may be mediated by a reduction in cell surface expression of TLR4 and its associated molecules CD14 and MD2, while enrichment of macrophages with a saturated fatty acid would have the opposite effect.**

The objective of this thesis was to use the murine macrophage cell line, RAW 264.7 to determine the effect of enriching the cell membrane with EPA, DHA, or a saturated fatty acid, myristic acid (MA), on TNFα and IL-6 production, cell surface expression of TLR4, and associated molecules CD14 and MD2 induced by ultra-pure LPS stimulation (a TLR4-specific agonist). The involvement of cAMP response element-binding protein (CREB), prostaglandin E_2 (PGE_2) and nuclear factor κB (NFκB) in mediating the differential effect of DHA on TNFα and IL-6 production were also studied.

EPA- and DHA-enrichment decreased the inflammatory response of RAW 264.7 cells to ultra-pure LPS stimulation relative to control cells: a reduction in TNFα, IL-6 and PGE_2 production, as well as NFκB activity was observed. In contrast, MA-enrichment did not potentiate the effect of ultra-pure LPS relative to control cells. EPA and DHA had a greater inhibitory effect on IL-6 compared to TNFα in both secretion and mRNA expression. This suggests an interference of signaling downstream of TLR4. Focusing on DHA, we found no effect on cell surface expression of TLR4, TLR4-MD2 complex or CD14, or the level of LPS-cell binding. Since NFκB is a major positive regulator of both TNFα and IL-6 gene transcription, we hypothesized that the weaker inhibitory effect of DHA on TNFα compared to IL-6 production may be due to the decrease in PGE_2 production, since PGE_2 has been previously reported to inhibit TNFα (possibly through the activation of CREB), and enhance IL-6 production. Addition of exogenous PGE_2 had a dose-dependent inhibitory effect on TNFα mRNA expression after 3 h of stimulation, but only at concentrations higher than that found to be secreted by our cells. However, inhibiting PGE_2 production by a cyclooxygenase 2 inhibitor also resulted in a small reduction in TNFα mRNA levels after 3 h but not 6 h of stimulation, suggesting that PGE_2 had a minor stimulatory effect (if any) on TNFα production under the conditions evaluated in our system. Neither increasing nor decreasing PGE_2 concentration had any effect on IL-6 mRNA expression. Although these data confirm differential regulation of

TNFα and IL-6 by PGE_2, it does not seem to be likely that a reduced PGE_2 production potentially induced by DHA is a significant contributing factor to the observed weak inhibitory effect of DHA on TNFα production. Since DHA had no significant effect on CREB activity, the involvement of this transcription factor in the DHA-induced inhibition of TNFα and IL-6 was not pursued. The effect of chemically reducing NFκB activity resulted in a larger inhibitory effect on IL-6 compared to TNFα mRNA expression, which is similar to the effect of DHA. These data suggest that the differential effect of DHA on TNFα and IL-6 mRNA expression may be mediated primarily by a reduction in NFκB activity, and that regulatory mechanisms are partially different between the TNFα and IL-6 genes.

The results of this research add to the current understanding of the effect of very-long chain polyunsaturated fatty acids and saturated fatty acids on TLR4 activation and signaling, and address the cytokine-specific effects of EPA and DHA in TLR4-activated macrophages. These data will advance the efforts to develop more specifically defined anti-inflammatory effects of EPA and DHA, which will lead to better understanding of the influence of EPA and DHA on atherosclerotic lesion progression.

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Introduction

1. Statement of Significance

Atherosclerosis is a primary pathogenesis underlying the development of ischaemic heart disease and stroke, the top two causes of death globally [1]. Atherosclerosis is an inflammatory disorder characterized by the accumulation of lipids and the development of fibrotic plaques within the arterial intima of large and medium size blood vessels [2]. Macrophages in the arterial intima contribute to all stages of the disease by accumulating LDL-derived cholesterol and by secreting pro-inflammatory factors [3]. Cytokines including TNFα and IL-6 augment the inflammatory response and enhance processes that promote the development of mature lesions and plaque rupture [4-6].

A key mediator of cytokine production in macrophages is toll-like receptor 4, (TLR4), a plasma membrane receptor that is activated by lipopolysaccharide (LPS), saturated fatty acids, oxLDL and heat shock proteins [7, 8]. Both TLR4 and its ligands have been positively associated with atherosclerosis [9]. Therefore, reducing cardiovascular disease by targeting TLR4 has been an area of increased interest in the research community [10, 11].

Limited evidence suggests that the very-long chain omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), inhibit TLR4 signaling and TLR4-mediated inflammation in macrophages [12-14] while saturated fatty acids activate TLR4 [15]. EPA and DHA, unlike saturated fatty acids, are cardio-protective and

have been reported to prevent cardiovascular events and cardiac death [16]. The inverse relationship between EPA and DHA on the severity of atherosclerotic lesions [17-19] may be partially explained by improvements in plasma lipid profile [20-22] and a general reduction in inflammation, indicated by a decrease in the concentration of plasma inflammatory markers [23-25].

Mechanistic evidence supporting a relationship between macrophage EPA or DHA content and inflammatory status, specifically with respect to TLR4 signaling, is not well established. EPA and DHA have been shown to reduce the pro-inflammatory profile of human and mouse macrophage models after stimulation with LPS *in vitro* [13, 24, 26-32]. This response has been related to the inhibition of the nuclear factor kappa B (NFκB) signaling pathway [13, 26, 27, 31, 32]. NFκB regulates the production of pro-inflammatory genes [33]. However, NFκB activation, commonly stimulated *in vitro* with impure LPS, is not specific for TLR4 activation, but is downstream of most TLRs including TLR2 [34, 35]. EPA and DHA effects have not been demonstrated using ultra-pure LPS, which specifically activates TLR4, and thus induces a unique pro-inflammatory gene expression profile not achieved by using the impure LPS [36]. Only one group to our knowledge have demonstrated that acute treatment (< 3 h) of macrophages with EPA and DHA inhibits TLR4 signaling rather than downstream signaling components that are not specifically related to TLR4 [12, 14]. This effect has not as yet been related to cellular EPA and DHA content. High intakes of EPA and DHA elevate the proportion of these fatty acids in the phospholipids of blood cells including monocytes [37]. Therefore, macrophage cell models in which both culture media and

cellular EPA and DHA are enhanced would better reflect *in vivo* conditions. Results of this study will aid in defining the role of DHA and EPA cell enrichment on TLR4 signaling and TLR4-mediated cytokine production in macrophages.

2. Specific Aims

Dietary and plasma concentrations of EPA and DHA have been negatively associated with cardiovascular events and death [16]. EPA and DHA have been shown to reduce biomarkers of inflammation, including tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) [38-40]. Macrophages are major producers of TNFα and IL-6. These cytokines promote cholesterol retention in the arterial intima and the development of mature and vulnerable plaque [4, 5]. TLR4 as a major mediator of macrophage production of pro-inflammatory cytokines, has the ability to recognize several atherogenic molecules, and is upregulated in plaque [7-9]. TLR4 activation triggers the activation of transcription factor NFκB, a major positive regulator of TNFα and IL-6 gene transcription [33], as well as other signaling molecules including prostaglandin E2 (PGE2) and phosphorylated cAMP response element-binding protein (P-CREB), which have been reported to differentially influence the production of TNFα and IL-6 [41-43].

The object of this thesis research was to use the murine macrophage cell line, RAW 264.7, to characterize the effect of EPA and DHA enrichment of cell membranes on TNFα and IL-6 production, plasma membrane receptors and

signaling pathways influencing the expression of TNFα and IL-6 genes induced by TLR4 ligand.

Specific Aim 1: To determine the effect EPA or DHA enrichment of cell membranes on TNFα and IL-6 secretion in response to ultra-pure LPS, cell surface expression of molecules essential for TLR4 activation including TLR4, CD14 and MD2, and LPS-cell surface binding in RAW 264.7 cells.

Hypothesis 1: EPA and DHA will inhibit TNFα and IL-6 secretion, cell surface expression of TLR4, MD2, and CD14, and LPS-cell surface binding.

Specific Aim 2: To determine the mechanisms responsible for the differential effects of DHA on TNFα and IL-6 secretion in RAW 264.7 cells stimulated with ultra-pure LPS.

Hypothesis 2: DHA enrichment will reduce IL-6 gene expression to a greater extent than TNFα gene expression. DHA will suppress the induction of NFκB activity, a positive regulator of both TNFα and IL-6 gene expression, and will also suppress the induction of PGE_2 and P-CREB, negative regulators of TNFα but not of IL-6 gene expression.

Results from these studies will aid in the understanding of the effect of EPA or DHA cell enrichment on macrophage inflammation as it pertains to TLR4 signaling.

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CHAPTER 2

Literature Review

1. Macrophage inflammation and atherosclerosis

The development of mature atherosclerotic plaque and the risk of clinical outcomes depend not only on elevated circulating levels of LDL combined with low HDL, but also on the inflammatory response of vascular and immune cells to the cholesterol burden [1]. Macrophages play an integral role in augmenting and sustaining the inflammatory response of vascular and immune cells throughout the process of atherosclerotic lesion development [4]. A major mediator of macrophage inflammation is the plasma membrane receptor, toll-like receptor 4 (TLR4) [5]. Activation of TLR4 triggers the nuclear factor κB (NFκB) and mitogen-activated kinase (MAPK) signaling pathways which regulate the transcription of various pro-inflammatory cytokines, chemokines, growth factors and prostaglandins [5-7]. Pro-inflammatory cytokines, particularly, TNFα, IL-1β and IL-6, have been shown to enhance the production of atherogenic molecules by endothelial cells, smooth muscle cells and immune cells that promote key processes including recruitment of immune cells to the arterial intima [8-10], differentiation of monocytes to macrophages [11, 12], foam cell formation [13, 14], smooth muscle cell migration and proliferation [15-17], apoptosis and necrosis of advanced lesions [17-19], and plaque rupture [20-22].

2. Toll-like receptor 4 signaling

TLR4 is a major mediator of macrophage inflammatory response and is implicated in the initiation and progression of atherosclerotic lesions [4-6]. TLR4 is a

pattern recognition receptor (PRR) expressed on the surface of macrophages, endothelial and dendritic cells [23]. Activation of TLR4 triggers NFκB and MAPK pathways which regulate the expression of various pro-inflammatory cytokines including TNFα, IL-1, IL-6 and interferon gamma (IFNγ) [7]. The most well studied TLR4 agonist is lipopolysaccharide (LPS), a major constituent of the outer cell membrane of Gram-negative bacteria. Endogenous TLR4 agonists have also been identified, including saturated fatty acids, oxLDL and heat-shock proteins [24-26]. LPS-TLR4 binding and downstream signaling is depicted in Fig 2. [2, 3]. Briefly, LPS bound to LPS binding protein (LBP) in the serum binds to CD14. LPS-bound CD14 forms a complex with MD2-bound TLR4 which is recruited to lipid rafts [27, 28]. This is followed by dimerization of two MD2-TLR4 complexes. Signaling proteins are recruited to the cytoplasmic portion of the receptor complex, engaging either the MYD88-dependent pathway which triggers NFκB and MAPK pathways or the MYD88-independent, TRIF-dependent pathway, which activates the IFN regulatory factor 3 (IRF3) transcription factor that controls IFN and IFN-inducible genes.

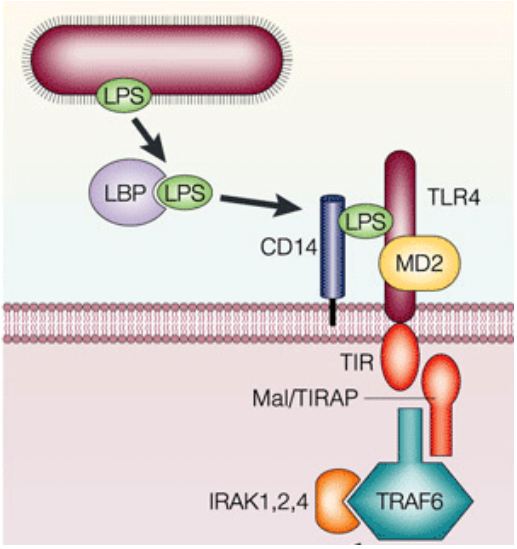
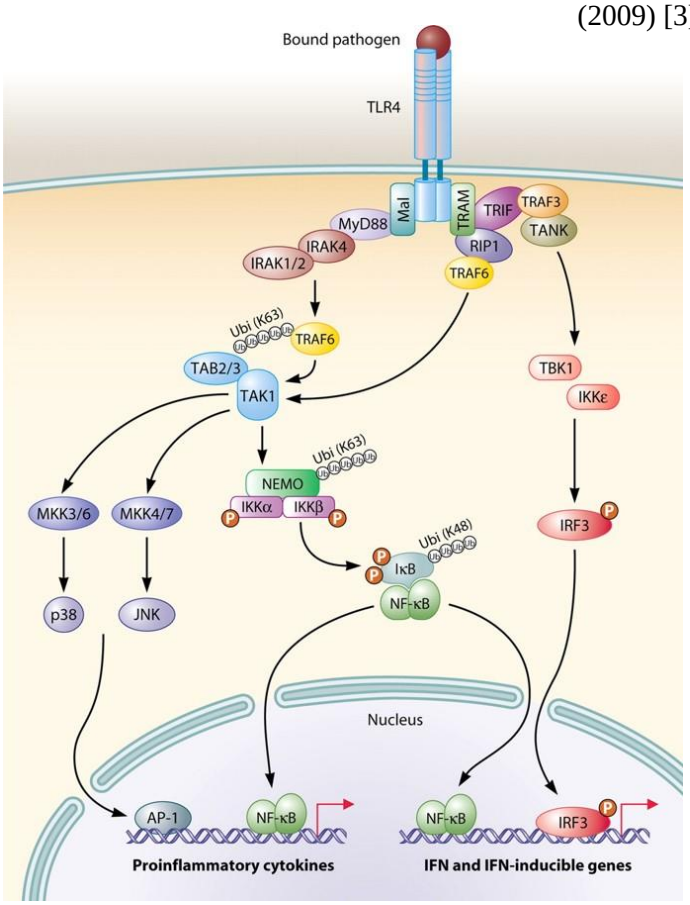


Figure 1. Toll-like Receptor 4 (TLR4) signaling. *Top*: LPS bound to LPS binding protein (LBP) in the serum, binds to plasma membrane CD14, which then forms a complex with TLR4 and MD2. *Bottom*: Bound to LPS, TLR4 molecules homodimerize in lipid rafts, which initiates the recruitment of signaling molecules to the cytoplasmic portion of the dimerized receptors. Two major signaling pathways are activated downstream of TLR4: the MYD88-dependent pathway which triggers NFκB and MAPK pathways and the MYD88-independent, TRIF-dependent pathway, which activates the IFN regulatory factor 3 (IRF3) transcription factor which controls IFN and IFN-inducible genes. *Top:* Adapted from Buer J. and Balling R. (2003) [2]. *Bottom:* Adapted from Mogensen TH (2009) [3].



3. Role of TLR4 in atherosclerotic lesion development

A prominent role of TLR4 signaling in the development of atherosclerotic plaque is supported by both human and animal data. Examination of atherosclerotic plaque of human [29, 30] and apoE-null mice [30] have revealed that TLR4 expression is markedly elevated compared to unaffected human arteries and mice aortic tissue. In atherosclerotic lesions, TLR4 is primarily expressed by macrophages and endothelial cells [29]. However, studies of the relationship between TLR4 polymorphisms (associated with LPS hyporesponsiveness) and atherosclerosis have reported both protective [31] and null effects [32, 33] on lesion development. The role of TLR4 signaling in lesion development is best evidenced by studies of apoE-null mice deficient in TLR4 or MyD88 (an adaptor molecule common to other TLRs, IL-1R and IL-18R). In these studies, deficiency in TLR4 or MyD88 was associated with a significant reduction in aortic plaque size, lipid content and macrophages infiltration [34, 35]. This evidence combined with the role of TLR4 in macrophage inflammation has created a compelling case for TLR4-mediated signaling in atherosclerotic lesions. Hence, the potential for TLR4 as a therapeutic target for reducing cardiovascular disease progression has been a topic of interest within the research community [36-38].

4. EPA and DHA and atherosclerosis

Eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) are very long-chain omega-3 fatty acids that have been reported to decrease the risk of cardiovascular events,

and cardiac death [39]. Beneficial effects on various risk factors for cardiovascular disease include decreased triglyceride and increased high density lipoprotein concentrations [40-42], and improved indicators of cardiovascular and vascular function, including blood pressure, inflammation, hemostasis, resting heart rate and flow-mediated dilation. [42, 43].

An inverse relationship between EPA and DHA intake and atherosclerotic lesion formation is supported by both human and animal data. Erkkilä, *et al* found a negative association between plasma DHA levels and progression of coronary atherosclerosis in women with established coronary artery disease [44]. In another study, serum EPA and DHA was found to be inversely associated with intima media thickness [45]. However, interventional studies have produced varied results. At least two studies in which patients were supplemented with fish oil containing EPA and DHA found no change in diameter of atherosclerotic coronary arteries [46, 47], and at least one study reported no change in intima media thickness of carotid arteries [48]. In contrast, more recent studies have reported protective effects of EPA and DHA supplementation. Diabetic men supplemented with EPA for over two years were reported to have decreased carotid intima media thickness compared to control group [49]. Similarly, Cawood *et al* reported that supplementation with n-3 PUFA ethyl esters (Omacor®) containing EPA and DHA for a median of 21 days reduced the number of foam cells and markers of plaque instability in advanced human plaque [50]. Similarly, mouse models of atherosclerosis fed an EPA and DHA-rich diet have shown to develop smaller lesions in some studies [51-54] while showing no difference in lesion severity in others [55, 56] .

5. Effect of EPA and DHA on inflammation in lesions

EPA and DHA are anti-inflammatory and may slow lesion development by reducing inflammation in the intima. [57, 58]. An association between the proportion of EPA and DHA in the diet and/or plasma and inflammation in lesions has been found in a small number of human and animal studies. These studies have reported the following changes in the markers of inflammation in atherosclerotic lesions due to EPA and/or DHA or fish oil supplementation: a reduction in the number of T-cells [50], macrophages and monocytes [51, 52, 54], an increase in the number of smooth muscle cells [52, 54], and a decrease in the expression of matrix metalloproteinase [50] and endothelial adhesion molecules [53].

6. Effect of EPA and DHA on macrophage inflammation

Macrophages are a primary source of pro-inflammatory factors in the arterial intima [4]. Due to the difficulty in studying lesions *in vivo*, *in vitro* studies using primary and immortalized cells lines have been instrumental in providing the bulk of evidence supporting a role for EPA and DHA in reducing macrophage inflammation in lesions. Peripheral blood mononuclear cells (PBMCs) [59, 60] and elicited peritoneal macrophages [51, 61] isolated from human and mice, respectively, fed an EPA and DHA-rich diet, and human THP-1 cells [62-67] and mouse [68-70] monocyte/macrophage cell lines treated with EPA and DHA *in vitro* have demonstrated an anti-inflammatory effect. Reduction in inflammation in these studies was determined based on a decrease in TNFα,

IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1) secretion, and/or prostaglandin E_2 (PGE_2) secretion in response to LPS. In human intervention trials, the effect of EPA and DHA on outcomes, particularly for inflammation, has been less consistent, and the number of studies that found no difference far outweigh the number of studies that have found an inhibitory effect on pro-inflammatory cytokine production. (reviewed by Sibjen and Calder [59]). In contrast, the majority of studies using macrophage cell lines have reported a reduction in one or more pro-inflammatory factors. Therefore, the data as a whole favors an anti-inflammatory effect of EPA and DHA on macrophages.

7. Effect of EPA and DHA on TLR4 signaling

Since TLR4 is positively associated with atherosclerosis, down-regulation of TLR4 and TLR4-mediated inflammation would provide mechanistic evidence supporting an anti-inflammatory role of EPA and DHA in reducing atherosclerotic lesion development. Limited evidence suggests that EPA and DHA reduce macrophage inflammation via TLR4 activation. Reduction in pro-inflammatory cytokine production in LPS-stimulated macrophage systems, as discussed previously [59, 60, 62-66, 68-70], implies involvement of TLR4. However, impure LPS has been shown to activate both TLR4 and TLR2, and induce a different gene profile than ultra-pure LPS [71, 72]. Whether EPA and DHA suppress TLR2 or TLR4 signaling is an important distinction because the evidence for the role of TLR2 in atherosclerotic lesion formation is relatively weaker than TLR4 [73]. Since prior work did not use ultra-pure LPS, an inhibitory effect

of DHA on TLR4-mediated production of pro-inflammatory cytokines is suggestive but inconclusive.

Few studies have provided direct, molecular evidence for inhibition of TLR4 signaling in macrophages by EPA and DHA. Chu *et al*. reported a decrease in LPS-cell binding and a decrease in CD14 expression in THP-1 monocytes treated with EPA and DHA for 72 h [67]. Similarly, PBMCs isolated from patients with severe trauma and who were supplemented with an omega-3 fish oil fat emulsion containing EPA and DHA for 1 to 7 days, showed significantly reduced TLR4 mRNA and protein expression after a minimum of 5 days [74]. In both studies, reduced CD14 and TLR4 cell surface expression by EPA, DHA and omega-3 fish oil fat emulsion was observed in unstimulated cells, while no information is available for stimulated cells. Therefore, how this effect relates to the reduction in cytokines production in LPS-stimulated cells remains unresolved. Additionally, the biological relevance of a change in CD14 and TLR4 expression in monocytes as opposed to macrophages is not known.

The strongest evidence for direct inhibition of TLR4 signaling after exposure to EPA and DHA comes from a series of studies conducted by Hwang and colleagues [75-77]. They first demonstrated that the molecular target of DHA is TLR4 and not downstream signaling components by showing DHA inhibition of NFκB activation in cells transfected with constitutively active TLR4 but not constitutively active MYD88 (a downstream signaling component) in both RAW 264.7 cells and human embryonic kidney cells (293T) [76, 77]. These “ligand-independent” systems eliminated the

possibility of impure LPS activating multiple receptors. Next, they demonstrated DHA inhibition of LPS-cell binding, LPS-induced recruitment of TLR4 into lipid rafts of RAW 264.7 cells, and LPS-induced dimerization in lipid rafts of Ba/F3 cells (pro-B, murine cell line) [75]. Of note, the exposure of the cells to DHA was relatively short, 0-3 h, and cell fatty acid profiles before and after treatment were not reported. Therefore, it is not clear whether the treatment period was adequate to see the maximal effect, the effect was due to external exposure of the cells to DHA, or if DHA was actually incorporated into the cells. Since increased consumption of EPA and DHA elevates the proportion of these fatty acids in the phospholipids of blood cells including monocytes [78], macrophage cell models in which both culture media and cellular EPA and DHA are enhanced would better reflect *in vivo* conditions. Furthermore, TLR4 expression was only measured 7 min post LPS stimulation. Therefore, it is not evident whether the effect of EPA and DHA is transient or sustained. A transient effect would be difficult to observe in *in vivo* models.

The primary objective of this thesis research was to further define the inhibitory effect of cellular enrichment with EPA and DHA on the production of pro-inflammatory cytokines specifically related to TLR4 signaling, and to determine whether the effects are primarily due to inhibition of TLR4 signaling or down-stream signaling components using the RAW 264.7 murine macrophage cell line.

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CHAPTER 3

Manuscript A:
Effect of EPA and DHA on TLR4-mediated secretion of pro-inflammatory cytokines in RAW 264.7 cells

Effect of EPA and DHA on TLR4-mediated secretion of pro-inflammatory cytokines in RAW 264.7 cells

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Abstract

Background: Relative to saturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) slow the initiation and progression of atherosclerotic lesions, in part through anti-inflammatory effects on macrophages. Limited evidence suggests that toll-like receptor 4 (TLR4) plays a major role in macrophage inflammation. The effect of enriching macrophage cell membranes with a saturated fatty acid (myristic acid, MA), EPA or DHA, on the secretion of pro-inflammatory cytokines initiated by TLR4 activation was determined.

Methods: RAW 264.7 cells were pretreated with 100 μM MA, EPA or DHA for 24 h and then stimulated with ultra-pure lipopolysaccharide (LPS), a specific ligand and activator of TLR4. Tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) secretion was determined by enzyme-linked immunosorbent assay. Cell surface abundance of TLR4, TLR4-MD2 and CD14, and fluorescein isothiocyanate (FITC)-LPS-cell were determined by flow cytometry.

Results: The cell membrane content of MA, EPA and DHA increased by 4.5-fold, 20.6-fold and 8.9-fold, respectively, as a result of pretreatment. EPA and DHA, but not MA, reduced TNFα and IL-6 secretion from ultra-pure LPS-stimulated RAW 264.7 cells ($P < 0.05$). The reduction in IL-6 was significantly greater than TNFα. Unexpectedly, the abundance of cell surface TLR4, TRL4-MD2, CD14, and FITC-LPS was not significantly altered by any fatty acid.

Conclusion: DHA-, and to a lesser extent EPA-enriched RAW 264.7 cells had reduced inflammatory response to TLR4 activation, while no significant changes were

observed in MA-enriched cells. The DHA-associated interference in TLR4 signaling may occur downstream of the receptor.

1. Introduction

Inflammation is a major contributing factor to the development of atherosclerotic lesions. Among the different types of immune cells that have been found in lesions, macrophages are a prominent cell type, and play a critical role in initiating and promoting lesion development [1]. Macrophages infiltrate the arterial wall where they accumulate cholesterol and secrete pro-inflammatory factors (e.g., cytokines, chemokines, eicosanoids) which supports the recruitment of additional immune cells, thereby augmenting the inflammatory response. This sustained and heightened inflammatory state promotes the formation of mature, unstable plaque. Hence, lowering macrophage pro-inflammatory activity may delay atherosclerotic lesion progression.

Toll-like receptor 4 (TLR4) is a primary mediator of macrophage inflammatory activity. Bacteria-derived lipopolysaccharide (LPS) [2] and endogenous lipid species such as oxidized LDL (oxLDL) and saturated fatty acids activate TLR4 [3-5]. Activation of TLR4, which requires the formation of a receptor complex of TLR4 and its associated molecules, myeloid differentiation 2 (MD2) , and cluster of differentiation 14 (CD14), in turn activates nuclear factor κB (NFκB) and mitrogen-activated protein kinase (MAPK) signaling pathways, both which control the transcription of pro-inflammatory cytokines [6]. The relationship between TLR4 and arterial lesion development has been reviewed extensively [7] and is supported by the finding that TLR4 protein is prominently expressed by macrophages in both human and murine lipid-rich lesions but not in healthy

regions [8]. In addition, knock-out and knock-in mouse models have demonstrated a causative role of TLR4 expression in lesion development [9].

Epidemiological evidence suggests that diets rich in omega-3 fatty acids, particularly the very long-chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are atheroprotective [10]. Plasma phospholipid EPA and DHA concentrations are inversely associated with intima-media thickness [11], prospectively with narrowing of coronary artery diameter [12], and intercranial atherosclerotic stenosis [13]. The available data suggest that the anti-inflammatory effects of EPA and DHA on macrophages play a role in their protective function. Administration of EPA and DHA supplements or fish-rich diets to humans [14-20], animals [21, 22], and macrophage cell lines [21, 23-28] lowers circulating inflammatory marker concentrations and/or LPS-induced pro-inflammatory cytokine and prostaglandin secretion. In the majority of the aforementioned *in vitro* studies, macrophages were incubated in EPA or DHA-supplemented culture media, but little is known about the effects of EPA and/or DHA cell membrane enrichment on inflammatory effects, particularly as related to TLR4 activation. In a murine microglial cell line, incorporation of DHA into cells was associated with a decrease in the cell surface expression of TLR4 and CD14, as well as attenuated NFκB activity and pro-inflammatory cytokine synthesis induced by LPS [29]. To date, studies that link anti-inflammatory effects of EPA or DHA to disrupted TLR4 activation and signaling in macrophages have been limited to relatively short cell exposure times to EPA or DHA (< 3 h) and in some cases EPA or DHA cellular incorporation was not confirmed [30-32]. Since dietary EPA and DHA are

incorporated into macrophage cell membranes, the biological relevance for the *in vitro* effect of EPA and DHA on TLR4 protein expression would be further supported by models in which cell membrane incorporation is confirmed. An additional point that requires clarification is whether EPA and DHA cell membrane enrichment inhibits inflammatory responses exclusively due to the activation of TLR4, since the purity of LPS has not reported in previous studies. Impure LPS has been shown to independently activate both TLR2 and TLR4 [33].

The aim of these studies was to compare the effects of cell membrane enrichment with EPA, DHA and a saturated fatty acid on the secretion of pro-inflammatory cytokines and factors relating to LPS-induced TLR4 activation. We hypothesized that enrichment of cell membranes with EPA or DHA together with a compensatory change in the membrane fatty acid composition will be associated with a reduction in pro-inflammatory factors through the decreased cell surface expression of TLR4 and its associated molecules, MD2 and CD14, and that enrichment of cell membranes with a saturated fatty acid would have the opposite effect.

2. Methods

2.1. *Cell culture*

Murine macrophage-like cell line RAW 264.7 cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, endotoxin < 25 EU/mL; Sigma-Aldrich, St. Louis, MO), 100 U/mL of penicillin, and 100 µg/mL streptomycin (MP Biomedicals, LLC, Santa Anna, CA) at 37°C in a 5% CO_2 humidified incubator. Cells were pretreated with 100 µM of the fatty acid combined with endotoxin-free bovine serum albumin (BSA; Sigma-Aldrich) at a 2:1 molar ratio or BSA alone for 24 h. After fatty acid pretreatment, cells were stimulated with 100 ng/mL of ultra-pure LPS (Invivogen, San Diego, CA) of the *E.* coli 0111:B4 strain for the indicated times in DMEM containing 10% FBS in the presence or absence of 100 µM fatty acid used in the pretreatment. Cellular protein concentration was measured by the bicinchoninic acid method (Pierce Inc., Rockford, IL).

2.2. Fatty acid profile of cells

Cells were collected by scraping in phosphate buffered saline (PBS). A portion of the cell suspension was used for protein determination. The remaining cells were put into cryotubes and stored at -80℃. At the time of analysis, samples were thawed over ice and after osmotic haemolysis, the cell membranes were washed thrice with sodium chloride

(0.9% buffered to pH 7.4) using high speed centrifugation. Lipids were extracted and the fatty acids methylated using boron trifluoride. The resulting fatty acid methyl esters were quantified using an established gas chromatography method as previously described [34, 35]. Peaks of interest were identified by comparison with authentic fatty acid standards (Nu-Chek Prep. Inc. Elysian, MN) and expressed as molar percentage (mol%) proportion of total fatty acids.

2.3. TNFα and IL-6 secretion

Enzyme-linked immunosorbent assays (ELISA) were used to measure tumor necrosis factor α (TNFα), and interleukin-6 (IL-6; R&D Systems, Minneapolis, MN) concentrations in culture media.

2.4. Flow Cytometry

Flow cytometry was used to detect cell surface receptors and fluorescein isothiocyanate (FITC)-LPS-cell association as previously described with minor modifications [36]. Briefly, 1 x 10^6 cells were pretreated with fatty acid as indicated above with or without additional ultra-pure LPS stimulation and blocked with 1 µg/100 µL anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA) for 5 min at 4°C. Cells were then labeled with anti-TLR4-APC (R&D Systems; 0.25 µg/100 µL), anti-TLR4/MD2-APC (eBioscience; 0.5 µg/100 uL), anti-CD14-PE (eBioscience; 0.5 µg/100 uL) or their isotype controls in the blocking solution for 30 min at room temperature. To assess the

effect of each fatty acid on LPS-cell association, fatty acid-treated cells were harvested and resuspended in the original culture media including the treatment fatty acid and incubated with LPS-FITC for 1 h at 37°C. Fluorescent labeled cells were washed and resuspended in staining buffer (R&D Systems), and detected by Accuri Flow Cytometer (BD Biosciences).

2.5. Statistical Analysis

Differences among mean values were tested using one- or two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons (GraphPad Prism 6, La Jolla, CA). P values < 0.05 were considered to be statistically significant.

3. Results

3.1. *Ultra-pure LPS-induced secretion of TNFα and IL-6*

Ultra-pure LPS was chosen to stimulate RAW 264.7 cells because it is selective for TLR4. Neither TNFα nor IL-6 was detectable in the culture media of RAW 264.7 cells at the basal (unstimulated) condition. Exposure to ultra-pure LPS induced the secretion of both TNFα and IL-6 into the culture media, indicating TLR4 activation (Fig. 1). TNFα and IL-6 secretion differed in both induction time (TNFα secretion was induced earlier than IL-6 secretion) and magnitude (TNFα secretion was approximately twice that of IL-6 through the majority of the 24-h incubation period).

3.2. Effect of fatty acid pretreatment on total cell fatty acid profiles

In the current study, we wanted to compare the effects of EPA and DHA enrichment to that of a saturated fatty acid. The saturated fatty acids evaluated included myristic (MA), lauric (LA), and palmitic acids (PA). We selected MA on the basis of greatest cell membrane enrichment after a 24-h incubation period (8.6-fold compare to 7.9-fold for LA and 1.2-fold for PA (mol %) (Supplementary Table). RAW 264.7 cells were pretreated with 100 μM MA, EPA, or DHA for 24 h to enrich cell membranes with the specific fatty acid. Pretreatment with MA, EPA and DHA resulted in a 4.5-fold, 20.6-fold, and 8.9-fold, increase, respectively (all at $p < 0.05$), all primarily at the expense of oleic acid and to a lesser extent arachidonic acid (AA) (Table 1). Pretreatment with EPA

also resulted in a 6.0-fold increase ($p < 0.05$) in docosapentaenoic acid (DPA), an elongation product of EPA.

3.3. EPA and DHA but not MA attenuate TLR4-mediated TNFα and IL-6 secretion

The effect of MA, EPA and DHA enrichment on TLR4-mediated TNFα and IL-6 secretion was assessed 6 and 24 h post-exposure to ultra-pure LPS which corresponded to the early and later stages of TNFα and IL-6 protein induction as determined by secretion curves in our time-course experiments (Fig. 1). Without stimulus (time 0), TNFα and IL-6 concentrations were below the detection limit in all fatty acid treatment groups (data not shown). After 24 h of ultra-pure LPS stimulation, EPA and DHA pretreatment resulted in 36% and 41% less TNFα secretion, respectively (both $p < 0.05$) (Fig. 2A). The effect of MA, relative to BSA, was not significantly different. A similar pattern was observed in the samples collected after a shorter incubation period, 6 h, however, the difference did not reach statistical significance. In contrast, cells pretreated with EPA or DHA, resulted in significantly less IL-6 secretion after both 6 h (67% and 72%, respectively) and 24 h (69% and 76%, respectively) of stimulation, compared to BSA (Fig. 2B). Again, as in the case of TNFα, relative to BSA, MA had no significant effect on IL-6 secretion. Removing the fatty acids from the culture media prior to ultra-pure LPS stimulation did not change these results (Fig. 2C and 2D). When change in TNFα and IL-6 secretion for each time point was assessed using a 2-way ANOVA after both 6 and 24 h of stimulation, with and without the fatty acid in the culture media during stimulation, EPA and DHA reduced IL-6 to a greater extent than TNFα ($p < 0.05$).

3.4. DHA does not alter receptor abundance or LPS-cell association

Because DHA had a slightly greater inhibitory effect on IL-6 secretion compared to EPA, we focused on DHA to further investigate the mechanism. DHA enrichment in the culture media and cell membrane could inhibit TLR4 signaling by altering cell LPS recognition. Therefore, we measured cell surface expression of TLR4, TLR4-MD2 complex and CD14. MA or DHA membrane enrichment did not significantly alter expression levels of cell surface TLR4, TLR4-MD2 complex, or CD14 before or after ultra-pure LPS stimulation (10 min – 360 min; Fig. 3A – 3C). We also assessed the effect of MA and DHA cell membrane enrichment on LPS-cell surface association, as an additional measure of LPS recognition. There was no significant difference in either groups relative to the BSA control (Fig. 3D).

4. Discussion

In the present study, we found that pretreating RAW 264.7 cells with MA, EPA, or DHA for 24 h enhanced the proportion of these fatty acids in the cell membrane. However, only EPA and DHA treatments were associated with reduced pro-inflammatory activity specific to the activation of TLR4 induced by ultra-pure LPS. Our data also suggest that the interference with TLR4 signaling occurs downstream of the receptor.

It is likely that the effects of EPA and DHA on TNFα and IL-6 production are associated with inhibition of signaling pathways initiated by TLR4 activation because ultra-pure LPS is specific for TLR4, unlike impure LPS, which can also activate TLR2 [33]. The effect of each fatty acid on TNFα and IL-6 secretion in cells stimulated with ultra-pure LPS was similar between the two conditions tested: with and without the respective fatty acid in the culture media during stimulation. This implies that inhibition of TNFα and IL-6 secretion was unlikely caused by an interaction of EPA or DHA with ultra-pure LPS or TLR4 in the culture media. We cannot exclude the possibility that cellular EPA and DHA could have been released into the culture media, perhaps locally, due to membrane turnover or in response to TLR4 activation. However, in either case, the back release would be much lower than the concentrations initially used [37]. Hence, our data suggest that inhibition of the TLR4 signaling pathway by EPA or DHA in our model is more likely to be related to their incorporation into cell membrane and modification in cell membrane fatty acid composition.

We hypothesized that MA pretreatment and cell membrane incorporation would enhance TNFα and IL-6 secretion induced by ultra-pure LPS on the basis of previous reports implicating saturated fatty acids as activators of both TLR4 and TLR2 signaling in murine cell lines [38-40]. However, we found that MA only slightly augmented ultra-pure LPS-induced IL-6 secretion. The discrepancy between the current and previous data may imply that TLR4 signaling is augmented by only certain saturated fatty acids. Induction by lauric, palmitic and stearic acids has been previously reported. Additionally, fatty acid exposure time between the current and previous study (24 h vs. 3h, respectively) may contribute to the difference [31]. Increase in TNFα gene expression by palmitic and stearic acid after 1 h but not after 6 h or 12 h of treatment has been reported [38]. It is also possible that TNFα and IL-6 reached maximum induction with the concentration of ultra-pure LPS used in our study.

It has been proposed that increasing the proportion of very long-chain omega-3 fatty acids in the cell membrane modulates immune cell function such as in T cells, by influencing membrane receptor distribution and activity [41, 42]. However, evidence from the current work suggests EPA and DHA exert anti-inflammatory effects in macrophages downstream of TLR4 activation. Inhibition of TNFα secretion by either EPA or DHA was much weaker than that of IL-6. A similar observation has been reported in peripheral blood mononuclear cells isolated from subjects who consumed EPA and DHA supplements [17, 36], and after addition of the fatty acids to the culture media of human THP-1 [43, 44] and murine J774 [24, 26] macrophage cell lines. If EPA or DHA treatment decreased TNFα and IL-6 secretion primarily through inhibition of

TLR4 activation a similar relative decline induced by the two fatty acids would be predicted because TLR4 initiates the signaling pathway. Since this was not the case, we hypothesize that inhibition likely occurs downstream of TLR4 activation, at a point in the signaling pathway that has differential influences on TNFα and IL-6 production.

The lack of an effect of DHA on cell surface expression of TLR4 receptor, TLR4-MD2 receptor complex, CD14, and LPS-cell association in RAW 264.7 cells further supports the hypothesis that disruption in TLR4 signaling occurs downstream of TLR4 activation. Our findings are not consistent with previous reports demonstrating that DHA decreases the abundance of membrane TLR4 and CD14 in BV-2 microglial cells [29] and LPS binding to THP-1 cells [30]. These studies differed from ours in cell type, vehicle in which DHA was added, and DHA concentration. Our findings are also inconsistent with previous work demonstrating decreased TLR4 abundance in the lipid raft fraction of the membrane in DHA-treated BA/F3 cells (a murine pro-B cell line) [32]. Since we measured TLR4 expressed on the surface of intact cells, we cannot rule out the possibility that the effect of DHA on TLR4 abundance is detectable only when measured in lipid raft fractions where activation of TLR4, engagement with associated molecules, and signaling occurs. Our studies also differ from prior work in DHA exposure time. The initial report used a relatively short exposure time, 1 to 3 h, prior to LPS stimulation, and did not report changes in DHA cellular content [32]. The current study used 24 h, a length of time shown to be sufficient to alter DHA cellular content. Since dietary EPA and DHA are incorporated into macrophages *in vivo*, the effects of EPA or DHA on TLR4 cell

surface expression observed after these fatty acids have been incorporated into the cells may have more biological relevance than the effects observed after an acute treatment.

In summary, our study provides evidence that in RAW 264.7 cells, a monocyte macrophage cell line, cell membrane enrichment with EPA and DHA resulted in attenuation of inflammatory activity initiated by TLR4 activation. However, the ability to reduce the secretion of specific cytokines varied significantly. This, combined with the lack of influence on factors influencing LPS recognition and TLR4 activation suggest that DHA exerts a greater influence downstream of TLR4 activation. Future studies should determine whether the inhibitory effect of DHA on TLR4 cell surface expression occurs only in lipid rafts and/or is dependent on factors such as treatment duration, the extent of DHA enrichment, and cell type, in order to resolve current discrepancies. Finally, the effect of DHA supplementation on macrophage TLR4 expression should be verified in humans.

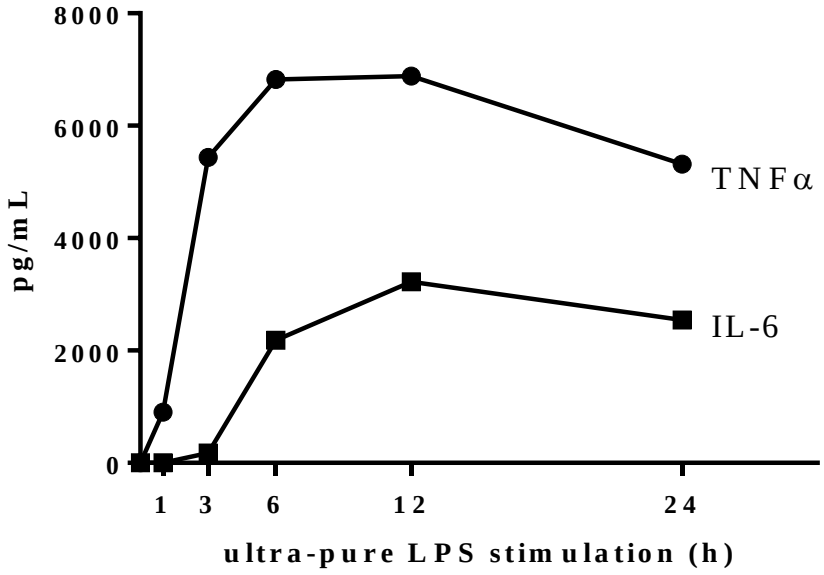


Fig. 1. Time course of ultra-pure LPS-induced TNFα and IL-6 secretion. RAW 264.7 cells were stimulated with ultra-pure LPS (100 ng/mL) for a 24 h period. TNFα and IL-6 in the culture media were determined by ELISA.

Table 1. Selected fatty acid composition (mol%) after 24-h fatty acid pretreatment and prior to ultra-pure LPS stimulation

Fatty acid	Control		MA		EPA		DHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA	47.52	6.26	53.96	1.68	47.46	5.42	45.93	1.21
14:0	3.69[a]	2.36	16.53[b]	4.39	2.46[a]	0.14	2.69[a]	0.45
16:0	22.56[ab]	1.76	20.03[a]	1.51	24.00[b]	1.92	23.71[b]	0.34
18:0	18.89	3.01	15.62	1.30	18.65	3.12	17.24	0.93
MUFA	35.28	6.99	31.29	2.40	25.05	2.72	26.32	6.01
16:1n-9	3.60	0.77	3.19	0.34	2.92	0.87	2.62	0.29
16:1n-7	3.31	1.18	4.15	1.11	2.32	0.54	2.39	0.71
18:1n-9	17.28	4.45	13.55	0.63	12.61	1.15	13.05	0.51
181n-7	9.31	1.25	9.63	2.10	6.69	3.43	7.66	4.46
PUFA								
n-6 PUFA	9.10	1.38	7.86	1.62	4.64	0.29	6.54	1.06
18:2	2.39	0.26	1.98	0.40	1.42	0.61	2.12	0.57
20:4	5.58[a]	1.85	4.79[a]	1.14	2.51[b]	0.35	3.61[ab]	0.66
22:4	0.44	0.13	0.34	0.06	0.32	0.04	0.26	0.05
n-3 PUFA	6.46	1.14	4.43	1.23	20.42	8.58	19.02	9.62
20:5	0.41[a]	0.21	0.46[a]	0.15	8.39[b]	3.70	0.45[a]	0.16
22:5	1.72[a]	0.96	1.52[a]	0.52	10.39[b]	5.70	1.08[a]	0.05
22:6	1.91[a]	1.28	1.86[a]	0.65	0.69[a]	0.26	16.92[b]	9.88

MA, myristic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Fatty acids that comprised less than 1 mol% of total fatty acids are not included, but are included in the calculations. Values are mean ± SD of 3 independent experiments. Mean values within a row without common letters statistically differ at $p < 0.05$ determined by one-way ANOVA adjusted with Tukey post-hoc test for multiple comparisons.

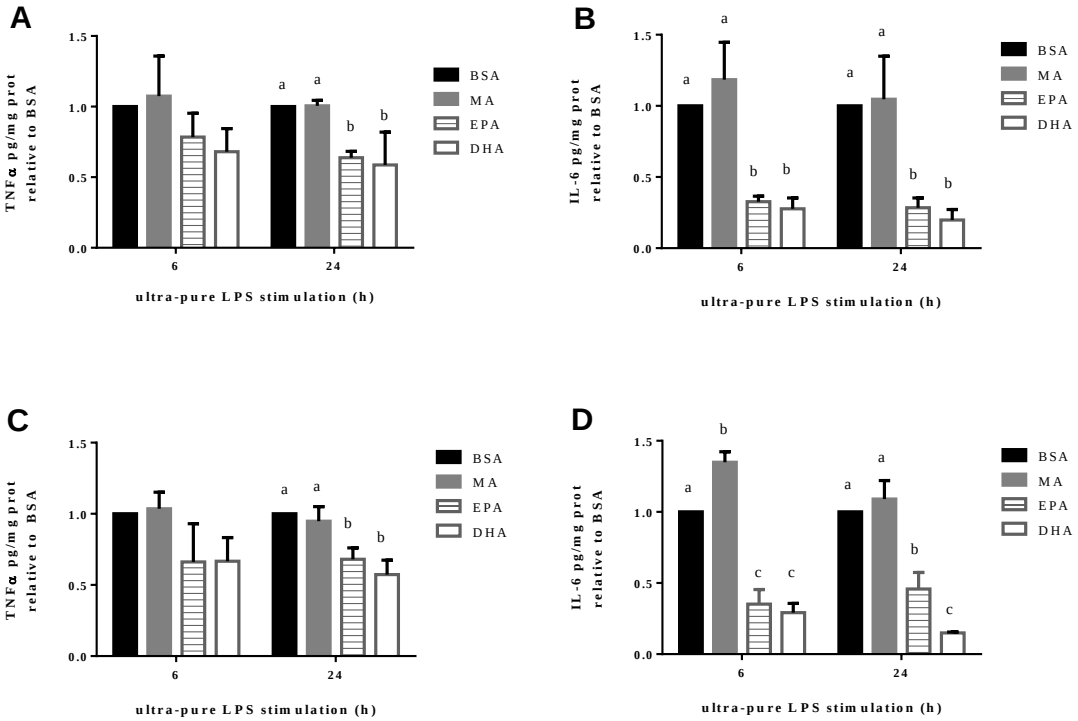
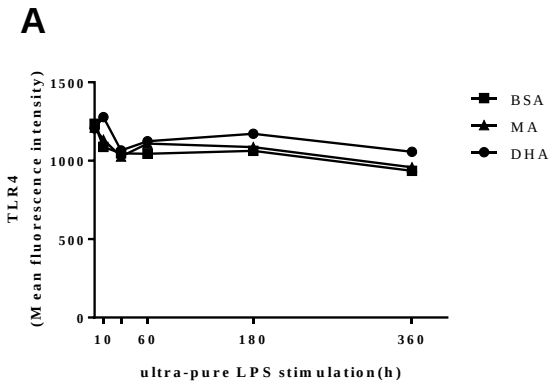
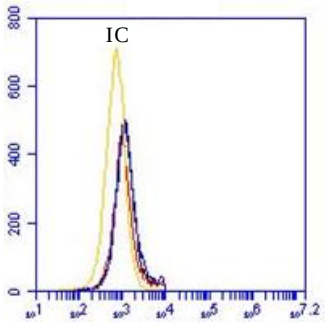
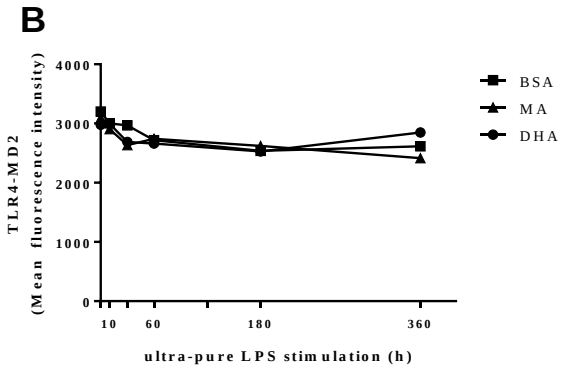


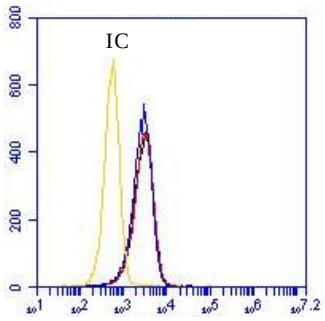
Fig. 2. Ultra-pure LPS-induced secretion of TNFα and IL-6 from fatty acid-pretreated RAW 264.7 cells. RAW 264.7 cells were pretreated with the respective fatty acids (100 μM) for 24 h and then stimulated with ultra-pure LPS (100 ng/mL) for 6 or 24 h. Production of TNFα (A) and IL-6 (B) with the respective fatty acid in the culture medium and production of TNFα (C) and IL-6 (D) without the respective fatty acid in the culture media during ultra-pure LPS stimulation were determined by ELISA. Values are mean ± SD of 3 independent experiments normalized to BSA-treated cells. For each time point, bars without common letters statistically differ at $p < 0.05$ determined by one-way ANOVA adjusted with Tukey's post-hoc test for multiple comparisons.



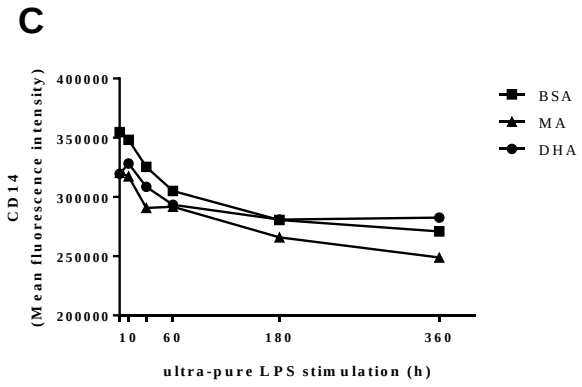


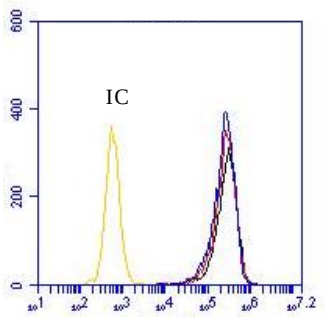
TLR4 fluorescent intensity



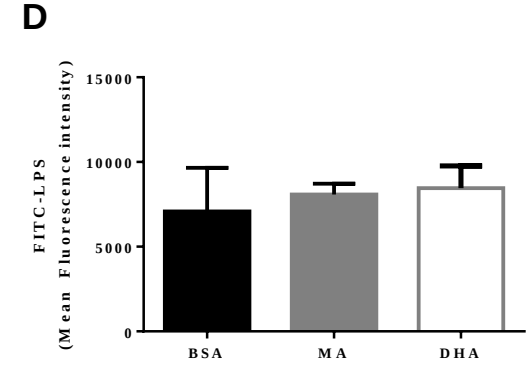


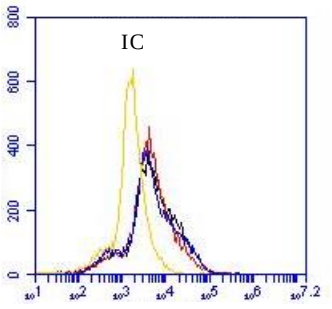
TLR4-MD2 fluorescent intensity





CD14 fluorescent intensity





FITC-LPS fluorescent intensity

Fig. 3. *Previous page.* Cell surface expression of TLR4 (A), TLR4-MD2 (B), and CD14 (C), and LPS-cell surface association (D) in RAW 264.7 cells pretreated BSA, MA or DHA (100 μM, 24 h) were determined by flow cytometry. Representative histograms for indicated markers under different fatty acid treatments (BSA: black, MA: red, DHA: blue) compared with corresponding isotype control (IC) in unstimulated cells (right panels) and mean fluorescence intensities (MFI, left panels) of BSA-, MA- and DHA-treated cells stimulated with ultra-pure LPS for the times indicated (A-C), or with FITC-LPS for 1 h (D) are shown. Values are expressed as MFI ± SD, or just MFI for clarity (A-C) of 3 independent experiments.

Supplementary Table. Selected fatty acid composition (mol%) after 24-h saturated fatty acid pretreatment

Fatty acid	Control	LA	MA	PA
	Mean	Mean	Mean	Mean
SFA	38.55	43.72	52.05	41.27
12:0	0.34	2.70	0.29	0.35
14:0	2.35	5.97	20.17	1.72
16:0	21.55	21.98	19.83	25.90
18:0	14.31	13.06	11.76	13.30
MUFA	42.76	39.39	34.57	43.41
16:1n-9	4.12	3.93	3.39	5.16
16:1n-7	4.43	4.82	5.13	6.54
18:1n-9	26.15	22.93	19.03	23.94
181n-7	8.00	7.71	7.02	7.77
PUFA	13.11	11.98	10.13	12.53
n-6 PUFA	9.45	8.17	7.32	9.33
18:2	1.25	1.23	1.15	1.32
20:4	3.96	3.48	3.26	3.46
22:4	0.574	0.525	0.384	0.495
n-3 PUFA	3.66	3.81	2.81	3.20
20:5	0.408	0.378	0.305	0.384
22:5	1.55	1.45	1.16	1.35
22:6	1.60	1.81	1.19	1.33

The degree of incorporation into the cell membrane of three different saturated fatty acids were compared to control cells. LA, lauric acid; MA, myristic acid; PA, palmitic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Fatty acids that comprised less than 1 mol% of total fatty acids are not included, but were included in the calculations. Values are mean of 2 samples of 1 experiment.

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CHAPTER 4

Manuscript B:

DHA differentially affects IL-6 and TNFα expression in LPS-stimulated murine macrophage cell line RAW 264.7

DHA differentially affects IL-6 and TNFα expression in LPS-stimulated murine macrophage cell line RAW 264.7

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Abstract

Background: The cardio-protective effects of docosahexaenoic acid (DHA) may in part be related to its anti-inflammatory properties. Exposure of macrophages to DHA has been shown to reduce the inflammatory profile, but how DHA influences individual cytokines is not well established. We previously reported differential effects of DHA on TNFα and IL-6 production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells (Thesis, Chapter 3). The current study was conducted to determine the effect of DHA on nuclear factor κB (NFκB), a central transcription factor for regulating genes coding for TNFα and IL-6, and prostaglandin E_2 (PGE_2), a lipid pro-inflammatory mediator that may impact TNFα and IL-6 production by activating transcription factor cAMP-response element binding protein (CREB).

Methods: PGE_2 secretion, phosphorylated CREB (P-CREB), and NFκB-DNA binding were determined by ELISA in ultra-pure LPS-stimulated RAW 264.7 cells pretreated with 100 μM DHA for 24 h. TNFα and IL-6 mRNA levels in the culture media were assessed by real-time PCR after inhibiting PGE_2 production (NS-398), or inhibiting NFκB activity (BAY-11-7082 or SN50).

Results: DHA reduced PGE_2 secretion by 41% ($p < 0.05$), NFκB-DNA binding by 32% ($p < 0.05$) but not P-CREB protein levels in stimulated cells. Exogenous PGE_2 decreased TNFα but not IL-6 mRNA levels in a dose-dependent manner. Blocking PGE_2 production also decreased TNFα mRNA. NFκB inhibitors reduced IL-6 mRNA to a greater extent than TNFα mRNA.

Conclusion: Differential effects of DHA on TNFα and IL-6 are likely mediated by a partial inhibition of NFκB and are independent of changes in PGE_2 secretion.

1. Introduction

Docosahexaenoic acid (DHA) is a very long chain omega-3 fatty acid derived from marine animals and algea. In contrast to saturated fatty acids, DHA down-regulates toll-like receptor 4 (TLR4)-stimulated production of pro-inflammatory cytokines. These effects are suggested to be primarily mediated by a reduction in nuclear factor κB (NFκB) activity as evidenced by decreased IκB phosphorylation and reduced nuclear levels of NFκB p65-p50 dimers [1]. However, because DHA has been shown to reduce individual inflammatory cytokines by varying degrees, it appears that DHA's effect on cytokine production cannot be attributed to a global inhibition of NFκB alone. We have previously reported that in cultured RAW 264.7 cells DHA supplementation decreases interleukin 6 (IL-6) secretion to a greater extent than tumor necrosis factor α (TNFα), while the saturated fatty acid, myristic acid, has no significant effect on either cytokine (Thesis, Chapter 3). TNFα and IL-6 influence the development of atherosclerotic plaque by promoting immune cell recruitment, macrophage foam cell formation, and destabilization of mature plaque [2-7]. Despite the importance of TNFα and IL-6 in atherosclerosis lesion progression, the effect of DHA on production of these cytokines in macrophages, as well as the regulatory mechanisms have not been well established. Although NFκB is a central regulator of TNFα and IL-6 production, other regulatory molecules that are up-regulated in TLR4-activated macrophages, including prostaglandin E_2 (PGE_2) and the transcription factor cAMP response element-binding protein (CREB) may also be involved in regulating the production of these cytokines through their specific pathways.

PGE_2 is perhaps the most prominent pro-inflammatory lipid mediator. PGE_2 promotes inflammation and causes redness, swelling and pain in affected tissues [8], and its synthesis has long been a pharmaceutical target for controlling inflammation. There is general consensus that endothelial inflammation plays a key role in the development of atherosclerosis. Over-expression of the enzymes that synthesize PGE_2 from its precursor, arachidonic acid (AA), cyclooxygenase 2 (COX2) and PGE synthase, have been reported in plaque and blood mononuclear cells of patients with carotid atherosclerosis [9-11]. PGE_2 may play a role in plaque destabilization by up-regulating the expression of matrix metalloproteinases by vascular cells [12, 13]. PGE_2 also modulates the production of cytokines by macrophages including TNFα and IL-6, which is thought to occur in an autocrine/paracrine-like manner [14, 15]. Activation of TLR4 by LPS increases PGE_2 production by macrophages by inducing a series of steps including the release of AA from membrane phospholipids, increased activity of the rate limiting enzyme, COX2, which converts AA into the intermediate product PGH_2, and subsequent conversion of the latter into PGE_2 by action of PGE synthase [16]. Through engagement of E prostanoid receptor 2 and/or 4 (EP2/EP4) expressed on the surface of macrophages, PGE_2 has been reported to decrease TNFα production and increase IL-6 production [17-21]. These effects have been shown to be mediated through activation of cAMP/PKA system [22, 23].

Interestingly, studies have suggested that triggering cAMP/PKA may be independently associated with inhibition of NFκB-mediated transcription of specific genes, including TNFα in THP-1 and RAW 264.7 cells [24-26]. Transcription factor

CREB, which can be phosphorylated and activated by PKA, may mediate the suppression and enhancement of TNFα and IL-6 mRNA, respectively, due to cAMP/PKA activation [27]. Activated CREB inhibits transcription of select NFκB genes by binding to the cAMP- responsive element (CRE) in the promoter region and limiting the interaction between NFκB and the transcriptional co-activator of CREB binding protein, CBP [28, 29]. However, CREB has been shown to enhance the transcription of some NFκB target genes including IL-6, which may occur through cooperative recruitment of CBP with NFκB, facilitated by the proximity of their binding sites [30]. Since CREB is phosphorylated by PKA, the effect of PGE_2 on TNFα and IL-6 gene transcription may be mediated through the cAMP/PKA/CREB pathway [31-33].

The ability of DHA to reduce PGE_2 production has been reported in a variety of cell types including LPS-stimulated RAW 264.7 cells [34-37]. Using this model, the aim of the present study was to determine the effect of DHA on PGE_2 production and CREB and NFκB activities, and the role of PGE_2 and NFκB in DHA-induced change in TNFα and IL-6 gene expression. We initially hypothesized that a reduction in PGE_2 by DHA may decrease the repressive effects of PGE_2 on TNFα gene expression and thus diminish the inhibitory effect of DHA on TNFα but not IL-6 production. Our results suggest that PGE_2 secreted from LPS-stimulated RAW 264.7 cells may not be a significant regulator for TNFα and IL-6 gene expression and rather, a reduction in NFκB activity independent of a change in PGE_2 secretion may account for the differential effects of DHA on TNFα and IL-6 gene transcription.

2. Methods

2.1. Cell culture

RAW 264.7 cells, a murine, macrophage-like cell line (ATCC, Manassas, VA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin and 100 µg/mL streptomycin (MP Biomedicals, LLC, Santa Anna, CA) at 37°C in a 5% CO_2 humidified incubator.

2.2. Fatty acid treatment and LPS stimulation

RAW 264.7 cells were pretreated with 100 µM of DHA or MA, a saturated fatty acid comparator, both complexed to endotoxin-free, bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) at a 2:1 molar ratio for 24 h. BSA without fatty acid was used as a control. After the 24-h pretreatment, cells were stimulated with 100 ng/mL of ultrapure LPS (Invivogen, San Diego, CA) from *E.* coli 0111:B4 strain for 3, 6, and 24 h in the presence of pre-treatment fatty acids. Cells were harvested and cellular protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce Inc., Rockford, IL).

2.3. TNFα and IL-6 gene transcription

RNA was isolated from RAW 264.7 cells using an RNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized from RNA using a Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. Real Time PCR was performed using SYBR green and Quantitect primer assays (Qiagen, Valencia, CA) for mouse TNFα, IL-6, beta (β) actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (QT00104006, QT00098875, QT01136772, QT01658692) on real-time PCR 7300 (Applied Biosystems, Foster City, CA). Relative quantification (ΔΔCt) was used to assess expression of target genes, using β-actin or GAPDH as an endogenous control.

2.4. Enzyme-linked immunosorbant assays (ELISA)

ELISA kits were used to determine CREB phosphorylated at S133 in cell lysates (R&D Systems, Minneapolis, MN), and PGE_2 concentration in the culture media (Cayman Chemical Company, Ann Arbor, MI).

2.5. Exogenous PGE_2 treatment

PGE_2 (Cayman Chemicals, Ann Arbor, MI) dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) was added to the culture media to achieve a final concentration of 2, 10, 50, 100 or 1000 µM. RAW 264.7 cells were pre-incubated in this PGE_2 supplemented culture media for 45 min. Cells were then stimulated with ultra-pure LPS (100 ng/mL) for an additional 3 h at 37°C. TNFα and IL-6 gene transcription was determined as described above.

2.6. Inhibition of NFκB and COX2

To inhibit nuclear translocation of NFκB subunit p50, RAW 264.7 cells were pre-treated with the p50 inhibitor peptide, SN50, (Imgenex, San Diego, CA) dissolved in phosphate buffered saline (PBS) for 15 min at 37°C. The concentration of SN50 in the culture media was 40, 80, or 120 μM concentration. PBS was added to the control group. Thereafter, cells were stimulated with ultra-pure LPS (100 ng/mL) for an additional 3 h.

RAW 264.7 cells were pretreated for 16 h with 10 μM of BAY-11-7082 (BAY) or 10 μM NS-398 (Cayman Chemicals, Ann Arbor, MI) dissolved in DMSO to inhibit NFκB and COX2, respectively, and then stimulated with ultra-pure LPS (100 ng/mL) for 3 and/or 6 h. The final concentration of DMSO in the medium did not exceed 0.1%. TNFα and IL-6 gene transcription was determined as described above.

2.7. Western blotting for nuclear NFκB p50 and p65 proteins

RAW 264.7 cells were pretreated with BSA (fatty acid vehicle) for 24 h, and then treated with SN50 dissolved in PBS for 15 min at 37°C at 10 and 100 μM. Cells were then stimulated with LPS (100 ng/mL) for 30 min. Nuclear protein was extracted using NE-PER® nuclear extraction reagents (Thermo Scientific, Rockford, IL). Nuclear protein (10 μg) were separated by SDS-PAGE through a 4-20% Criterion® Tris-HCL gradient gel (Bio-Rad, Hercules, CA) and transferred onto a nitrocellulous membrane (Bio-Rad, Hercules, CA). After blocking, the membrane was incubated with primary antibodies for

NFκB p50 (cat# ab32360, Abcam, Cambridge, MA), p65 (cat# 8242, Cell Signaling, Danvers, MA), and TATA binding protein (TBP; cat# ab818, Abcam, Cambridge, MA), a nuclear loading control, and peroxidase-conjugated detection antibody (goat anti-mouse IgG-HRP [sc-2005] and goat anti-rabbit IgG-HRP [sc-2030] Santa Cruz Biotechnology, Inc., Dallas, TX). Signals were visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA).

2.8. NFκB-DNA binding assay

The nuclear extracts prepared as mentioned above was used to determine NFκB p50 binding to target DNA using with a TransAM NFκB ELISA kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol.

2.9. Statistical analysis

The significance of the differences among mean values from 3 independent experiments, each in triplicate unless otherwise noted, was determined by one-way analysis of variance (ANOVA) or two-way ANOVA, when both treatment and time were factors. Tukey's or Sidak's test for multiple comparisons followed each analysis. The repeated measures method was included in the analysis when within treatment group values varied among repeated experiments. Student's t-test was used to determine the significance in the difference in PGE_2 concentration in the culture media between control

cells and cells treated with NS-398. The statistical software GraphPad Prism 6 (La Jolla, CA) was used for statistical calculations. Significance was set at $P < 0.05$.

3. Results

3.1. Effect of MA and DHA on TNFα and IL-6 gene expression

Prior work documented that DHA pre-treatment of RAW 264.7 cells caused a greater reduction in LPS-induced IL-6 secretion compared with TNFα secretion (Thesis, Chapter 3). This effect was observed during both the early (6 h) and late (24 h) phases of protein induction. To further determine the mechanism for this differential effect, we measured the mRNA levels of TNFα and IL-6 before and 3, 6 and 24 h after stimulation with ultra-pure LPS (Fig. 1). Pretreatment with DHA reduced IL-6 mRNA levels under stimulated conditions ($p < 0.05$) compared to the control and MA-treated cells. DHA reduced IL-6 mRNA by 77% compared to the control cells (non-significant) and 97% compared to MA-treated cells ($p < 0.05$) under non-stimulated conditions. In contrast, DHA did not significantly affect TNFα mRNA expression compared to the control or MA-treated cells in LPS-stimulated conditions, while it reduced TNFα mRNA compared to MA-treated ($p < 0.05$) but not the control cells under non-stimulated conditions.

3.2. Effect of DHA on PGE_2 production and CREB activity

In unstimulated cultures PGE_2 levels in the culture media were below the detection limit. PGE_2 concentration reached approximately 3,000 pg/mL after stimulation with ultra-pure LPS for 6 h. DHA pretreatment reduced PGE_2 production by 41% (Fig. 2A, $P < 0.05$), while MA had no significant effect. In response to ultra-pure

LPS, P-CREB levels increased by approximately 3.5 fold after 30 min (Fig. 2B), which was consistent with previous reports [38, 39]. Pretreatment of the cells with DHA reduced basal levels of P-CREB ($p < 0.05$), but did not alter P-CREB at 30 min or 60 min post-stimulation compared to BSA. Based on these data we ruled out a possible role of CREB in mediating the effect of DHA on TNFα and IL-6 gene transcription.

3.3. Differential effect of PGE_2 on TNFα and IL-6 gene transcription

Since DHA reduced PGE_2 production in stimulated cells, we next investigated how PGE_2 affected TNFα and IL-6 gene expression. Cells were pre-incubated with exogenous PGE_2 at a wide range of concentration: 0, 2, 10, 50, 100 and 1000 nM (10 nM = 3525 pg/mL) and then stimulated with ultra-pure LPS. PGE_2, at concentrations 10 nM and higher, suppressed TNFα mRNA expression (all $p < 0.05$, Fig. 3A). The decrease in TNFα mRNA expression was dose-dependent ($p < 0.01$ for linear trend). PGE_2 had no significant effect on IL-6 mRNA expression (Fig. 3B).

To confirm these findings we inhibited PGE_2 production in the cells using NS-398, a specific COX2 inhibitor, and found that NS-398 reduced PGE_2 secretion by 98% (Fig. 4A). TNFα and IL-6 gene expression was also measured in the NS-398-treated cells 3 and 6 h post-stimulation, which corresponded to the times when PGE_2 concentration in the culture media was low (below detection) and high (> 3000 pg/mL), respectively. Contrary to our expectation, at 3 h post-stimulation with ultra-pure LPS, exposure to NS-398 resulted in decreased, rather than increased TNFα mRNA expression (21%, $p < 0.05$)

(Fig. 4B). This effect was no longer present 6 h post-stimulation with ultra-pure LPS NS-398 (Fig. 4B). NS-398 had no significant effect on IL-6 mRNA expression (Fig. 4C). Based on these data, it is unlikely that endogenous PGE_2 inhibits TNFα or IL-6 gene expression in LPS-stimulated RAW 264.7 cells. Therefore, it is unlikely that the reduction in PGE_2 production observed with DHA accounts for the overall effect of DHA on TNFα or IL-6 gene expression.

3.4. Differential effect of NFκB on TNFα and IL-6 gene expression.

Since PGE_2 and CREB were unlikely to mediate the differential effect of DHA on TNFα and IL-6 mRNA levels under LPS-stimulated conditions, we further evaluated the influence of NFκB activity on TNFα and IL-6 gene expression. As expected, NFκB activation was induced after exposure to ultra-pure LPS as indicated by an increase in nuclear levels of p65 protein (Fig. 5A). Of note, DHA reduced NFκB-DNA binding activity by 32% compared to the control cells ($p<0.05$) (Fig. 5B). To assess the relationship between NFκB activity and TNFα or IL-6 gene expression, we blocked NFκB activation using two NFκB inhibitors. First, we pre-incubated cells with SN50, a p50-specific inhibitor that prevents the nuclear translocation of p50 subunit by acting as a p50 decoy. Pretreatment of ultra-pure LPS-stimulated cells with 100 μM SN50 reduced nuclear p50 and p65 protein by 46% and 64%, respectively (Fig 6). However, while SN50 treatment decreased IL-6 mRNA expression in a dose-dependent manner, it had no significant effect on TNFα mRNA expression. These data suggest a greater dependence on NFκB activity by IL-6 than TNFα gene expression. We further confirmed these effects

using another NFκB inhibitor, BAY-11-7082 (BAY). BAY inhibits the phosphorylation of IκB, resulting in decreased IκB degradation which in turn reduces the release of NFκB p50-p65 heterodimer and its subsequent translocation into nuclei [40]. At 10 μM concentration, in ultra-pure LPS-stimulated RAW 264.7 cells BAY reduced NFκB activity by 41% (Fig. 7A). BAY was toxic to cells at 50 μM concentration as indicated by the detachment of cells from the plate. At 10 μM BAY had no significant effect on PGE_2 secretion (Fig. 7B). Similar to the results obtained with SN50, pretreatment with BAY resulted in a stronger inhibition of IL-6 (62%) compared to TNFα (32%) mRNA expression 3 h post-ultra-pure LPS stimulation. However, after 6 h of ultra-pure LPS stimulation, the inhibitory effect of BAY was only significant for IL-6 mRNA.

4. Discussion

This study demonstrates that DHA pretreatment for 24 h decreases IL-6 but not TNFα mRNA expression in RAW 264.7 cells stimulated with ultra-pure LPS, a TLR4 agonist. Furthermore, the observed gene-specific effects of DHA may be due to a greater sensitivity of IL-6 compared to TNFα to a partial reduction in general NFκB activity. This was an unexpected finding since NFκB regulates both TNFα and IL-6 downstream of TLR4 activation [41, 42]. We initially investigated the potential roles of PGE_2 and CREB in mediating the differential response to DHA treatment in cellular production of TNFα and IL-6. Our data suggest that PGE_2 produced by RAW 264.7 cells in response to ultra-pure LPS may not be sufficient to suppress TNFα mRNA generation. Moreover, DHA did not decrease CREB activity in ultra-pure LPS-stimulated RAW 264.7 cells, making it an unlikely mediator as well.

We previously observed a greater reduction in IL-6 than TNFα production in DHA-treated RAW 264.7 cells stimulated with LPS (Thesis, Chapter 3). In line with this, we observed a significant reduction in mRNA expression of IL-6, but not TNFα, after LPS stimulation. These results are also consistent with the study by Wang *et al.* reporting a significant reduction in IL-6 but not TNFα mRNA expression in human THP-1 macrophages treated with 100 µM DHA for 2 h and stimulated with of LPS for 24 h [43]. Similarly, Roche HM and coworkers reported that pre-treatment with DHA for 48 h followed by 6 h of LPS stimulation reduced IL-6 but not TNFα mRNA expression in THP-1 cells. [44]. However, several other studies have shown down-regulated secretion

or mRNA expression of both TNFα and IL-6 in THP-1 cells [45] [46] or RAW 264.7 cells [47] using similar or different treatment and stimulation conditions. The inconsistency between our findings and those reported previously may be partially related to the differences in the purity of LPS. Impure LPS containing lipoproteins has been reported to stimulate TLR2 signaling pathways at high concentrations used in the aforementioned studies. DHA has been shown to inhibit TLR2 activity and TNFα production induced by a TLR2 agonist [48]. The duration and dose of DHA and LPS treatments may also affect the relative potency in inhibiting TNFα versus IL-6 production.

There is limited data to assess the biological implications of our findings. Sijben and Calder, 2007, reviewed twenty-four studies published between 1991 and 2006, examining the effect of DHA and EPA supplementation in healthy humans on the secretion of TNFα, IL-6 and/or IL-1β from LPS-stimulated isolated peripheral blood monocytes (PBMCs) [49]. Limiting to only those studies that measured both TNFα and IL-6, two reported a significant reduction in both [50, 51], two reported a significant reduction in IL-6 but not TNFα [52, 53], one reported a significant reduction in TNFα but not IL-6 [54], and six reported no significant effect on either IL-6 or TNFα [55-60] . More recently, the OmegAD study reported a significant decrease in IL-6 but not TNFα mRNA from LPS-stimulated PBMCs [61].

Our goal was to identify the regulatory mechanism(s) not shared between TNFα and IL-6 gene expression, which may underlie the differential effect of DHA. We

initially evaluated the influence of DHA on PGE_2 production and CREB activity as they have each been shown to influence the transcription of TNFα and IL-6. Consistent with previous reports [34-36], DHA reduced PGE_2 production in stimulated RAW 264.7 cells. However, PGE_2 did not appear to control TNFα or IL-6 gene transcription in our *in vitro* system as predicted. We found that pre-incubating cells with exogenous PGE_2 reduced TNFα mRNA induced after 3 h of LPS stimulation, but pre-treating cells with a COX2 inhibitor, which blocked LPS-induced PGE_2 production also reduced TNFα mRNA induced after 3 h of stimulation and had no effect on TNFα after 6 h. It is possible that the nature of PGE_2's effect on TNFα may differ depending on the concentration as suggested by Renz et al. who reported that low PGE_2 concentrations (0.1 – 10 ng/mL) stimulated, whereas high concentrations (>10 ng/mL) suppressed TNFα release in primary mice macrophages [62]. In the current study, the lowest concentration of exogenous PGE_2 found to suppress TNFα was 10 nM (3525 pg/mL), which is comparable to the average endogenous PGE_2 concentration in the media (~ 3088 pg/mL) after 6 h of stimulation. Taken together, it is reasonable to speculate that PGE_2 produced by cells between 0 and 6 h of LPS stimulation may not be sufficient to down-regulate TNFα. In this study we used COX2 inhibitor to block *de novo* synthesis of PGE_2, however, these results may not necessarily represent a consequence solely due to reduced PGE_2 production because COX2 inhibition reduces production of other prostaglandins and increases production of lipoxygenase products such as leukotrienese [63]. Determining the effect of inhibiting PGE_2 receptors EP2/EP4 on TNFα expression, as previously done in primary mouse cells [17, 20], may help verify the regulatory potential of endogenous PGE_2.

In contrast to the large body of evidence supporting the role of CREB in regulating the transcription of NFκB-target genes including TNFα and IL-6, little is known about effect of DHA on CREB activity [64]. In the only study reported to date, peritoneal macrophages isolated from DHA-fed mice had attenuated CREB activity and IL-6 expression in response to *ex vivo* treatment with deoxynivalenol (a fungus-derived mycotoxin found in wheat, barley, corn, rice and oats [65]); however, *in vitro* treatment of peritoneal macrophages with DHA did not affect deoxynivalenol-induced CREB activity [66]. Largely in agreement with that study, we only observed a modest reduction in the basal level of P-CREB and no effect on LPS-induced P-CREB in RAW 264.7 cells pretreated with DHA compared to control. However, there was a small but significant decrease in stimulated P-CREB levels in DHA- compared to MA-pretreated cells. Of note, Avni *et al.* found that LPS-induced P-CREB in the absence of a cAMP inducer is transcriptionally inactive and is not necessary for LPS-induced TNFα production in RAW 264.7 cells [38]. Considering the available data we did not further investigate the role of CREB on TNFα and IL-6 expression.

We also investigated the influence of NFκB itself. The magnitude of observed inhibition on NFκB-DNA binding by DHA (approximately 30%) in our cell system was within the range reported in previous studies [37, 44, 45, 67]. Interestingly, we found that partial inhibition of NFκB by SN50 or BAY resulted in a much greater reduction in IL-6 compared to TNFα, a pattern similar to the effect of DHA. Unlike SN50, BAY is a less specific NFκB inhibitor. BAY has been reported to inhibit the activation of multiple

kinases including MAP kinases that activate nuclear transcription factors such as AP-1 which up-regulate TNFα and IL-6 transcription [41, 42, 68]. In comparison, while SN50 has also been reported to inhibit the nuclear import of AP-1 and other non-NFκB transcription factors, it targets further downstream than BAY, which makes it more explicit to attribute the consequences generated by using SN50 to suppressed NFκB activation [69].

One possible reason for the discrepancy in the response of IL-6 and TNFα gene expression to the general reduction in NFκB activity could be that other regulatory mechanisms come into play that upregulate TNFα gene expression to a greater extent than IL-6 gene expression. Transcription factors activated by the mitogen-activated protein kinase (MAPK) signaling pathway, such as AP-1, also regulate TNFα and IL-6 gene expression. In comparison to NFκB, few studies have examined how DHA impacts MAPK signaling pathways and/or AP-1 activation in stimulated macrophages. LPS-stimulated RAW 264.7 macrophages have been shown to have reduced MAPK signaling by DHA treatment [47], and similar observations have been made in other cell types including T-cells, endothelial cells and cancer cells [70-72]. Stronger compensatory mechanisms for TNFα compared to IL-6 gene expression may be explained by a more fundamental difference in the transcriptional regulation of these genes. This is exemplified by the temporal difference in their inductions in response to LPS. The early induction of TNFα relative to IL-6 in response to LPS is attributed to a "constitutively and immediately accessible" promoter region. In contrast, IL-6 induction lags behind TNFα due to a promoter with "regulated" and "late accessibility," which requires

stimulus-induced chromatin remodeling [73, 74]. Similar to what is observed in cell culture, plasma TNFα has been observed to increase before IL-6 in response to an intravenous administration of LPS in humans [75]. If triggering the NFκB pathway in turn activates proteins involved in chromatin remodeling at the IL-6 promoter, inhibition of this pathway would also limit the possibility of other transcription factors accessing the promoter and activating IL-6 gene transcription in a compensatory manner. The greater dependence of IL-6 compared to TNFα on NFκB may also be explained by the importance of early NFκB gene products for IL-6 promoter activation. A growing body of evidence suggests that one such NFκB gene product is IκBζ protein which has been shown to be indispensable for LPS-induced IL-6 but not TNFα gene transcription [76-78]. Therefore, it is plausible that the additional requirement of an NFκB gene product is contributing to the greater sensitivity of IL-6 to a moderate reduction in NFκB compared to TNFα. Targeting candidate NFκB gene products such as IκBζ will help answer this question.

In summary, this study demonstrated a differential effect of DHA on TNFα and IL-6 gene expression in LPS-stimulated RAW 264.7 cells and this effect of DHA may be mediated by a partial inhibition of the NFκB signaling pathway. DHA's effect on recruitment of NFκB as well as alternative transcription factors such as AP-1 to the promoter region of each cytokine gene, and the influence of alternative transcription factors on TNFα and IL-6 gene transcription during reduced NFκB activity will help elucidate gene-specific regulatory mechanisms at work. In addition to further *in vitro* mechanistic studies, future research is also needed to validate the biological relevance

and clinical implications of the unique effects of DHA on specific pro-inflammatory cytokines.

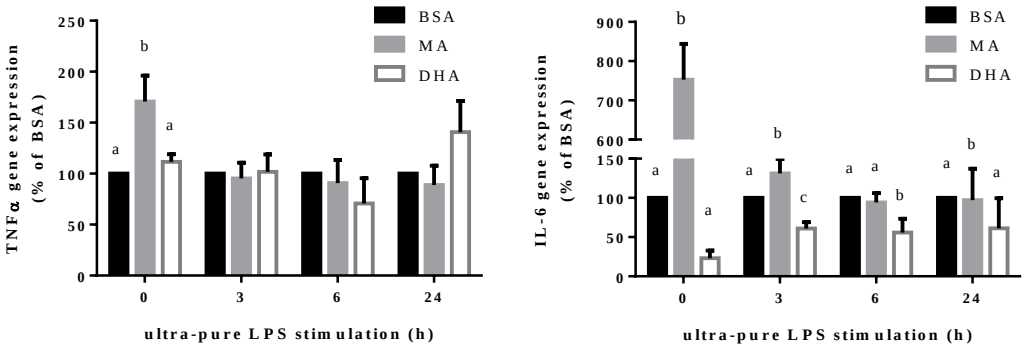

Fig. 1. Effect of fatty acid on TNFα (A) and IL-6 (B) gene expression. RAW 264.7 cells were pretreated with DHA or MA (100 μM, 24 h) then stimulated with ultra-pure LPS (100 ng/mL) in the presence of treatment fatty acid for the times indicated. Bars without common letters within the same time group statistically differ at $p < 0.05$ determined by one-way ANOVA, adjusted with Tukey's post-hoc test for multiple comparisons. Values are mean ± SD of three independent experiments.

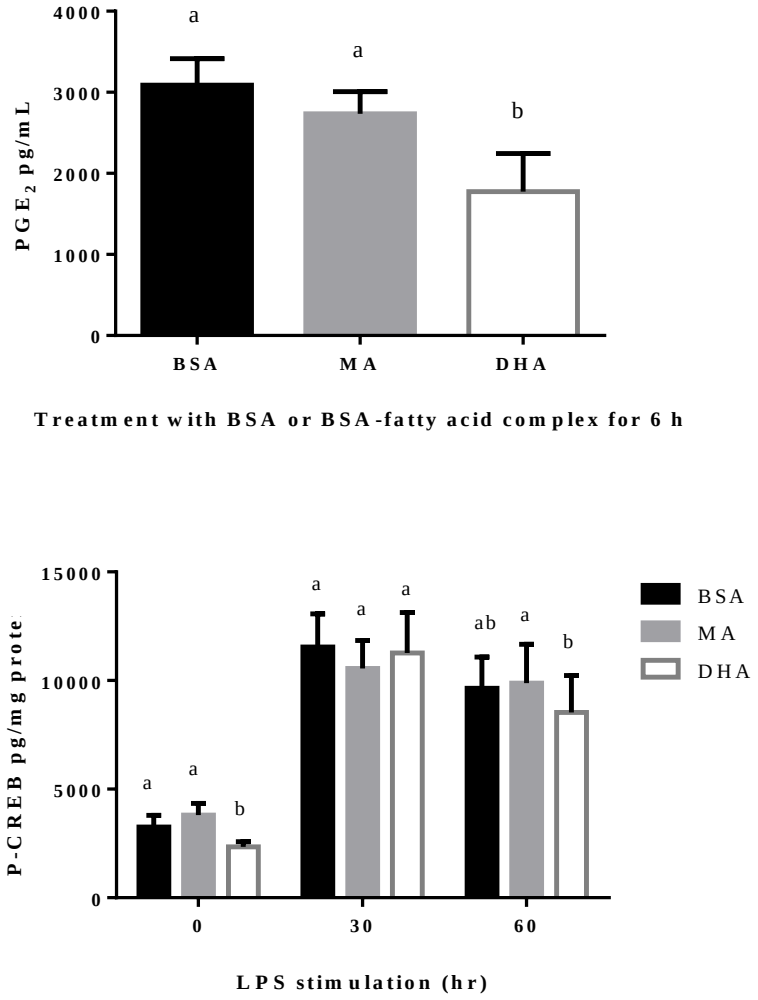

Fig. 2. Effect of fatty acid on PGE_2 secretion and CREB activity in RAW 264.7 cells. (A) Cells were pretreated with MA or DHA (100 μM, 24 h) and then stimulated with ultra-pure LPS (100 ng/mL, 6 h). PGE_2 concentration in culture media was determined by ELISA. Values are mean ± SD of three independent experiments. Bars without common letters within each time group statistically differ at $p < 0.05$ determined by one-way ANOVA adjusted with Tukey's post hoc test for multiple comparisons. (B) Cells were pretreated with MA or DHA (100 μM, 24 h) and then stimulated with ultra-pure LPS (100 ng/mL) for the times indicated. The concentration of P-CREB in whole cell lysates was determined by ELISA. Values are mean ± SD of four independent experiments. Bars without common letters within each time group statistically differ at $p < 0.05$ determined by two-way repeated measures ANOVA adjusted with Tukey's post-hoc test for multiple comparisons.

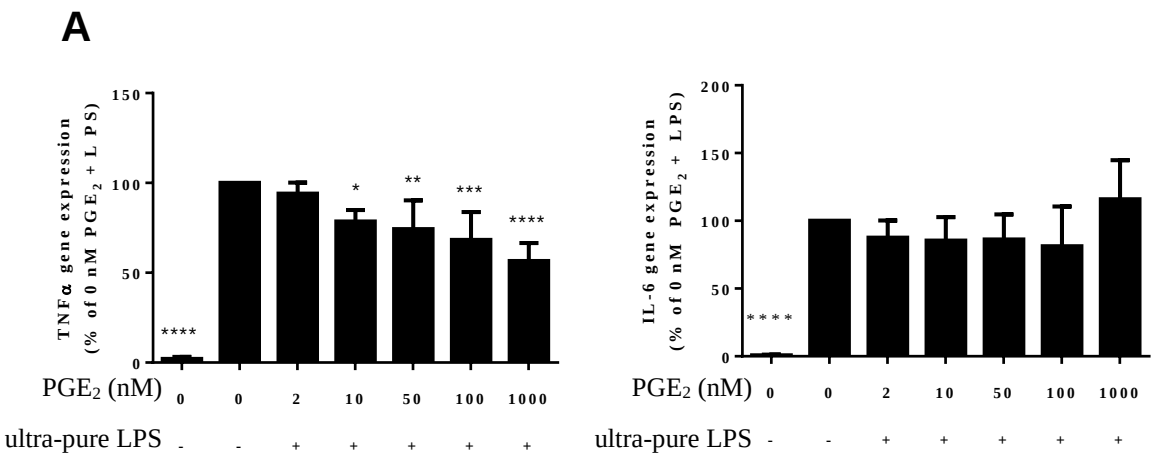

Fig. 3. Effect of exogenous PGE_2 on (A) TNFα and (B) IL-6 gene expression. RAW 264.7 cells were incubated with exogenous PGE_2 at the concentrations indicated for 45 min, and then stimulated with ultra-pure LPS (100 ng/mL, 3 h). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$ vs. 0 nM PGE_2 + LPS. Statistical difference determined by one-way ANOVA adjusted with Tukey's post-hoc test for multiple comparisons.

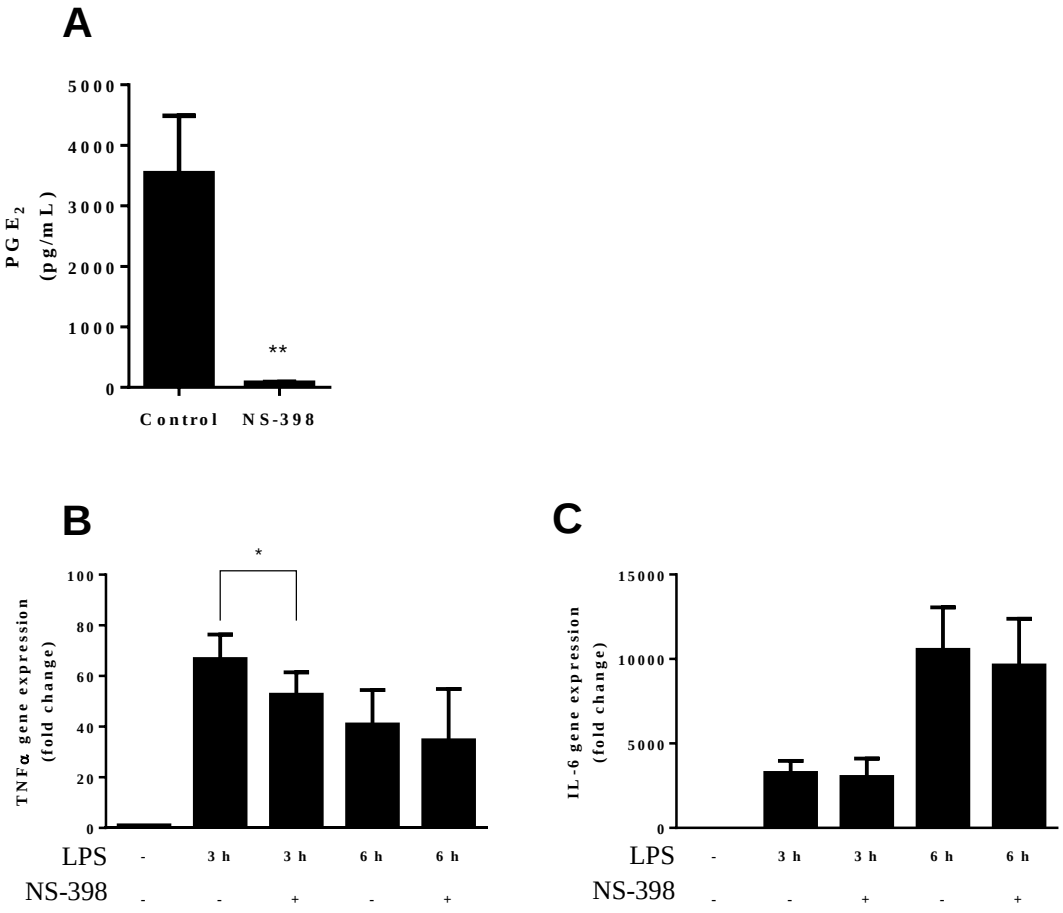

Fig. 4. Effect of NS-398 on (A) PGE_2 secretion, (B) TNFα and (C) IL-6 gene expression. RAW 264.7 cells were pretreated with NS-398 (10 μM, 18 h) then stimulated with ultra-pure LPS (100 ng/mL) for the times indicated. Values are mean ± SD of three independent experiments. * $p < 0.05$ determined by two-way, repeated measures ANOVA. ** $p < 0.01$ vs. control determined by t test.

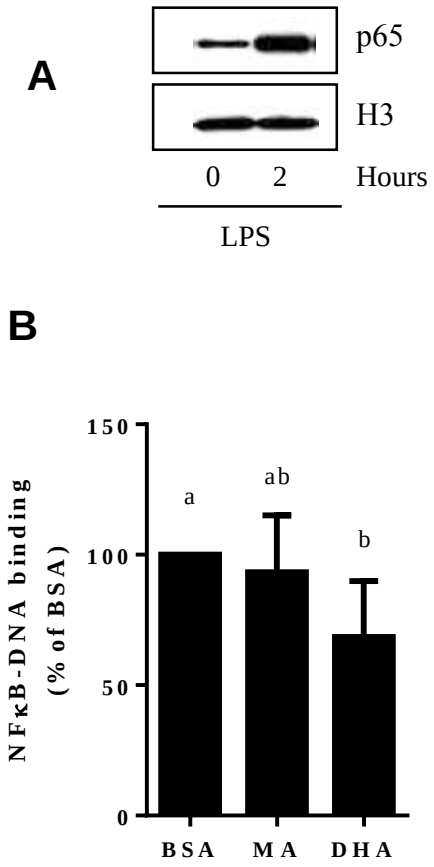

Fig. 5. NFκB activity in RAW 264.7 cells. (A) Western blot of nuclear p65 protein expression before and after 2 h of ultra-pure LPS exposure compared to histone 3 (H3) protein expression (nuclear loading control). One representative experiment is shown out of 3 independent experiments that had similar results. (B) Cells were pretreated with MA or DHA (100 μM, 24 h), then stimulated with ultra-pure LPS (100 ng/mL, 30 min). NFκB-DNA binding in nuclear extracts was determined by ELISA. Values are mean ± SD of five independent experiments. Bars without common letters statistically differ at $p < 0.05$ determined by one-way ANOVA adjusted with Tukey's post-hoc test for multiple comparisons.

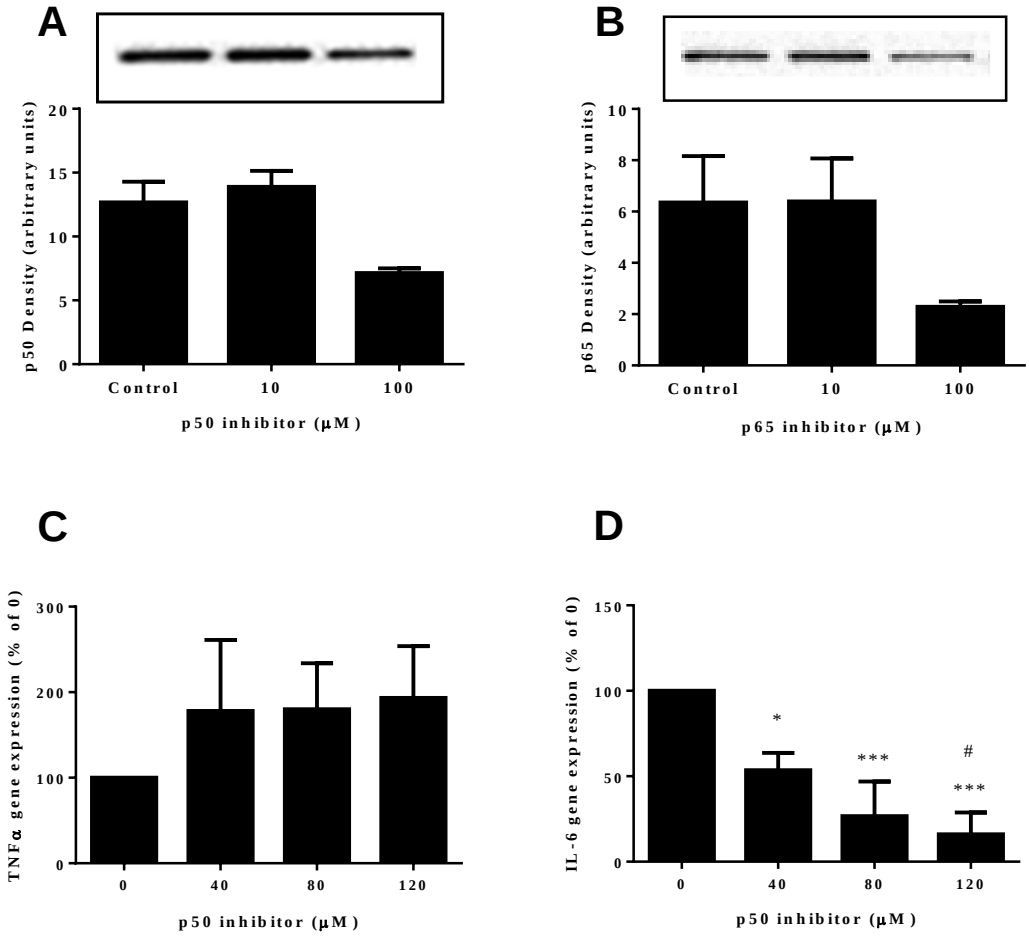

Fig. 6. Effect of SN50 in RAW 264.7 cells. Cells were pretreated with SN50 for 15 min at the concentrations indicated then stimulated with ultra-pure LPS (100 ng/mL, 3 h). Protein expression of p50 (A) and p65 (B) in nuclear extracts determined by western blot expressed as mean of duplicate samples from one experiment. (C) TNFα and (D) IL-6 mRNA expression values are mean ± SD of three independent experiments. * $p < 0.01$; *** $p \leq 0.001$ vs. 0; # $p \leq 0.01$ vs. 40, determined by one-way ANOVA, adjusted with Tukey's post-hoc test for multiple comparisons.

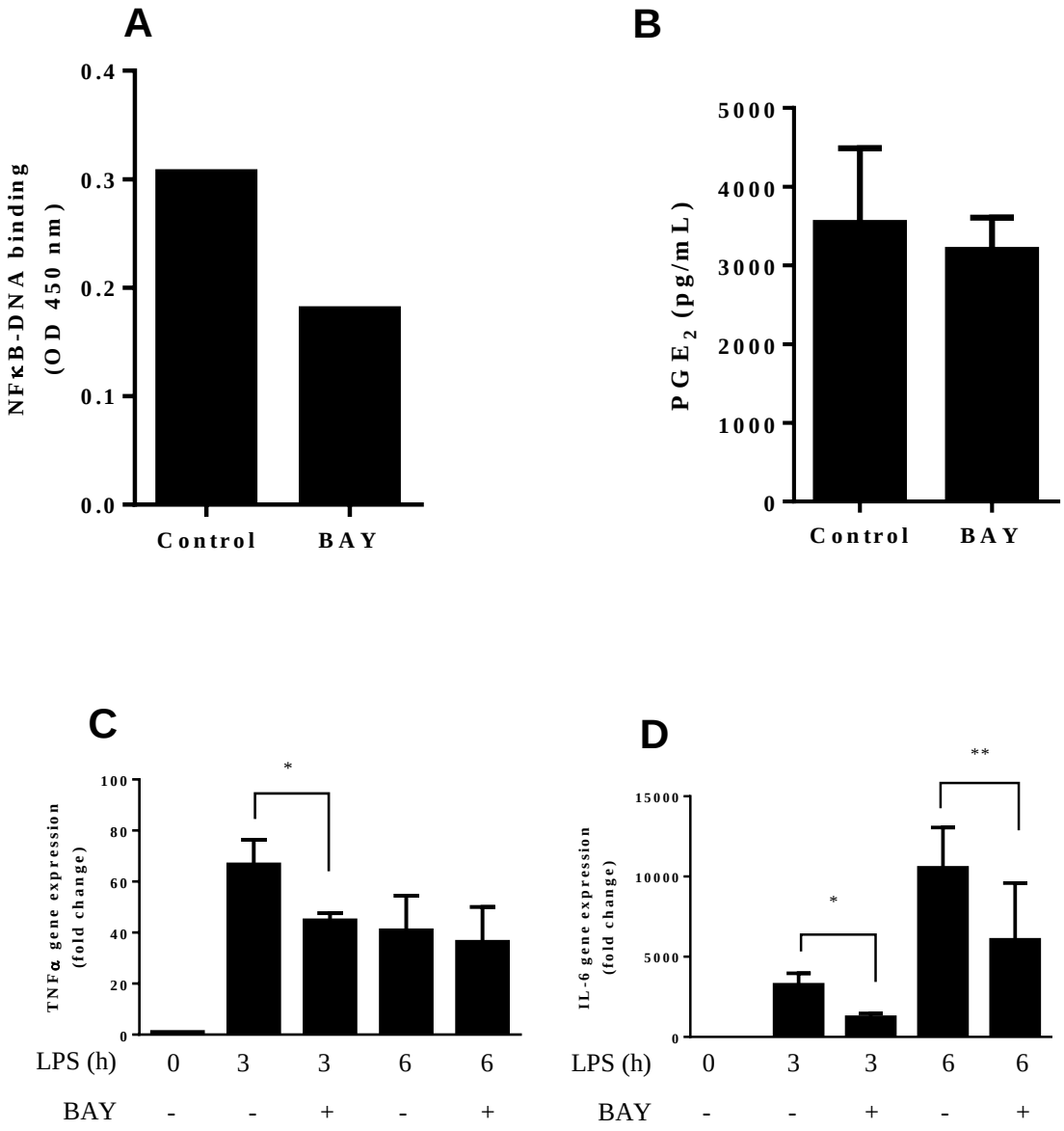

Fig. 7. Effect of BAY on RAW 264.7 cells. Cells were pretreated with BAY (10 μM, 18 h) then stimulated with ultra-pure LPS (100 ng/mL). (A) NFκB-DNA binding determined by ELISA. Values are mean of triplicate samples of one experiment. (B) PGE2 secretion after 6 h of LPS stimulation. (C) TNFα and (D) IL-6 gene expression. Values are mean ± SD of three independent experiments. * $p \leq 0.05$ vs. control; ** $p \leq 0.01$ vs. control determined by two-way ANOVA adjusted with Sidak's post-hoc test for multiple comparisons.

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CHAPTER 5
Summary and Discussion

1. Summary of results

Inflammation plays a critical role in the development of atherosclerotic lesions. Evidence supports a role of toll-like receptor 4 (TLR4) in heightening inflammatory conditions in arterial lesions. Long-chain omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are anti-inflammatory [1], and have been shown to decrease several cardiovascular risk factors [2]. However, the effect of EPA and DHA on TLR4-mediated pro-inflammatory cytokine production and TLR4 signaling is not well characterized in macrophages.

The overall aim of this research project was to determine the effect of EPA or DHA cell membrane enrichment on TLR4 activated tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) production, plasma membrane receptor expression, and signaling pathways influencing the expression of TNFα and IL-6 genes in lipopolysaccharide (LPS) stimulated RAW 264.7 cells.

EPA and DHA

Our results confirm that EPA and DHA reduce the inflammatory response of RAW 264.7 induced by TLR4 activation. EPA and/or DHA reduced TNFα, IL-6 and prostaglandin E2 (PGE2) production and nuclear factor κB (NFκB) activity in LPS stimulated cells. By using ultra-pure LPS to stimulate TLR4 in all of the experiments, we ruled out the possible involvement of TLR2, which can also be activated by impure LPS. EPA and DHA reduced IL-6 protein secretion significantly more than TNFα secretion

from cells stimulated with LPS for 6 or 24 h. DHA had a similar differential effect on TNFα and IL-6 mRNA expression. However, although, IL-6 mRNA expression was significantly reduced, DHA had no significant effect on TNFα mRNA expression. These data suggest that DHA may have affected post-transcriptional and post-translational regulation of TNFα. Future studies will need to investigate the influence of DHA on TNFα protein translation, TNFα protein stability and secretion, in order to pinpoint which regulatory step(s) were targeted by DHA.

The differential inhibitory effect on TNFα and IL-6 suggested that EPA and DHA largely targeted downstream regulatory components rather than TLR4 itself or associated molecules. We measured cell surface expression of TLR4, TLR4-MD2 complex and CD14 in DHA-pretreated cells before and after LPS stimulation for 10 min – 360 min. We found no difference in the cell surface expression of these receptors under any of these treatment conditions. The inability of DHA to affect the level of LPS binding to the cell surface also confirmed these results. Our results largely agree with those of Chang *et al* who recently published the results of a study investigating the effects of saturated (palmitic and stearic acids) and unsaturated (oleic and linoleic acids) fatty acids on TLR4-mediated pro-inflammatory response in RAW 264.7 cells [3]. They did not find any difference in either total or cell surface protein expression of TLR4 or CD14 (determined by western blot and flow cytometry, respectively) after 12 h of pretreatment with any fatty acid followed by LPS stimulation for 6 h. The expression pattern of TLR4 and CD14 were also similar between the two studies: relatively low levels of TLR4 while high levels of CD14 were detected. The results from the study by Chang *et al* and our

current study are not in agreement with Wong *et al* who reported a decrease in TLR4 expression in lipid rafts after cells were co-treated with DHA and LPS for a relatively short period of time, 7 min [4]. The combined data raise the question of whether changes in TLR4 expression due to DHA only take place in lipid rafts rather than in total plasma membrane, and whether the duration of exposure of cells to DHA and LPS before measuring TLR4 expression may also make a difference. Future studies are needed in order to resolve these open issues.

Myristic acid (MA)

Since saturated fatty acids are pro-inflammatory, we hypothesized that MA cell enrichment would potentiate the induction of TNFα, IL-6 and PGE2 production or NFκB activity by LPS, by upregulating cell surface TLR4 protein expression. However, MA enrichment did not further increase the pro-inflammatory profile of RAW cells. We also found that MA did not increase the cell surface expression of TLR4 and associated molecules or the level of LPS binding to the cell surface. However, MA did increase TNFα and IL-6 mRNA levels in unstimulated cells. These data suggest that similar to the saturated fatty acid, lauric and palmitic acids [5], MA may act mainly as a TLR4 agonist and not a TLR4 regulator. Our results are consistent with those reported by Wang *et al* who observed no further increase in TNFα, IL-6 and MCP-1 production in MA-pretreated THP-1 cells stimulated with LPS [6]. Varying the duration of MA pretreatment will aid in understanding the relationship between MA effects and MA cell membrane content. In

addition, the use of a TLR4-neutralising antibody would help determine whether MA's effects were mediated by TLR4 activation.

Differential regulation of TNFα and IL-6 gene by DHA

We found that EPA and DHA decreased IL-6 production to a greater extent than TNFα production in RAW 264.7 cells stimulated with LPS. Similar differential effects of omega-3 fatty acids on the production of individual cytokines observed in human intervention studies provided a rationale for investigating the differences between TNFα and IL-6 regulation *in vitro*. Both Vedin *et al* [7] and Wallace *et al* [8] reported a reduction in IL-6 but not TNFα secretion in stimulated PBMCs harvested from subjects supplemented with EPA or DHA, or fish oil containing these fatty acids, respectively. Wallace, *et al* duly noted "This suggests that there may be subtle differences in the mechanism by which *n*-3 PUFA influence the production of different cytokines [8]." No one to our knowledge has previously investigated differences in the regulation of TNFα and IL-6 by EPA or DHA. We examined the effect of DHA on PGE_2 and cAMP response-binding protein (CREB) because the available data suggested differential regulation of TNFα and IL-6 by PGE_2 [9-16] and CREB [17, 18], through common or independent pathways. DHA reduced PGE_2 production as expected, but did not change P-CREB levels in stimulated cells. Further investigation of PGE_2 revealed differential influence on TNFα and IL-6. However, our data did not implicate PGE_2 as a mediator of DHA's differential effects on TNFα and IL-6 gene transcription. Moreover, the influence of PGE_2 on TNFα mRNA expression was not straightforward. According to our results,

PGE_2 induced TNFα mRNA expression at low concentrations and inhibited TNFα mRNA expression at high concentrations. A similar effect on TNFα secretion was reported in rat resident peritoneal macrophages [19]. An inhibitor that specifically inhibits PGE_2 synthesis rather than a cyclooxygenase 2 inhibitor will be needed to confirm a concentration-dependent dual regulatory role of PGE_2 on TNFα expression. Exposing DHA-pretreated cells with exogenous PGE_2 cells may help determine whether the reduction in PGE_2 production influenced the overall effect of DHA on TNFα and IL-6 gene expression.

Because DHA reduced LPS-induced NFκB activity in RAW 264.7 cells, we investigated the effect of reduced NFκB activity on TNFα and IL-6 gene expression. We found that partial inhibition of NFκB resulted in a much greater reduction in IL-6 than TNFα, similar to the effect of DHA. This suggested that DHA's effects might be largely mediated by a reduction in NFκB activity alone, and that other regulatory mechanisms may come into play that upregulate TNFα gene expression to a greater extent than IL-6 gene expression. TLR4 signaling also activates mitogen-activated protein kinase (MAPK) pathways, which, through the activation of transcription factor activator protein 1 (AP-1) also regulate the production of pro-inflammatory cytokines [20]. Inhibiting AP-1 in DHA-treated cells would help determine the role of AP-1 in TNFα and IL-6 transcription. Additionally, the effect of DHA on the recruitment of NFκB, AP-1, or other potentially influential regulatory factors to the promoter regions of TNFα and IL-6 will provide more direct evidence for differences in the transcriptional influences of these factors between TNFα and IL-6 genes.

2. Limitations

The biological relevance of the results of our research is challenged by the high concentrations of fatty acid and LPS imposed on our cells. Pretreating cells with 100 µM of EPA or DHA resulted in cell membrane EPA and DHA levels of 8.4 % and 16.9% (total fatty acids), respectively. In comparison, peripheral blood mononuclear cells (PBMCs) isolated after subjects were supplemented with 1600 mg DHA/day for two weeks had a mean DHA level of 5.7% [21]. In Greenland Inuits, who are among the highest consumers of marine animals, serum phospholipid EPA and DHA levels were reported to be 4.9% and 7.9% (total fatty acids), respectively. In the same study, individuals living in Canada who were supplemented with EPA and DHA for 3 weeks surpassed the EPA levels in Greenland Inuits (7.1%), and achieve similar levels of DHA (6.9%) [22]. These data suggest that even with supplementation, it is improbable that EPA and DHA levels attained in our cell model can be replicated in *in vivo*. Similarly, the concentration of free EPA or DHA bound to BSA in human plasma is unlikely to reach 100 µM. However, Weldon *et al* observed anti-inflammatory effects of EPA and DHA at both 25 µM and 100 µM (after 48 h pretreatment) in THP-1 cells, demonstrating that the effect does not depend on very high concentrations (at least in this cell line) [23] .

Perhaps related to the high proportion of cellular EPA achieved with treatment, and differences in the activity of enzymes that elongate or hydrolyze fatty acids, the proportion of docosapentaenoic acid (DPA) in the RAW 264.7 cells increased by 6-fold. Similar observations were previously reported of both RAW 264.7 cells and human THP-

1 cells [6, 24]. However, the increase in DPA due to EPA supplementation is considerably less *in vivo*. After EPA supplementation mouse macrophage DPA content increased 2-fold [25] and human plasma phospholipids increased 1.3-fold [26]. Whether the substantial increase in cellular DPA observed in RAW 264.7 cells influenced the anti-inflammatory effects of EPA-treated cells are not known, but is a critical point in determining the limits of studying EPA effects in macrophage cell lines.

Finally, in the current studies, the concentration of LPS used to stimulate cells, 100 ng/mL (100 EU/mL), is very high compared to endogenous blood concentrations in human. LPS is typically <1 EU/mL in healthy subjects, whereas levels approaching 15 EU have been reported in subjects with non-alcoholic liver disease [27-29]. The influence of LPS levels on the anti-inflammatory effect of EPA and DHA is not fully explored. Mullen *et al* found that EPA and DHA reduced cytokine secretion in THP-1 cells induced by a range of LPS concentrations, from 10 ng/mL to 1000 ng/mL, but not at 1 ng/mL [30]. Similarly, Vedin *et al* observed a decrease in the release of IL-1β and granulocyte colony-stimulating factor from PBMCs stimulated by LPS at 10 ng/mL but not at 1 ng/mL, while IL-6 release was decreased at both concentrations (43% vs 41%, significance unreported) [7]. Interestingly, the findings of Weldon *et al* and Vedin *et al* are consistent with Sibjen and Calder who stated that "In general, the direction of immunomodulation in healthy subjects (if any) and in inflammatory conditions is the same…However, the extent of the effect might be very different in inflammatory conditions…" [31]. Human intervention trials are needed to specifically address the relationship between the inflammatory status of subjects and the efficacy of EPA and

DHA supplementation on reducing cytokine production. Data obtained from these studies will also aid in developing appropriate macrophage models for investigating EPA and DHA effects *in vitro*.

3. Conclusion and Future Direction

The anti-inflammatory effects of EPA and DHA cell enrichment in TLR4-activated RAW 264.7 cells were addressed in this thesis research. This is the first study, to our knowledge, to demonstrate that cell membrane enrichment of RAW 264.7 cells with EPA or DHA reduces the inflammatory response to a pure TLR4 ligand. Additionally, our data suggest that DHA inhibits signaling pathways downstream of TLR4 activation. There was no change in the cell surface expression of TLR4, TLR4-MD2 complex, or CD14, or alteration in the level of LPS binding to the cell surface resulting from DHA membrane enrichment. However, future work is needed to determine whether DHA affected TLR4 recruitment to lipid raft fractions of the membrane as Wong *et al* have previously reported [4]. Therefore, the effect of DHA on TLR4 signaling remains inconclusive. This study also addressed the differential effect of DHA on TNFα and IL-6. While we were unable to pinpoint the regulatory molecule(s) that mediated these effects, we found that reduction in NFκB activity could be in part attributable for the differential effect of DHA. Our findings also suggest that alternative mechanisms may exist to upregulate TNFα gene expression to a greater extent than IL-6 gene expression when NFκB activity is reduced. Future studies will need to determine what these mechanisms are. Finally, the differential effects of EPA and DHA on individual

pro-inflammatory cytokines observed *in vitro* must be defined *in vivo*, and whether these effects are meaningful or beneficial in reducing atherosclerotic lesion development. This information will lead to better dietary and supplement recommendations for prevention and treatment of cardiovascular disease.

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APPENDIX A

Supplementary Data

Supplementary Table 1. Selected fatty acid composition (mol%) after 24 h fatty acid pretreatment followed by 6 h of ultra-pure LPS stimulation in the absence of treatment fatty acid.

Fatty acid	Control		MA		EPA		DHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA	53.13	3.79	52.53	5.24	51.99	10.13	49.45	2.50
14:0	3.27	0.48	5.66	1.86	3.22	0.64	3.67	1.18
16:0	26.01	2.76	23.53	2.82	26.03	*	24.06	*
18:0	18.94	1.17	18.61	0.72	20.59	0.72	22.89	1.09
MUFA	29.01	6.30	31.63	7.57	30.45	6.72	27.64	5.39
16:1n-9	3.27	1.80	2.40	0.87	4.34	3.11	2.61	1.15
16:1n-7	3.20	1.70	2.58	0.96	1.85	0.56	2.52	0.73
18:1n-9	16.13	1.88	13.10	4.58	14.90	2.33	15.24	2.96
181n-7	4.67	6.85	7.83	6.74	7.80	7.34	4.43	5.82
PUFA								
n-6 PUFA	8.85	1.50	8.71	2.61	6.87	1.28	9.40	3.18
18:2	2.50	0.48	3.56	2.39	1.58	0.51	2.36	0.47
20:4	4.01	1.20	4.04	0.40	3.44	1.05	4.08	1.74
22:4	0.34	0.16	0.22	0.03	0.37	0.10	0.31	0.12
n-3 PUFA	4.79	1.39	3.59	0.48	12.41	4.89	7.95	0.20
20:5	0.54	0.22	0.32	0.19	2.83	0.45	0.34	0.19
22:5	1.34	0.26	0.71	0.28	6.07	5.44	0.95	0.68
22:6	1.30	0.97	1.20	0.23	1.08	0.58	3.34	2.77

MA, myristic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Fatty acids that comprised less than 1 mol% of total fatty acids are not included, but are included in the calculations. Values are mean ± SD of 3 independent experiments. * Average of two experiments.

Supplementary Table 2. Selected fatty acid composition (mol%) after 24 h fatty acid pretreatment followed by 24 h of ultra-pure LPS stimulation in the absence of pretreatment fatty acid.

Fatty acid	Control		MA		EPA		DHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA	52.94	*	52.94	3.83	55.71	3.30	49.36	1.46
14:0	3.80	0.63	5.51	1.45	3.75	0.24	3.12	0.27
16:0	27.02	*	24.09	2.03	30.13	2.91	25.65	0.16
18:0	21.77	4.33	19.48	1.66	20.19	0.87	17.47	0.41
MUFA	36.63	10.43	32.06	5.50	20.99	6.68	33.74	3.20
16:1n-9	3.22	0.95	2.77	0.83	2.20	0.66	2.63	0.52
16:1n-7	3.67	1.59	3.42	1.44	2.64	0.68	3.29	0.90
18:1n-9	19.29	1.77	18.44	2.20	12.77	6.25	16.39	3.73
181n-7	9.09	6.85	6.48	6.74	2.29	7.34	7.89	*
PUFA								
n-6 PUFA	7.00	0.84	8.16	1.40	7.70	1.18	6.84	0.36
18:2	1.93	0.64	2.39	0.74	2.51	0.65	2.77	0.09
20:4	4.01	1.20	4.04	0.40	3.44	1.05	4.08	1.74
22:4	0.34	0.17	0.34	0.04	0.34	0.15	0.33	0.05
n-3 PUFA	4.14	1.43	4.15	0.21	10.07	5.99	5.55	1.25
20:5	0.41	0.02	0.54	0.29	1.90	1.29	0.53	0.14
22:5	1.15	0.66	1.03	0.66	5.87	4.10	1.03	0.34
22:6	1.39	0.84	1.34	0.76	1.42	0.93	3.05	1.19

MA, myristic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Fatty acids that comprised less than 1 mol% of total fatty acids are not included, but are included in the calculations. Values are mean ± SD of 3 independent experiments. * Average of two experiments.

Supplementary Table 3. Selected fatty acid composition (mol%) after 24 h fatty acid pretreatment followed by 6 h of ultra-pure LPS stimulation in the presence of pretreatment fatty acid.

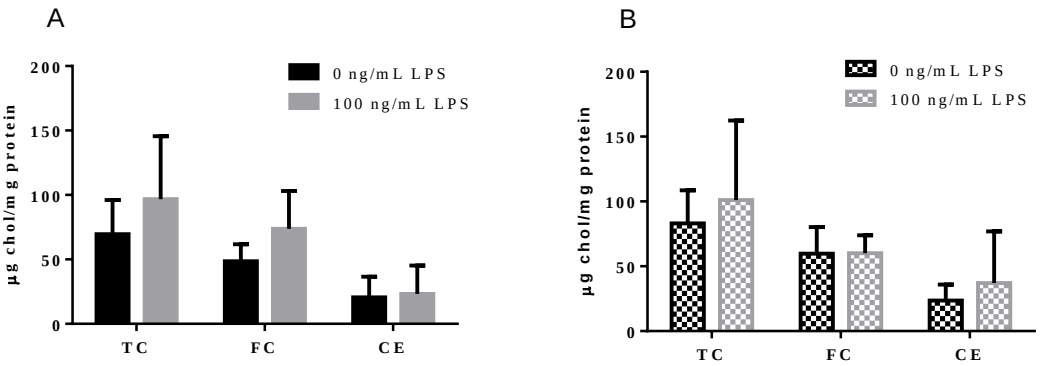
Fatty acid	Control		MA		EPA		DHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA	46.55	1.67	57.21	0.39	42.43	6.23	44.26	6.23
14:0	2.59	0.20	19.26	4.26	2.57	0.49	2.71	0.73
16:0	24.33	0.92	22.10	2.58	22.26	2.09	23.53	3.06
18:0	17.80	1.17	14.21	0.72	15.76	0.72	16.12	1.09
MUFA	39.94	0.50	30.76	1.85	25.60	4.50	26.40	7.20
16:1n-9	4.19	0.20	3.02	0.45	4.45	0.12	3.00	0.26
16:1n-7	4.54	0.92	4.30	1.02	2.05	0.10	2.33	0.95
18:1n-9	20.10	3.01	13.85	2.02	10.55	1.21	10.58	0.87
181n-7	10.28	2.82	8.50	2.06	9.03	4.21	8.86	4.83
PUFA								
n-6 PUFA	7.12	1.11	6.51	1.02	4.08	0.44	4.64	1.12
18:2	2.28	0.27	2.34	0.37	1.84	0.27	1.66	0.31
20:4	3.72	0.67	3.30	0.64	1.50	0.32	2.25	0.83
22:4	0.47	0.15	0.29	0.06	0.34	0.05	0.28	0.02
n-3 PUFA	4.37	1.34	3.64	1.33	26.10	7.59	22.52	14.9
20:5	0.44	0.17	0.45	0.28	8.74	2.83	0.32	0.11
22:5	1.60	0.64	1.22	0.47	16.02	5.68	0.98	0.21
22:6	1.96	0.74	1.59	0.64	0.97	0.24	20.79	14.82

MA, myristic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Fatty acids that comprised less than 1 mol% of total fatty acids are not included, but are included in the calculations. Values are mean ± SD of 3 independent experiments.

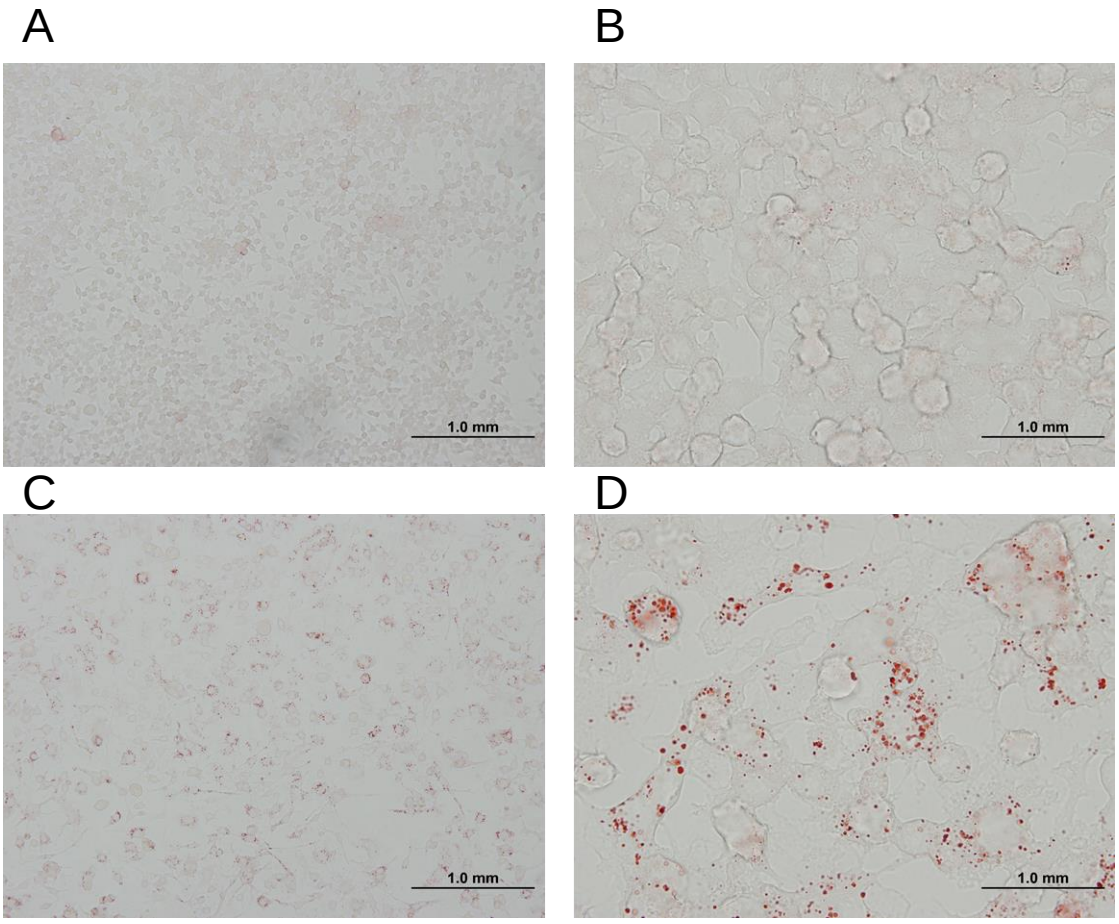
Supplementary Table 4. Selected fatty acid composition (mol%) after 24 h fatty acid pretreatment followed by 24 h of ultra-pure LPS stimulation in the presence of pretreatment fatty acid.

Fatty acid	Control		MA		EPA		DHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA	51.00	4.92	61.60	4.28	38.79	2.90	46.06	6.91
14:0	2.76	0.32	18.33	3.60	2.99	0.33	3.14	0.22
16:0	23.98	1.89	20.57	1.18	18.62	1.56	22.80	1.76
18:0	22.89	7.25	21.56	4.19	16.43	3.51	19.24	5.27
MUFA	37.73	3.80	28.89	3.49	17.71	2.79	22.10	8.33
16:1n-9	3.86	0.58	3.23	0.34	2.30	0.15	2.73	0.38
16:1n-7	4.80	0.77	4.43	0.82	1.56	0.09	2.31	1.42
18:1n-9	22.00	1.63	14.92	0.94	9.45	0.94	12.37	4.88
181n-7	6.70	0.94	6.09	1.40	4.16	0.88	4.82	1.84
PUFA								
n-6 PUFA	5.95	0.84	5.22	0.78	4.11	0.46	4.76	1.30
18:2	1.86	0.23	1.94	0.49	1.45	0.21	1.77	0.65
20:4	3.00	0.49	2.47	0.39	1.76	0.29	2.17	0.56
22:4	0.40	0.08	0.26	0.03	0.46	0.04	0.43	0.38
n-3 PUFA	4.11	0.62	3.29	0.51	38.64	0.39	26.34	15.46
20:5	0.40	0.06	0.37	0.10	11.11	0.38	0.27	0.05
22:5	1.48	0.25	1.15	0.17	25.98	1.21	1.41	0.22
22:6	1.85	0.32	1.52	0.30	1.28	0.32	24.23	15.26

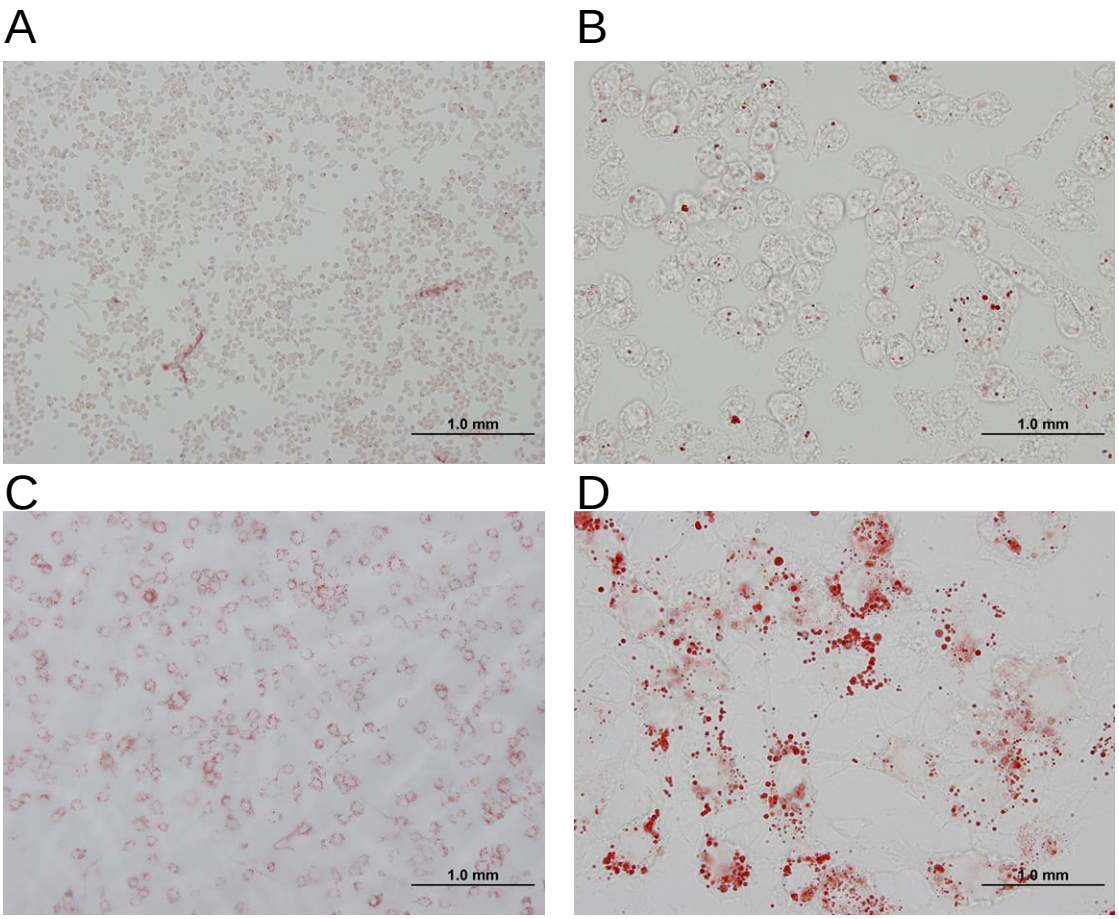
MA, myristic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Fatty acids that comprised less than 1 mol% of total fatty acids are not included, but are included in the calculations. Values are mean ± SD of 3 independent experiments.



Supplementary Fig. 1. Effect of LPS on cholesterol accumulation in RAW 264.7 cells. Total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE) were determined in (A) cells treated with or without ultra-pure LPS for 2 h, and (B) cells treated with 100 µg/mL of low density lipoprotein (LDL) with or without 100 ng/mL of LPS for 48 h. (Cells were pre-treated with LPS for 2 h followed by LPS + LDL for an additional 48 h.) Value are mean ± SD of 4 independent experiments (A) or 3 independent experiments (B).



Supplementary Fig. 2. Effect of LPS on lipid accumulation in RAW 264.7 cells. Neutral lipid accumulation was determined by Oil Red O staining. Control cells viewed under (A) 20X and (B) 100X magnification. Cells stimulated with LPS (100 ng/mL, 96 h) viewed under (C) 20X and (D) 100X magnification.



Supplementary Fig. 3. Effect of LPS on lipid accumulation in RAW 264.7 cells treated with low density lipoprotein (LDL). Neutral lipid accumulation was determined by Oil Red O staining. Control cells treated with LDL (100 µM, 72 h) without LPS viewed under (A) 20X and (B) 100X magnification. Cells pretreated with LPS (100 ng/mL, 24 h) followed by treatment with LDL (100 µM) plus LPS (100 ng/mL) for an additional 72 h, viewed under (C) 20X and (D) 100X magnification.

APPENDIX B

Research Methods

1. Culturing RAW 264.7 cells

Materials

RAW 264.7 cells (ATCC, TIB-71)
Dulbecco's modified eagle's medium (DMEM) (Invitrogen, 11995-065) – Light sensitive!
Fetal bovine serum (Sigma, F6178-500mL)
Penicillin/Streptomycin 10,000 U/mL (Invitrogen, 15140122)
100 mm x 15 mm polystyrene petri dish (Fisher, 0875713)
Corning™ cell lifter (Fisher, 3008)
2 mL sterile cryovials
Millipore Millex sterile syringe filters (0.22uM and Low Protein Binding) (Fisher, SLGV033RS)
Trypan Blue (Fisher, catalog No. ICN1691049)
70% alcohol
Dimethy sulfoxide (DMSO) high purity, sterile, for cell culture
100% isopropyl alcohol
Serological pipets (5 mL, 10 mL, 25 mL)
Autoclaved pipet tips
Autoclaved 1.5 mL microcentrifuge tubes
Autoclaved Pasteur pipets and bulbs

Equipment

Cryogenic Storage (6th floor, common room)
CO_2 incubator (Thermo Scientific Revco* Ultima II*, 11-700-136)
Biosafety cabinet (The Baker Company)
Microscope (Fisher, 12-561B)
Refrigerated Centrifuge (Sorvall RT6000B, Dupont)
Centrifuge Rotor- H-1000B
Water bath
Cell freezing container (stored at RT)
Cryogenic storage (6th floor, common room)

Things to do before starting

- Read the Product Information Sheet for ATCC® TIB-71
- Read an online introduction to cell culture manual (Example: http://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics.html/)
- Have the following autoclaved: 1L of diH2O, pipet tips, glass Pasteur pipets, and 1.5 mL microcentrifuge tubes.
- Turn on water bath set to 37℃.
- Turn on UV light of biosafety cabinet.

Protocol for starting a new culture

1. Clean CO2 incubator (see product manual).
2. Clean trays and tub with hot soapy water. Dry with paper towels. Clean with 70% alcohol. Wipe with large kimwipe. Place trays and tub in biosafety cabinet and turn on UV for at least 10 min.
3. Clean inside and door of incubator with 70% alcohol and Kimwipe.
4. Fill tub with 1 L autoclaved, sterile diH2O, and place in incubator.
5. Prepare biosafety cabinet for use.
6. Turn on blower and open cabinet up (maximum height is noted on cabinet side).
7. Clean inside of cabinet with 70% alcohol.
8. Clean all materials to be used (pipets, tubes, etc) with 70% alcohol, and place inside cabinet.
9. Make sure all pipet tip sizes are inside.
10. UV light must be on for at least 10 min before using the safety cabinet.
11. Make Complete Medium (biosafety cabinet lights off). *Complete Medium was used for both growing and for during experiments.*
12. Thaw a 50 mL vial of FBS in 37°C (or at 4°C overnight). Warm to 37°C. Clean tube with 70% alcohol.
13. Warm a bottle of 500 mL DMEM to 37° C. Clean bottle with 70% alcohol (especially cap area).
14. Remove 55 mL of DMEM and place in a new 50 mL Falcon tube. Label "sterile DMEM" cover with foil, and store in 4°C. *This aliquot of DMEM may be used to prepare 1 mM fatty acid stocking solutions. See section "2. Fatty acid preparation 1 mM."*
15. Add 50 mL of FBS and 5 mL of Penicillin/Streptomycin to make DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin. Mix by pipetting up and down several times with 25 mL pipet. Mark contents and date on bottle. Do not use after color changes (3-4 weeks).
16. Thaw cryovial of cells stored in cryogenic storage (6th floor).
17. Use key (attached to storage unit) to unlock door. Protecting hands (thick or double gloves) and eyes, remove shelf slowly, letting nitrogen drip off shelf and into tank before completely removing from storage unit. Place shelf on ground and remove box and 1 vial of cells.
18. Thaw cells by immersing the bottom-half of tube in warm tap water in a small container, while swishing.
19. Clean vial with 70% alcohol and place inside biosafety cabinet.
20. Add 9 mL of Complete Medium to 15 mL Falcon tube. Transfer vial contents using a glass pipet and bulb.
21. Centrifuge 125 x g for 5 min and remove liquid.
22. Add 8 mL complete medium to a 100 mm cell culture plate.
23. Resuspend cells using 1 mL of Complete Medium. Transfer to cell culture plate. Rinse tube with 1 mL of Complete Medium and transfer to cell culture plate. Tilt plate to distribute cells. Mark passage number (if new from supplier, "P1," if continuing, next passage number).

24. Place cells in CO2 incubator at 37℃ with 5% CO2 in air atmosphere. Incubate for 48 h, or, until culture reaches approximately 80% confluency.
25. Clean biosafety cabinet with 70% alcohol after use.

Protocol for cell subculture also called "splitting" or "passing" cells

1. Prepare biosafety cabinet for use (step 1 in "Protocol for starting a new culture").
2. Warm Complete Medium to 37℃ in water bath. Instead of warming up entire bottle, transfer amount needed into new, 50 mL Falcon tube (will need 10 mL per 100 mm dish for culturing and 20 mL per 100 mL dish for washing cells).
3. Wash cells with 10 mL Complete Medium twice. Remove liquid.
4. Add 5 mL of complete medium and dislodge cells from dish using cell lifter (slide cell lifter against dish bottom in one direction).
5. Pipet up and down to evenly suspend cells in media. Transfer to a 15 mL Falcon tube.
6. Count cells.
    - Transfer ~100 µL homogenous cell suspension into 1.5 microcentrifuge tube for cell counting.
    - In a new microcentrifuge tube, dilute cell suspension 8-fold with diH2O and 4-fold with Trypan Blue stock solution. (Example: 20 µL cell suspension + 40 µL Trypan Blue + 100 µL diH2O water. Be sure to mix cell suspension before taking out 20 µL.)
    - Pipet 10 µL of this mixture to each counting grid of clean, haemocytometer. For detailed instructions on counting cells using a haemocytometer, see http://cellculture.bitesizebio.com/articles/cell-counting-with-a-hemocytometer-easy-as-1-2-3/.
    - Count cells in each of four big corner squares and center big square (5 squares total). Repeat for second counting grid. Divide total cell count by two to get average count.
    - Calculate cell concentration (number of cells/mL of culture media) of original cell suspension. Total cells counted x dilution factor (8) x 2000. Multiply by total volume of cell suspension (5 mL) to determine total number of cells.
7. Add 1 x 10^6 cells and Complete Medium to each 100 mm dish (1 x 10^6 cells per 10 mL per 100 mm dish).
8. Determine volume of cell suspension needed for 1 x 10^6 cells (1 x 10^6 divided by cell suspension concentration).
9. Determine volume of Complete Medium needed for each dish (10 mL minus volume of cell suspension needed, step 8 above).
10. Add calculated volume of Complete Medium to each dish followed by calculated volume of cell suspension. Tilt dish to evenly distribute cells. Mark passage number. Incubate for 48 h.
11. Clean biosafety cabinet.

Protocol for expanding and freezing cells.

1. Subculture cells until you have enough cells to freeze, approximately 90 x 10^6 cells.
   - Freezing container can hold 18 cryovials.
   - Freeze 5 x 10^6 cells per cryovial. (Example: 5 x 10^6 cells/cryovial x 18 cryovials = 90 x 10^6 cells.)
2. Prepare cryovials and freezing container.
   - Wipe cryovials with 70% alcohol and place in biosafety cabinet. Label (number of cells, passage number, date and your ID).
   - Fill container with 100% isopropyl alcohol (up to mark).
3. Harvest and count cells (steps 1-6 in "Protocol for cell subculture…").
4. Prepare 1 mL of freezing media for each vial, or 18 mL for 18 vials.
   - Freezing media = 5% (v/v) DMSO Complete Medium. (Example: 18 mL x 0.05 = 0.9 mL DMSO)
   - Use sterile DMSO. To be safe, pass freezing media through Millipore Millex sterile syringe filters using a 10 mL syringe.
5. Transfer volume containing 5 x 10^6 cells per cryovial to a Falcon tube. (Example: for 18 cryovials transfer 90 x 10^6 cells). Pellet cells by centrifugation (125 x *g*, 5 min). Remove liquid.
6. Resuspend cell pellet in 18 mL freezing media.
7. Transfer 1 mL of cell freezing media solution to each cryovial. Place cryovial into the freezing container and store for 24 h at -80°C. (Do not store for more than a week at -80°C.)
8. Transfer cryovial from -80°C to cryogenic storage.

Protocol for plating a 3-day cell experiment

1. Determine number of wells and plates needed.
   - Number of treatments x 3 (replicates) + number of controls x 3 (replicates).
   - (Example: (3 treatments x 3) + (2 controls x 3) = 15 wells; for 35 mm well, three 6-well plates).
2. Prepare plates.
   - Remove plates from package and place in biosafety cabinet.
   - Label each well bottom with treatment or control type ID. Labeling lid top is helpful, but always label actual plate.
   - Label each plate lid top with experiment number, passage number, your ID, and date.
3. Determine number of cells and volume needed per well. (Example: 1 x 106 cells per 2 mL culture media per 35 mm well.)
4. Harvest and count cells (steps 1-6 in "Protocol for expanding and freezing cells") to determine the concentration of cell suspension (Example: 1.95 x 106 cells/mL.)
5. Make plating cell solution for experiment.
   - Determine cell density of plating cell solution needed. (Example: 1 x 10^6 cells per 2 mL culture media = 0.5 x 10^6 cells/mL)

- Determine volume of plating cell solution needed. (Example: 2 mL cell solution per well x 15 wells + 2 mL (extra) = 32 mL plating cell solution)
- Determine number of cells needed for volume of plating cell solution calculated above. (Example: 0.5 x 10^6 cells/ mL x 32 mL = 16 x 10^6 cells)
- Determine volume of cell suspension which contains number of cells calculated above. (Example: 16 x 10^6 cells/1.95 x 10^6 cells/mL = 8.2 mL cell suspension)
- Determine amount of Complete Medium needed to make volume of cell solution calculated above. (Example: 32 mL cell solution – 8.2 mL cell suspension = 23.8 mL)
- Prepare plating cell solution for experiment in 50 mL Falcon tube according to calculations above.

6. Pipet plating cell solution up and down to evenly distribute cells. Using a 10 mL pipet, quickly and carefully dispense volume of plating cell suspension calculated above for each well. (Example: 2 mL per 35 mm dish). Cells will settle quickly in solution, so be sure to pipet up and down before each draw.
7. Tilt plates to evenly distribute cell solution in wells. Cover plates and place in incubator.
8. Clean biosafety cabinet.

2. Fatty acid (FA):BSA preparation - 1 mM FA (2:1 FA to BSA ratio)

Materials/Equipment

Sodium myristate (MA) C14:0 (Nu-Chek Prep, S-1107) MW 264.40
Sodium eicosapentaenoate (EPA) C20:5 (Nu-Chek Prep, S-1144) MW 350.5
Sodium docosapentaenoate (DHA) C22:6 (Sigma, D8768-5MG) MW 300.48
Albumin from bovine serum (cell culture-tested, low endotoxin, fatty acid free) (Sigma, A8806)
Dulbecco's modified eagle's medium (DMEM) (Invitrogen, 11995-065)
1N NaOH (1M) MW 40.00, 1N HCl MW 36.46
Analytical balance
Weighing paper
Water bath
pH meter
Biosafety cabinet
Pipet and tips (10µL, 200µL and 1000µL), 10mL pipet, 10mL syringe (Fisher, 14-829-22A)
Microcentrifuge tubes (Fisher, 02-681-320), 15mL-sterile tubes (Fisher, 05-539-5)
Millipore* Millex* sterile syringe filters (0.22um, Low protein binding) (Fisher, SLGV 033 RS)

Things to do before starting

- Turn on water bath and set temperature to 37°C (for EPA and DHA) and 70°C (for MA)

Protocol

1. Make 0.5 mM (0.033 g/mL) albumin in DMEM (0.99 g into 30 mL or 1.32 g into 40 mL).
    - Invert conical tubes slowly and put onto rocker at RT to slowly dissolve BSA into DMEM. rock until albumin is completely dissolved (changes from pink to slightly yellow)
    - Keep albumin solution into water bath (37°C) while preparing 10 mg/mL fatty acid solution (step 2).
2. Make 10 mg/mL fatty acid (FA) solution.
    - MA and EPA: Weigh out between 5 and 10 mg of fatty acid. Place into 1.5 mL microcentrifuge tube. Add diH2O to FA to make 10 mg/mL FA solution. (Example: if weight is 8.5 mg, add 850 µL of diH2O.)
    - DHA comes pre-weighed (5 mg) in sealed glass vial, so add 500 µL of diH2O directly into vial and mix to dissolve completely. Transfer solution to 1.5 mL microcentrifuge tube.
    - Flush Sodium-FA powder with nitrogen and wrap container with parafilm. Store MA at RT, EPA and DHA at -20°C.)

- Vortex FA solution and put tube in water bath. To dissolve MA, 70°C water bath for about 10 min. After MA goes into solution, proceed to step 3. IMMEDIATELY before it percipitates. Place EPA and DHA solutions in 37°C, if they do not dissolve at RT.

3. Make 1mM FA.
    - Determine volume of FA needed to prepare 1 mM FA solution. Calculated volume turns out to be molecular weight of FA in µL. (Example: Molecular weight of MA is 264.40, so need 264.4 µL.) **Calculation:** Molecular weight of MA is 264.40 mg/mmol. 1 mmol/L x 264.40 mg/mmol = 264.4 mg/L. For 10 mL solution, need 2.644 mg total, which is 264.4 µL of 10 mg/mL FA solution. Add 264.4 uL to 9.7356 mL BSA solution.
    - Add 10 mL (minus volume of FA) of BSA solution into a 25 mL tube (easier to mix solution by swirling in 25 mL compared to 15 mL tube.)
    - Add calculated amount of 10 mg/mL FA solution to the above BSA solution, drop by drop and swirl every 10 drops.
    - Incubate at 37°C for 30min with occasion mixing/shaking (to help the FA, BSA mix).
4. Adjust pH of 1 mM FA to 7.6 by adding either NaOH or HCl.
5. In biosafety cabinet, 1mM fatty acid through 0.22µm Millipore filters by using 10 mL syringe, and into new 15mL-sterile tube.
6. Aliquot fatty acid into several autoclaved microcentrifuge tubes. **Gently flush with nitrogen for 10 s (outside biosafety cabinet), immediately close cap and seal with parafilm.** Store at −80°C. Use within 1-2 months, but best to use freshly-prepared FA solutions. Never refreeze fatty acid solutions aliquots that have been thawed.

Examples of 100 µM FA treatment and BSA control treatment

- 100 µM FA (50 µM BSA): Add 100 µL of **1 mM FA (0.5 mM BSA)** treatment solution to 900 µL Complete Media.

- 0 µM FA (50 µM BSA): Add 100 µL of **0 mM FA (0.5 mM BSA)** + 900 µL Complete Media.

3. Stimulation of RAW 264.7 cells with ultra-pure LPS

Materials/Equipment

Ultra-pure LPS from *E.coli* 0111:B4 strain (ultra-pure LPS) (Invivogen, tlrl-pelps)
Sterile diH2O
Autoclaved 1.5 mL microcentrifuge tubes
Millipore* Millex* sterile syringe filters (0.22um, Low protein binding) (Fisher, SLGV 033 RS)
Complete Medium: DMEM + FBS (10%) + Penicillin/Streptomycin (100 U/mL)

Things to do before starting

- Read Ultra-pure LPS product information sheet.
- Prepare sterile water: Pass autoclaved diH2O through sterile syringe filters to sterilize.
- Autoclave 1.5 mL microcentrifuge tubes and label.

Protocol for preparing ultra-pure LPS stocking solution (5 mg/mL)

1. Add 1 mL of sterile water to product vial to make 5 mg/mL stocking solution. Mix well.
2. Aliquot (20-40 µL) stocking solution to 1.5 mL tube. Store at -20℃. Thaw new tube for each experiment. Do not re-freeze stocking solution.

Protocol for stimulating cells with ultra-pure LPS (100 ng/mL)

For each experiment,a working solution of ultra-pure LPS was prepared and 10 µL of this working solution was added to each well to obtain a final concentration of 100 ng/mL. Therefore, the concentration of working solution depended on the volume of the culture media in each well or dish.

1. Determine concentration of working solution. (Example: for wells containing 2 mL total culture media, need 200 ng total ultra-pure LPS. So, need a working solution of 200 ng/10 µL, or 20 ng/µL).
2. Determine dilution factor required to make working solution. (Example: for 20 ng/µL working solution, must dilute 5 mg/mL stock solution 250-fold)
3. Dilute ultra-pure stocking solution with Complete Medium. (Example, combine 2 µL of stocking solution and 480 µL of Complete Medium). Vortex.
4. Add 10 µL of ultra-pure LPS working solution to culture media of each well or dish (*without washing cells or refreshing culture media).*
5. Tilt plates to distribute evenly. Incubate at 37℃ in incubator for determined stimulation time.

4. Extraction and methylation of fatty acid in cell membranes

Chemicals

Boron tri-fluoride 14% solution in methanol (BF_3-MEOH) (Sigma, B-1252)
-MEOH) (Sigma, B-1252) – Highly toxic. Store and transport in secondary container, store at 4°C.
Butylated hydroxytoluene (BHT) (Sigma, B-1378)
Chloroform-HPLC grade
De-Ionized water (diH2O)
Hexane-HPC grade
Heptadecanoic acid (Nu-Chek, N-17-M) – Store at -20°C after opened.
Methanol-HPLC grade
Potassium chloride (KCL) (Sigma, P-2911)
Sodium hydroxide (NaOH) (Sigma, S-5881)
0.9% sodium chloride solution (LabChem Inc., LC23460-2) – Stored at 4°C. Keep cold during protocol.
10x phosphate buffered saline (PBS) (Fisher, 17-517Q).

Glassware

Amber vials with inserts (0.3 mL) (Fisher, 03-337-47)
Aluminum seal caps TFE/Sil (Supelco, 27360-U)
Glass screw-top tubes 16 x 100 mm (Fisher, 74-959-65AA)
Glass tube with teflon cap 25 x 150 mm (Fisher, 14-930-10J)
PTFE-lined caps (Fisher, 14-930-15E)
250 mL glass bottle with teflon stopper
Watch glass (small)
Borosillicate glass disposable Pasteur pipet 5 ¾ in. (Fisher, 13-678-20B)
Homemade GC vial holders (5 mL plastic pipet tip surrounded by glass beads in a 10 mL glass bottle)

Equipment

Refrigerated centrifuge (Sorvall, RT6000B, Dupont) set to 4°C.
Centrifuge rotor H-1000B
Dry-heat bath
Nitrogen evaporator N-EVAP 112 (Organomation Associates, Inc.)
Prepurified nitrogen gas (Medical Technical Gases, Inc., 13J24)
SMI micropipet (50-250 µL)
Replacement capillaries for SMI micropipet (Fisher, 21-379-10)
Multi-tube vortexer (VWR, Vx-3500)
Teflon tape
Crimper (Fisher, 0640618F)
De-crimper (Fisher, 0334057B)
Rubber bulb 1 cc (Fisher, 0344825)

Solutions and reagents to prepare before starting

1x PBS

Chloroform methanol (CM) mixture

- Into a 1000 mL glass bottle with Teflon stopper, add chloroform, methanol, and butylated hydroxytoluene (BHT) in a 2:1:0.1 (v/v/w) ratio. (Example: 500 mL:250 mL:25 mg).

0.5N methanolic NaOH: *Prepare every 2 days.*

- Into 250 mL glass bottle with Teflon stopper, add 1 g NaOH: 5 mL DiH2O: 45 mL methanol (Add diH2O very slowly because this is an exothermic reaction. Methanol should be added last. This will be a cloudy solution).

Methanol/diH2O

- Into a 1000 mL glass bottle with Teflon stopper, add methanol and diH2O in a 1:1 (v/v) ratio. (Example: 100 mL:100 mL)

KCl/diH2O [1]

- Into a 250 mL glass bottle with Teflon stopper, add 0.88 g KCl to 100 mL of diH2O.

Internal standard (C17:0)

- Make enough to last through entire study.
- Concentration of C17:0 in chloroform is 1.0 mg/mL. (Example: 10 mg C17:0 + 10 mL chloroform.)
- Prepare in volumetric flask.
- Flush with nitrogen, cap flask and wrap parafilm around cap. Store at 4°C.

Harvest cells

1. Reserve culture media for ELISA analysis (refer to ELISA protocol).
2. Wash cells twice with PBS. Remove liquid completely.
3. Add 900 µL PBS to each well, scrape cells and transfer to 1.5 mL microcentrifuge tube.
4. Transfer 600 µL to new 1.5 mL microcentrifuge tube (reserve remainder for protein measurement). Pellet cells by centrifugation (125 x g, 5 min). Remove liquid, flush with nitrogen and wrap with parafilm. Store at -80°C.

Lipid extraction using modified Folch method [1]

1. On day of extraction, transfer cell pellet to new 16 x 100 mm glass tube.
    - Dislodge pellet from bottom of tube by forcefully dispensing100 µL diH2O. (Pellet should be intact but floating in water.)

- Transfer water and intact pellet with disposable glass pipet and dispense into 16 x 100 mm glass tube. *Cell pellet will stick to sides of a plastic pipet tip.*

2. Wash cells with 0.9% NaCl 3 times.
    - Resuspend cell pellet with 1 mL of 0.9% NaCl, vortex lightly.
    - Centrifuge 2500 rpm for 5 min to pellet cells; remove supernantant.
    - Repeat 2 more times.
3. Add 12.5 µL of internal standard.
4. Add 6 mL of CM mixture.
5. Place tubes on multi-tube vortexer for 10 min at level 1. Flush with nitrogen, cap and refrigerate overnight.
6. The next day, vortex tubes again and centrifuge at 3000 rpm (~1800 x g) for 10 min at 4°C. Preheat heat bath to 95°C.
7. Using a volumetric glass pipet, transfer 6 mL of supernatant into a clean tube (16 x 100 mm).
8. Using a 1000 µL pipet with plastic tip, add 1.5 mL of 0.88% KCl and cap.
9. Shake vigorously by hand for 30 s (solution will appear milky white upon shaking) and let the layers settle for 5 min (top layer should now be clear).
10. Pipet upper layer into a waste container. Use new Pasteur pipet and transfer lower layer into clean tube avoiding any remaining upper layer.
11. Add 1 mL methanol:diH2O and cap. Shake vigorously by hand for 30 s (solution will be milky white upon shaking) and let layers settle for 15 min (top layer should now be clear).
12. Pipet upper layer into a waste container. Use new pipet and transfer lower layer into clean tube avoiding any remaining upper layer.
13. Evaporate lower layer under nitrogen at 30°C.
14. To dried lipid extract, add 2 mL 0.5 N methanolic NaOH into tube using 5 mL graduated glass pipet. Cap tightly to avoid evaporation during incubation.
15. Vortex and incubate in dry heat bath at 90-95°C (<100°C) for 15 min. (Add more methanol if it evaporates. Samples should be pink-tinted after reaction has occurred).
16. Cool tubes on ice for 5 min.

FA methylation using Morrison and Smith [2]

17. Add 2 mL of 14% BF3-MeOH into each tube using 5 mL graduated glass pipet. Flush samples and BF3-MeOH bottle with nitrogen before capping. Seal BF3-MeOH bottle with Teflon tape and refrigerate.
18. Vortex and incubate in dry heat bath at 90-95°C (<100°C) for 1 h.
19. Cool tubes on ice for about 5 min.
20. To cooled tubes, add 2 mL hexane followed by 1 mL diH2O.
21. Place tubes on multi-vortexer (level 1) for 2 min and centrifuge tubes at 2500 rpm (~ 1300 x g) for 5 min at 4°C).
22. Using a Pasteur pipet, transfer the supernatant containing the FAMEs (fatty acid methyl esters) into a clean screw-top tube and dry down under nitrogen (Do not over-dry).

23. Re-suspend with 100 µL hexane using the SMI micropipet with glass tip, rinsing sides of tube.
24. Using Pasteur pipet, transfer (very carefully) into labeled amber GC vial with insert.
25. Repeat steps 23 and 24.
26. Dry down under nitrogen. (Do not over-dry; this should take 1-2 min.)
27. Re-suspend with 50 µL hexane.
28. Cap and store at -20°C.

Calculation

$$\text{RRF (external standard)} = \frac{\text{Fatty acid area/fatty acid amount}}{\text{C17:0 area/C17:0 amount}}$$

Internal standard concentration = 1.0 µg/µL
Internal standard volume = 12.5 µL
Inject volume = 0.5 µL
Final volume = 50 µL
ISTD = (1µg/µL x 12.5 µL) x 0.5 µL/ 50 µL = 0.125 µg
Fatty acid (µg) = (Fatty acid area/C17:0 area) x (ISTD/RRF/Injected volume) x Vial volume
Fatty acid (µmol) = Fatty acid (µg)/MW x 1000000
Fatty acid (mol%) = Fatty acid (µmol)/Total fatty acid ((µmol) x 100

5. RNA Isolation from RAW 264.7 cells

Materials/Equipment

RNeasy® Mini Kit (Qiagen, 74106)
70% EtOH
Sterile, RNase-Free DNase Set (Qiagen, 79254)
PBS
Cell lifter (Corning)
RNAse-free microcentrifuge tubes (1.5 mL)
20-gauge needle (0.9 mm)
Syringe
RNase-free pipet tips
Microcentrifuge
Vortexer

Things to do before starting

- Read the RNeasy® Mini Handbook supplied in the kit.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96-100%) as indicated on the bottle to obtain a working solution.
- Set microcentrifuge temperature to 4˚C, speed to ≥8,000 x g (≥10,000 rpm).
- Prepare DNase I stock solution as described below.

Prepare DNase I stock solution

- Prepare DNAse I stock solution before using the RNAse-Free DNAse Set for first time. Dissolve lyophilized DNase I (1500 Kunitz units) in 550 µl of RNase-free water provided. To avoid loss of DNase I, do not open vial. Inject RNase-free water into vial using RNase-free needle and syringe. (*RNase-free needle and syringe were not used.)* Mix gently by inverting vial. Do not vortex.
- For long-term storage of DNase I, remove stock solution from glass vial, divide it into single-use aliquots, and store at -20℃ for up to 9 months. Thawed aliquots can be stored at 2-8℃ for up to 6 weeks. Do not refreeze aliquots after thawing. *I stored DNase I stock solution in -4℃ for up to 1 week.*

Protocol (adapted from RNeasy® Mini Handbook)

1. Harvest cells (no more than 1 x 10^7 cells). Remove culture media and wash adherent cells twice with PBS. Completely aspirate PBS and proceed immediately to step 2.
2. Disrupt cells by adding Buffer RLT (350 µl for <6 cm diameter plate, 600 µl for 6- 10 cm diameter plate. Add Buffer RLT directly to the culture dish. Collect cells using cell lifter. Pipet lysate into a 1.5 mL RNAse-free microcentrifuge tube. Vortex or pipet to mix and ensure that no clumps are visible before proceeding to step 3.
3. Homogenize lysate. Pass lysate at least 5 times through 20-gauge needle (0.9 mm diameter) fitted to syringe (RNAse-free syringe was not used).
4. Add one volume of 70% ethanol to homogenized lysate, and mix well by pipetting. Do not centrifuge.
    - Note: The volume of lysate may be less than 350 µl or 600 µl due to loss during homogenization. (*The volume of 70% ethanol added was the same as the volume of Buffer RLT added.)*
    - Note: Precipitates may be visible after addition of ethanol. This does not affect procedure.
5. Transfer up to 700 µl of sample, including any precipitates that may have formed (especially gelatinous clumps), to RNeasy spin column placed in 2 mL collection tube (supplied). Close lid gently, and centrifuge for 15 s at ≥8,000 x g (≥10,000 rpm). Discard flow-through. Reuse collection tube in step 6. If sample exceeds 700 µl, centrifuge successive aliquots in same RNeasy spin column. Discard flow-through after each centrifugation.
6. DNase digestion.
    - D1. Add 350 µl RW1 to RNeasy spin column. Close lid gently, and centrifuge for 15 s at ≥8,000 x g (≥10,000 rpm) to wash spin column membrane. Discard flow-through. Reuse collection tube in D4.
    - D2. Add 10 µl DNase I stock solution to 70 µl Buffer RDD (provided in the set) per sample. Mix by gently by inverting tube, and centrifuge briefly to collect residual liquid from sides of tube. (Make enough DNAse I for all samples in one 1.5 mL microcentrifuge tube).
    - D3. Add DNase I incubation mix (80 µl) directly to RNeasy spin column membrane, and place on benchtop (20-30℃) for 15 min. Note: Be sure to add DNase I incubation mix directly to RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to walls or O-ring of spin column.
    - D4. Add 350 µl Buffer RW1 to RNeasy spin column. Close lid gently, and centrifuge for 15 s at ≥8,000 x *g* (≥10,000 rpm). Discard flow-through.
7. Add 500 µl Buffer RPE to RNeasy spin column. Close lid gently, and centrifuge for 15 s at ≥8,000 x g (≥10,000 rpm) to wash spin column membrane. Discard flow-through. Reuse collection column in step 8.
8. Add 500 µl Buffer RPE to RNeasy spin column. Close lid gently, and centrifuge for 2 min at ≥8,000 x g (≥10,000 rpm) to wash spin column membrane. Note: After centrifugation, carefully remove RNeasy spin column from collection tube so that column does not contact flow-through. Otherwise, carryover of ethanol will occur.

9. Optional: If RPE carry-over is suspected or if residual flow-through remains on outside of column, place column in new 2 mL collection tube and discard old collection tube with flow-through. Close lid gently, and centrifuge full speed for 1 min.
10. Place RNeasy spin column in new 1.5 mL collection tube (supplied). Add 30-50 µl RNase-free water directly to spin column membrane. Close lid gently, and centrifuge for 1 min at ≥8,000 x g (≥10,000 rpm) to elute RNA.
11. If expected RNA yield is >30 ug, repeat step 10 using another 30-50 µl RNase-free water, or using eluate from step 10 (if high RNA concentration is required). Reuse collection tube from step 10.
12. Immediately quantify by nanodrop. Store in -20C.

6. Quantification of RNA

Materials/Equipment

Nanodrop and software
Sterile, RNase-free pipet tips
RNase-free water
Ice

Protocol

1. Put freshly isolated RNA samples and RNase-free water on ice.
2. Double Click “ND-1000”, then Click “Nucleic Acid” (confirm setting is “RNA”)
3. Clean equipment by applying 2 µL RNase-free water to reader and reader lid. Wipe with Kimwipes™.
4. Apply 2µl RNase-free water to reader, Click “Blank”
5. Enter sample name. Apply 2 µl sample to reader, Click “Measure.” (*Two to three measurements were taken of each sample. An average of at least 2 similar readings were were used to determine sample concentration.*)
6. Repeat for next sample.
7. Record the 260/280 ratio and the 260/230 ratio, and concentration of each sample.
8. Clean the equipment as in step 3.

7. Reverse Transcription

Materials/Equipment

Reverse Transcription System (Promega, A3500)
Sample RNA
Sterile RNase-free pipet tips
Sterile RNase-free microcentrifuge tubes
Centrifuge (Eppendorf 5415R)
Hot water baths (3)

Important points before starting

- Do not vortex AMVRT (enzyme) – it is very sensitive. Do not remove from freezer until ready to add to master mix.
- Usually use 1ug RNA samples and bring them up to 10 µl with water. If your sample is too dilute to do this, bring it up to 15 µl (bring all your samples up to the same volume).
- Heat water baths to 42˚C, 70˚C, 95˚C (use thermometer)
- Thaw master mix buffers on ice

Master Mix Preparation

	per 10 µL sample	per 15 µL sample
MgCl2	4.0 µL	5.0 µL
10xRT	2.0 µL	2.5 µL
dNTPs	2.0 µL	2.5 µL
RNasIN	0.5 µL	0.625 µL
AMVRT	0.6 µL	0.75 µL
Rand. Prim	1.0 µL	1.25 µL
Total vol.	10.1 µL	12.625 µL

Protocol

1. Place 1 µg of RNA in 1.5 mL RNase-free microcentrifuge tube and add nuclease-free water to bring total volume up to 10 µL. If RNA is too dilute, bring volume up to 15 µL.
2. Incubate RNA samples at 70°C for 10 min, then on ice for at least 5 min.
3. Prepare master mix according to the table above.
4. Add 10.1µl (for 10 µL RNA samples) or 12.6 µL (for 15 µL RNA samples) master mix to RNA samples, mix well.
5. Incubate at RT for 10 min.
6. Incubate at 42°C for 15 min.
7. Incubate at 95°C for 5 min.
8. Incubate on ice for 5 min (+).
9. Store cDNA in -20C.

8. Real Time PCR analysis of TNFα and IL-6 mRNA expression

Materials/Equipment

Applied Biosystems® 7300 machine
QuantiTect SYBR Green PCR Master Mix (Qiagen, 204145)
QuantiTect Primer Assays
- Mouse TNFα (Qiagen, QT00104006)
- Mouse IL-6 (Qiagen, QT00098874)
- Mouse βactin (Qiagen, QT01136772)
- Mouse gapdh (Qiagen, QT01658692)

Tris EDTA buffered solution, pH 8 (Sigma-Aldrich, 93283)
Sample cDNA
RNase-free water
1.5 mL microcentrifuge tubes
96-well PCR plates

Important points before starting

- Read "QuantiTect® Primer Assay Handbook" provided by Qiagen.
- PCR must start with initial incubation step of 15 minutes at 95°C to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green Master Mix).
- Always readjust threshold value for analysis of every run.
- Do not use final reaction volumes of less than 25 µL when using this instrument.
- Must run primer efficiency assays to determine appropriate cDNA dilutions: see Kaori Honda's lab notebooks for details.
- Volume of cDNA added (from undiluted RT reaction) should not exceed 10% of final PCR volume.
- Due to hot start, it is not necessary to keep samples on ice during reaction setup or while programming real-time cycler.
- No optimization of Mg2+ concentration is required. Final Mg2+ concentration of 2.5 mM provided by 2X QuantiTect SYBR Green PCR Master Mix gives optimal results.

Things to do before starting

- Reconstitute 10X QuantiTect Primer Assays to make 5 µM solution. Briefly centrifuge product tube, then add 1.1 ml Tris EDTA buffered solution and mix by vortexing the tube 4 to 6 times; if necessary, gently warm the tube to help the primers dissolve. Store reconstituted primers in aliquots at –20°C, to avoid repeated freeze-thaw cycles. Discard primers after 18 months from date of receipt.
- Thaw 2x QuantiTect SYBR Green PCR Master Mix and prepare 1.5 mL aliquots in 1.5 mL microcentrifuge tubes. Store at -20°C.

Protocol

1. Thaw 2X QuantiTect SYBR Green PCR Master Mix, 10X QuantiTect Primer Assay aliquot, template cDNA, and RNase-free water. Mix individual solutions
2. Prepare Reaction Mix for each sample:
   - 2X QuantiTect SYBR Green PCR Master Mix*: 25 µL.
   - Template cDNA: 5 µL.
   - RNase-free water: 15 µL.
   - Total volume 45 µL.
3. Mix Reaction Mix thoroughly, and pipet 5 µL into each well (*dispense on side of well so as to leave visible drop*).
4. Add 10X QuantiTect Primer Assay (≤100 ng/reaction) to each well (*dispense on side of well opposite of Reaction Mix so as to leave visible drop*).
5. Seal plate with optical adhesive covers, and centrifuge plate at 800 x g to bring down liquid.
6. Place plate in real-time cycler.
7. Program and start real-time cycler (described below).

Cycling conditions for two-step RT-PCR

- PCR initial activation step -- 15 min at 95°C
- 3-step cycling (35-40 cycles):
  - Denaturation 15 s 94°C
  - Annealing 30 s 55°C
  - Extension 34 s 72°C (fluorescence data collection step)
- Number of cycles: 40
- Click "Add Dissociation Stage" for melting curve analysis*.

* Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier.
Note: The *T*m of a PCR product depends on buffer composition and salt concentration. *T*m values obtained when using QuantiTect SYBR Green PCR reagents may differ from those obtained using other reagents.

9. Nuclear and cytoplasmic extraction

Materials/ Equipment

NE-PER® (Thermo Scientific, 78833)
Kit contents:
- Cytoplasmic Extraction Reagent I (CER I), 10 mL
- Cytoplasmic Extraction Reagent II (CER II), 500 µL
- Nuclear Extraction Reagent (NER), 5mL

Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, 78444)
PBS
Cell lifter (Corning)
PBS
Bucket of ice
Tray of ice
1.5 mL microcentrifuge tubes
Pasteur pipet
Refrigerated centrifuge (Sorvall, RT6000B, Dupont)
Centrifuge rotor H-1000B
Microcentrifuge (Eppendorf 5415R)
Vortexer

Things to do before starting

- Read instructions included in NE-PER® kit.
- Set both centrifuges to 4°C.
- Put kit reagents and protease/phosphatase on ice.
- Put PBS on ice.
- Label and put all 1.5 mL microcentrifuge tubes to be used on ice.

Cell culture preparation

This protocol was adapted for an experiment in which 1 x 10^6 cells were plated per well in a 6-well plate (35 mm diameter per well), and harvested 48 h later. About 2-4 x 10^6 cells per sample were processed for this assay.

1. Take out cell culture plates from incubator and set on tray of ice.
2. Wash cells 2 times with ice-cold PBS. Remove liquid.
3. Add 1 mL of ice-cold PBS and scrape cells, using cell lifter. Transfer to 1.5 mL microcentrifuge tube.
4. Centrifuge 500 x *g* 2 min at 4°C for in Sorvall centrifuge.
5. Remove supernatant. Want pellet to be dry as possible.

Cytoplasmic and nuclear extraction

1. Add proteinase/phosphatase inhibitor to Cytoplasmic Extraction Reagent (CER I) at 1:100 dilution.
2. Add 200 µL of this CER to a 20 uL volume cell pellet. (See instructions Table 1. for volume of CER to add according to volume of cell pellet.)
3. Vortex tube vigorously on highest setting for 15 s to fully suspend pellet. Incubate on ice for 10 min.
4. Add ice-cold CER II to the tube (11 µL for 20 uL cell pellet).
5. Vortex tube for 5 s on highest setting. Incubate on ice 1 min.
6. Vortex tube for 5 s on highest setting. Centrifuge tube for 5 min at maximum speed in microcentrifuge (16,000 x *g*).
7. Immediately transfer supernatant (cytoplasmic extract) to clean, pre-chilled tube. Place tube on ice.
8. Add protease/phosphatase inhibitor to NER (1:100) dilution in 1.5 mL microcentrifuge tube.
9. Add this NER to insoluble pellet fraction containing nuclei.
10. To break apart pellet, scrape bottom of tube across top of a 1.5 mL microcentrifuge tube rack 3 times. (*Notice that pellet is suspended intact in solution, but spread out like a cloud. Vortexing alone will not sufficiently break up the pellet.*)
11. Vortex on highest setting for 15 s. Place sample on ice and continue vortexing for 15 s every 10 min, for total of 40 min.
12. Centrifuge tube at maximum speed (16,000 x *g*) in microcentrifuge for 10 min at 4°C.
13. Immediately transfer supernatant (nuclear extract) fraction to a clean pre-chilled tube. Place on ice.
14. Store extracts at -80°C until use.

10. BCA™ protein assay

Materials/Equipment

BCA™ Protein Assay Kit (Pierce, 23225)
Kit contents:
- BCA™ Reagent A
- BCA™ Reagent B
- Albumin standard, 2mg/mL

diH2O
Microcentrifuge tubes
96-well clear, flat-bottom microplate (Fisher, 08-772-5)
Adhesive cover (Fisher, 08-408-240)
Water bath
Microplate reader

Things to do before starting

- Read Instructions included in BCA™ protein assay kit.
- Set water bath to 37°C.
- Samples on ice.

Protocol

1. Prepare diluted albumin (BSA) standards
   - Use Table 1 in Instructions as a guide to prepare a set of protein standards.
   - Use diH2O as a diluent.
   - Store diluted standards in 4°C for up to 2 weeks.
2. Dilute samples
   - Dilute samples with diH2O (dilution factor depends on experiment and treatment)
   - *Nuclear protein extract was diluted 2-fold* (10 µL sample + 10 µL diH2O).
3. Prepare BCA working reagent (WR)
   - Use following equation to determine total volume of WR required:
     (# standards + # samples) x (# replicates) x (volume of WR per sample)
   - Round up total volume of WR to nearest mL to account for loss.
   - Mix 50 parts reagent A with 1 part reagent B.
4. Pipet 25 µL of each standard or sample (diluted) into microplate well in duplicate.
5. Add 200 µL WR to each well. Move plate back and forth on bench to mix. Cover with adhesive cover. (To conserve sample, pipet 20 µL of sample and 160 µL WR.)
6. Incubate in 37° water bath with gentle shaking for 30 min.
7. Cool plate to RT.
8. Measure absorbance at or near 562 nm on plate reader.
9. Calculation based on standard curve and dilution factor.

11. Western blot analysis of nuclear NFκB p65 and p50 protein expression

Materials

Criterion Tri-HCL Precast gel (4-20%, 45 µL well) (Biorad, #345-0032)
10X Tris/Glycine/SDS Buffer ("Running Buffer") (BioRad, 161-0772)
10X Tris/Glycine ("Transfer Buffer") (BioRad, 161-0071)
Methanol (VWR, 9093-03)
Gel-Loading pipet tips
Laemmli Sample Buffer (Biorad, 161-0737)
Beta Mercapto Ethanol, BME (Sigma, M7154-25mL)
Precision Plus Prot Kaleidoscope standards (Biorad, 161-0375)
Thick blot paper (BioRad, 1704085)
Nitrocellulose Membrane (BioRad, 162-0094)
Ponceau S. Stain (Sigma, P7170-1L)
Powder Infant Formula (CVS, 691968)
Tween 20 (Sigma, P1379)
Sodium Chloride (Sigma, S7653-1KG)
Anti-NFκB-p50 rabbit (Abcam, ab32360)
Anti-NFκB-p65 rabbit (Cell Signaling, 8242)
Anti-TATA binding protein (TBP) mouse (Abcam, ab818)
Anti-Histone 3 (H3) rabbit (Cell Signaling, 9715)
Goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005)
Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2030)
SuperSignal West Pico Chemiluminescent Substrate (ECL) (ThermoScientific, 34080)
kodak x-omat LS film, 5 x 7 in. (Sigma, F1274-50EA)

Equipment

Hot plate
1L glass beaker filled about a quarter full of water
Mini, benchtop centrifuge
Criterion Pre-Cast System (running and transfer tanks, electrical source)
Bio-Rad Sponges (2 per gel) (BioRad, 170-4086)
Magnetic Stirring bar (1 per transfer box)
Razor blade
Blot roller
Tray (for assembling gel and membrane sandwich)
Ice pack
Magnetic stirrer
Rocker
Cold room
6 x 4 in., plastic, clear ziplock bags
Cassette for film exposure
Darkroom, developer

QuantOne scanner and software

Important points before starting

- Refer to "Protein Blotting Guide" which can be found on BioRad website (http://www.bio-rad.com/).
- TBS-T: Tween can settle and build up in container bottom of TBS-T solution. Mix solution before using, and use new, clean bottle for each preparation.

Things to prepare before starting

Running Buffer (can reuse)
100 ml buffer (SDS 10X "Running Buffer" box)
900 ml diH2O

Transfer Buffer (make fresh for each transfer)
20% MeOH: 100 mL 10X buffer ("Transfer Buffer" box)
200 mL Methanol
700 mL diH2O

TBS-T (can make up 10 L stock in big Nalgene – minus Tween 20)

	1 L diH2O	x10	10 L diH2O
(10mM)	10 mL Tris (pH 8)	x10	100 ml Tris (pH 8)
(150mM)	8.76 g NaCl	x10	87.66 g NaCl

1010 ml of stock + 1 mL Tween 20 (0.1% Tween 20)

Protocol for electrophoresis and transfer

1. Turn on hotplate to boil samples.
2. Place precast gel into running tank – peel off sticker, remove comb, rinse wells with diH2O, insert into box.
3. Fill running tank with running buffer to cover wells.
4. Prepare 2X loading buffer: 475 µL Laemeli + 25 µL BME (do in hood).
5. Prepare samples (do in hood).
    - Thaw samples and precision plus ladder on ice.
    - Add equal volume of 2X loading buffer to sample (use sample volume containing 10 µg protein).
    - Boil 5 min, allow to cool at RT for 5 min.
    - Spin down samples briefly using mini, benchtop centrifuge.
    - Load each sample in duplicate and 10 uL precision plus ladder.
6. Run at 80-100 volts until dye is near bottom of gel (1-2 h)

7. Remove gel.
    - Pry open cassette, cut off wells with razor blade, rinse gel with water (to remove gel debris).
    - Use razor or fingers to separate gel from cassette. Slip gel into a container (large pipet tip box lid) filled with transfer buffer.
8. Soak gel in transfer buffer for 5 min to equilibrate.
9. Prepare gel and membrane sandwich tray.
    - Fill tray with transfer buffer.
    - Open gel holder cassette and submerge cathode (black) side into transfer buffer.
    - Wet one sponge in transfer buffer and place it on submerged side of cassette.
    - Wet one piece of filter paper in transfer buffer and place it on top of sponge. Use blot roller to remove trapped air.
    - Place equilibrated gel on top of filter paper. Pick up gel by two top corners using fingers. If needed, gently use blot roller to remove trapped air.
    - Wet second piece of filter paper in transfer buffer and place on top of membrane. Again, roll to remove trapped air.
    - Soak second sponge in transfer buffer and place on top of filter paper, then close and lock cassette.
10. Place one magnetic stirring bar and one ice pack into transfer tank. Insert cassette(s) and fill tank with transfer buffer to cover cassette(s).
11. Place transfer tank on magnetic stirrer in cold room and set to mid-strength. Make sure stirring bar is stably spinning in middle of tank.
12. Transfer 20 volts overnight (~16 h).
13. Strong appearance of ladder on membrane is first sign of successful transfer.
14. To visualize transferred proteins, stain with Ponceau S (1-5% in diH2O).
    - Rinse membrane with TBS-T.
    - Pour Ponceau S working solution into a pipet tip box lid and slide in membrane face up. Rock gently using hands. Should see horizontal red lines of varying intensity and thickness.
15. Store membrane in clear, plastic ziplock bags at -20℃ or proceed to blotting.

Blotting (p65, H3 and TBP)

Due to similarity in weight, p65 was blotted and developed first, followed by p50.

1. Using the lines of stained proteins as guide, cut membrane between 37 KD and 50 KD. Cut lower-right-hand corners of each membrane piece to indicate right-side up.
2. To remove stain, rinse membrane with diH2O, then rock in 5% infant formula in TBS-T (5-10 min).
3. Block in 5% infant formula in TBS-T. Rock for 1 h at RT (shield from direct sunlight). Remove liquid.
4. Blot with primary antibody.

- Top half of membrane: Add 15 mL of 5% infant formula in TBS-T. Add 3 uL anti-NFκB p65, swirl to mix.
- Bottom half of membrane: Add 15 mL of 5% infant formula in TBS-T. Add 8 uL anti-TBP, or 15 uL anti-H3, swirl to mix.
- Rock 1 h at RT.

5. Wash (Repeat the following steps 3 times)
    - Remove liquid.
    - Add TBS-T, swirl and dump. Add TBS-T.
    - Rock 5 min, RT.
6. Blot with secondary antibody.
    - Top half of membrane: Add 15 mL of 5% infant formula in TBS-T. Add 3 uL goat anti-rabbit, swirl to mix.
    - Bottom half of membrane: Add 15 mL of 5% infant formula in TBS-T. For probing TBP, Add 3 µL goat anti-mouse, or for histone 3, add 6 µL of goat anti-rabbit, swirl to mix.
    - Rock 1 h, RT.
7. Wash as described in step 5.
    - During last wash, go to the dark room to reserve developer and turn on red lamp.

Signal development using ECL (lights off).

1. Make plastic folder for each membrane piece by cutting off three sides of a clear, plastic ziplock bag.
2. For 1 membrane, mix 2 mL of ECL buffer 1 and 2 mL of ECL buffer 2.
3. Using tweezers, hold membrane by one corner, blot membrane on Kimwipe thoroughly and place in a dry container (pipet tip box lid).
4. Add 1-2 mL of ECL solution to each membrane piece, to cover completely.
5. Incubate 2 min.
6. Blot each membrane on Kimwipe thoroughly and place in individual clear, plastic folder.
7. Tape each folder containing membrane to inside of cassette for film exposure, to prevent shifting.

Film exposure and development (Dark room)

1. Bring scissors, marker and timer to dark room.
2. Light should already be on and you should be signed up (so that no one turns off light).
3. Cut right-hand corner of film to indicate right-side up. Quickly place film in cassette on top of membranes – no readjusting.
4. Push button on machine to get it moving (periodically stops).
5. Place film in machine

6. Note: If it takes more than 3 minutes then your film is probably stuck and you will need to press button to move it through or open machine and find your film (pressing button while film is inside will likely over expose your film so only do this if it really is stuck!)
7. If band quality is poor, repeat step 4, adjusting film exposure time.
8. Write down exposure time on each piece of film.
9. Before leaving dark room, shut off machine (if not currently reserved by others) and sign off.
10. Trace ladder onto film and write down corresponding weights.
11. Identify bands and mark sample ID for each well.
12. Rinse membrane with TBS-T, put in clear, plastic ziplock bag and store at -20°C.

Re-blotting (p50)

1. For NFκB-p50 detection, re-blot top half of membrane according to steps 4-7 in "Blotting."
    - Primary antibody blot: 15 mL of 5% infant formula in TBS-T + 1.9 uL anti-NFκB-p50.
    - Secondary antibody blot: 15 mL of 5% infant formula in TBS-T + 3 uL goat anti-rabbit.
2. To detect bands, follow steps in "Signal development" and "Film exposure."

Scan film

1. Turn on scanner. Warm up for 15 min.
2. Open QuantOne. Select "GS-710."
3. Put film face down on scanner. Select "Blue," and "Transmissive" film type.
4. Click "Preview." Move box over image. Click "Acquire."
5. Save image. Click "File export to Tiff," "Publishing," "Same as scan," "Export."
6. Subtract background: Move background and click "Autoscale". Click "OK."
7. Volume: Draw a volume rectangle around a band. Be sure it can encompass every band. Copy paste rectangle on top of each band. Alternatively, draw a line around each band manually. This is appropriate when bands have merged, or band size vary greatly from one sample to another.
8. Draw a volume rectangle that encompasses an example "background" density that is similar to the density around the perimeter of the bands. Double-click, "Label as background."
9. Volume analysis report export data (box, bottom right-hand corner), Excel. Adjust volume ODmm2.

12. ELISA analysis of IL-6 and TNFα protein in culture media

Materials/Equipment

Mouse TNFα DuoSet ELISA Development Kit (R&D Systems, DY410)
Mouse IL-6 DuoSet ELISA Development Kit (R&D Systems DY406)
Each kit includes
- Capture Antibody
- Detection Antibody
- Standard
- Streptavidin-HRP

ELISA microtiter plates (Costar, 2592)
Disposable plate sealers (Costar, 3095)
Disposable reagent reservoirs (Baxter, 5082-128)
Large tub lined with paper towels
Wash bottle
8 or 12 channel multichannel pipet
Assorted volume pipets
PBS
Wash Buffer (R&D Systems, WA126)
Reagent Diluent (R&D Systems, DY995)
Substrate Solution (R&D Systems, DY999)
Stop Solution (R&D Systems, DY994)
Refrigerated centrifuge (Sorvall, RT6000B, Dupont) set to 4°C
Centrifuge rotor H-1000B
Microplate reader

Things to do before starting

- Read kit insert/instructions.
- Read "ELISA Developing Guide" found on R&D website (http://www.rndsystems.com/resources/images/5670.pdf)

Preparation of reagents

- Bring all reagents to RT before use.
- Prepare Capture Antibody, Detection Antibody, Standard and Streptavidin-HRP as directed on kit instructions.

Cell culture preparation

1. Plate 1 x 10^6 cells per well in 6-well plate (total volume per well is 2.0 mL). Place in incubator for 2 h.
2. Treat with MA, EPA or DHA (100 µM final concentration) or BSA for 24 h.
3. Stimulate with ultra-pure LPS for 0, 6 or 24 h.

- Group 1: Wash cells two times with DMEM, add 2 mL new DMEM, then stimulate with ultra-pure LPS (100 ng/mL final concentration).
- Group 2: Add ultra-pure LPS directly to each well without changing media (100 ng/mL final concentration).

4. Collect culture media from each well. Centrifuge 1500 rpm for 10 min at 4°C. Transfer supernatant into new tube and store at -80°C until use.
5. Determine total protein from one third of cells by BCA™ protein assay.
6. Determine cell fatty acid profile from two thirds of cells by GC.

ELISA protocol (based on kit instructions)

1. Dilute Capture Antibody to working concentration in PBS without carrier protein. Immediately coat 96-well microplate with 100 µL per well of diluted Capture Antibody. Seal plate and incubate overnight at room temperature. *For each incubation, put plate in an empty ice bucket with lid to keep even temperature.*
2. Empty wells
    - Invert plate over a large bin lined with paper towels, and flick to remove as much liquid from wells. Blot plate onto clean paper towels until no more liquid comes out.
3. Wash wells with Wash buffer.
    - Fill each well completely with wash buffer using a 25 mL serological pipet and pipet aid. *Hold plate over bin to catch drips*.
    - Empty wells as in step 2. Complete removal of liquid is essential for good performance.
4. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at RT for at least 1 h.
5. Repeat empty/wash (steps 2 and 3). Plates are now ready for sample dilution.
6. Dilute samples with Reagent Diluent. (Samples were diluted 10-fold or 15-fold.)
7. Add 100 µL of sample or standards diluted in Reagent Diluent, per well. Cover with adhesive cover and incubate 2 h at RT.
8. Repeat empty/wash (steps 2 and3).
9. Add 100 µL of Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive cover and incubate 2 h at RT.
10. Repeat empty/wash (steps 2 and 3).
11. Add 100 µL of working dilution of Streptavidin-HRP to each well. Cover plate and incubate 20 min at RT. Protect from direct light.
12. Repeat empty/wash (steps 2 and 3).
13. Add 100 µL Substrate Solution to each well. Incubate 20 min at RT. Protect from direct light.
14. Add 50 µL of Stop Solution to each well. Gently tap plate to ensure thorough mixing.
15. Determine optical density of each well immediately, using microplate reader set to 450 nm. Set wavelength correction to 540 nm or 570 nm.
16. Calculations based on standard curve and dilution factor.
17. Normalize to total protein content.

13. ELISA analysis of phosphorylated CREB in whole cell lysate

Materials/Equipment

Human/Mouse/Rat Phospho-CREB (S133) DuoSet ELISA (R&D Systems, DY2510-2)
Kit contents:
- Phospho-CREB (S133) Capture Antibody
- Phospho-CREB (S133) Detection Antibody
- Phospho-CREB (S133) Standard
- Streptavidin-HRP

Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, 78444)
Aprotonin, leupeptin, pepstatin cocktail (kind gift from Dr. Stefania Lamon-Fava)
PBS
Wash Buffer (R&D Systems, WA126)
Reagent Diluent Concentrate 2(R&D Systems, DY995)
Sample Diluent Concentrate 1 (R&D Systems, DYC001)
Substrate Solution (R&D Systems, DY999)
Stop Solution (R&D Systems, DY994)
96-well microplates (R&D Systems, DY990)
Plate sealers (R&D Systems, DY992)
8 or 12 channel multichannel pipet
Disposable reagent reservoirs (Baxter, 5082-128)
Serological pipets
Stop Solution (R&D Systems, DY994)
Refrigerated centrifuge (Sorvall, RT6000B, Dupont)
Centrifuge rotor H-1000B
Microplate reader

Things to do before starting

- Read kit insert/instructions.
- Read "ELISA Developing Guide" found on R&D website (http://www.rndsystems.com/resources/images/5670.pdfRead kit instructions.

Prepare solutions and reagents as explained in kit instructions.

Solutions	Reagents
Block Buffer	Phospho-CREB (S133) Capture Antibody
IC Diluent #1	Phospho-CREB (S133) Detection Antibody
IC Diluent #8	Phospho-CREB (S133) Standard
IC Diluent #3	Streptavidin-HRP
IC Diluent #7	
Lysis Buffer #6 (without inhibitors)	

Cell culture preparations

1. Plate 1 x 10^6 cells per well in 6-well plate (total volume per well is 2.0 mL). Place in incubator for 24 h.
2. Treat with MA, EPA or DHA (100 µM final concentration) or BSA for 24 h.
    - Add 1 mM FA or BSA directly to each well without changing media.
3. Stimulate with ultra-pure LPS (100 ng/mL) for 0, 30 or 60 min.
    - Add ultra-pure LPS directly to each well without changing media. Place plate in incubator during stimulation.
4. Rinse cells with PBS two times and remove liquid completely.
5. Cell lysates
    - Add protease/phosphatase inhibitor cocktail at 1:100 dilution and Aprotinin, Leupeptin, Pepstatin cocktail to Lysis Buffer #6. Lyse cells with Lysis Buffer #6 and allow samples to sit on ice for 15 min.
    - Assay immediately or store at -80°C.
    - Before use, centrifuge samples at 2000 x *g* for 15 min and transfer supernatant to a clean tube. For assaying, dilute lysates 6-fold with IC Diluent #8 and make further serial dilutions in IC Diluent #3.

ELISA Protocol (based on kit instructions)

1. Dilute Capture Antibody to working concentration of 4.0 µg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 µL per well of diluted Capture Antibody. Seal plate and incubate overnight at RT.
2. Empty wells
    - Invert plate over a large bin lined with paper towels, and flick to remove as much liquid from wells. Blot plate onto clean paper towels until no more liquid comes out.
3. Wash wells with Wash Buffer.
    - Fill each well completely with Wash Buffer using a 25 mL serological pipet and pipet aid. *Hold plate over bin to catch overflow*.
    - Empty wells as in step 2. Complete removal of liquid is essential for good performance.
4. Block plates by adding 300 µL of Block Buffer to each well. Incubate at RT for 1- 2 h.
5. Repeat empty/wash (steps 2 and 3). The plates are now ready for sample addition.
6. Add 100 µL sample or standard in IC Diluent #3 per well. Use IC Diluent #3 as zero standard. Cover with plate sealer and incubate 2 h at RT.
    - Note: A seven point standard curve using 2-fold serial dilutions and a high standard of 2000 pg/mL is recommended.
7. Repeat empty/wash (Steps 2 and 3)
8. Dilute Detection Antibody to working concentration of 500 ng/mL in IC Diluent #1 before use. Add 100 µL of diluted Detection Antibody to each well. Cover with new plate sealer and incubate 2 h RT.

9. Repeat empty/wash (steps 2 and 3).
10. Immediately before use, dilute Streptavidin-HRP to working concentration specified on vial label using IC Diluent #1. Add 100 µL of diluted Streptavidin-HRP to each well. Incubate 20 min at RT. Protect from direct light.
11. Repeat empty/wash (steps 2 and 3)
12. Add 100 µL Substrate Solution to each well. Incubate 20 min at RT. Protect from direct light.
13. Add 50 µL of Stop Solution to each well. Gently tap plate to ensure thorough mixing.
14. Determine optical density of each well immediately, using microplate reader set to 450 nm. Set wavelength correction to 540 nm or 570 nm.
15. Calculations based on standard curve and dilution factor.
16. Quantify protein from cell lysate using BCA™ protein assay. Dilute samples 10-fold.
17. Normalize Phospho-CREB (S133) values to total cell protein content.

14. ELISA analysis of PGE_2 in culture media

Materials/Equipment

Prostaglandin E_2 Express EIA kit – Monoclonal (Cayman Chemical, 500141)
Kit contents
- Prostaglandin E_2 Express EIA Monoclonal Antibody
- Prostaglandin E_2 Express AChE Tracer
- Prostaglandin E_2 Express EIA Standard
- EIA Buffer Concentrate (10X)
- Wash Buffer Concentrate (4000X)
- Polysorbate 20
- Goat Anti-Mouse IgG Coated Plate
- 96 Well Cover Sheet
- Ellman's Reagent
- EIA Tracer Dye
- EIA Antiserum Dye

Plastic film (Cayman Chemical, 400012)
8 or 12 channel multichannel pipet
Disposable reagent reservoirs (Baxter, 5082-128)
Refrigerated centrifuge (Sorvall, RT6000B, Dupont) set to 4℃.
Centrifuge rotor H-1000B
Orbital shaker
Microplate reader

Things to do before starting

- Read kit insert/instructions.
- Read "ELISA Developing Guide" found on R&D website (http://www.rndsystems.com/resources/images/5670.pdf

Cell culture preparation

1. Plate 0.5 x 10^6 cells per well in 24-well plate (1 mL culture media per well). Incubate 24 h.
2. Add MA, DHA or BSA directly to each well without changing culture media (100 µM final concentration). Incubate 24 h.
3. Add ultra-pure LPS directly to each well without changing culture media (100 ng/mL final concentration. Incubate 6 h.
4. Collect culture media. Centrifuge 1500 rpm for 5 min at 4℃. Transfer to new tube and store at -80℃ until use.

ELISA protocol (based on kit instructions)

1. Sample preparation. Dilute sample 10-fold with EIA Buffer.
2. Prepare PGE_2 standards according to instruction manual.
3. Reconstitute 100 dtn Prostaglandin E2 Express AChE Tracer by reconstituting with 6 mL EIA Buffer. Add 60 µL Tracer Dye. Store at 4 °C and use within 4 weeks.
4. Reconstitute PGE_2 Express Monoclonal Antibody with 6 mL EIA Buffer. Add 60 µL of Antiserum Dye. Store at 4°C and use within 4 weeks.
5. Plate Set Up: Refer to manual instructions and sample plate format.
    - Include 2 blanks, 2 non-specific binding wells, two maximum binding wells, 1 total activity well, and eight point standard curve run in duplicate.
    - Run samples in triplicate.
6. Add 100 µL EIA Buffer to non-specific binding wells. Add 50 µL EIA Buffer to maximum binding wells.
7. Add 50 µL of PGE_2 standard per well starting with lowest concentration and ending with highest concentration. Use same pipet tip to dispense all standards. Before pipetting each standard, be sure to equilibrate pipet tip in that standard (i.e., slowly fill the tip and gently expel the contents, repeat several times).
8. Add 50 µL sample per well.
9. Add 50 µL PGE_2 Express AChE Tracer to each well except Total Activity and Blank wells.
10. Add 50 µL PGE_2 Express Monoclonal Antibody to each well except Total Activity, Non-Specific Binding, and Blank wells.
11. Cover each plate with plastic film and incubate 60 min at RT on orbital shaker (165 rpm).
12. Reconstitute Ellman's Reagent immediately before use: Reconstitute 100 dtn vial Ellman's Reagent with 20 mL of diH2O. Protect from light and discard after use.
13. Empty wells and rinse 5 times with Wash Buffer. Blot plate onto clean paper towel after emptying wells until no liquid comes out.
14. Add 200 µL of Ellman's Reagent to each well.
15. Add 5 µL of Tracer to Total Activity wells.
16. Cover plate with plastic film. Incubate plate for 60 - 90 min on orbital shaker at 165 rpm until maximum binding wells are $\geq$ 0.3 A.U. (blank subtracted). Protect from light.
17. Wipe bottom of plate with clean tissue to remove fingerprints, dirt, etc.
18. Remove plastic film carefully without splashing Ellman's Reagent on plastic film. Return any reagent that splashed onto the plastic film back to original well. If too much Ellman's Reagent has splashed on the cover, wash plate three times with wash buffer and repeat development with fresh Ellman's Reagent.
19. Read plate at wavelength between 405 and 420 nm. Absorbance may be checked periodically until maximum absorbance wells have reached minimum of 0.3 A.U. (blank subtracted). Read plate when maximum absorbance wells are between 0.3 and 1.0 A.U. If absorbance exceeds 1.5, wash plate, add fresh Ellman's Reagent and develop again.

15. ELISA analysis of NFκB activation

Materials/Equipment

TransAM® NFκB p50 (Active Motif, 02512025)
Kit contents
- NFκB p50 antibody
- Anti-rabbit HRP-conjugated Antibody
- Wild-type Oligonucleotide AM20
- Mutated Oligonucleotide AM20
- Positive Control Nuclear Extract (Jurkat Nuclear Extract)
- Dithiothreitol (DTT) (1M)
- Protease Inhibitor Cocktail
- Herring Sperm DNA
- Lysis Buffer AM2
- Binding Buffer AM3
- 10X Wash Buffer AM2
- 10X Antibody Binding Buffer AM2
- Developing Solution
- Certificate of Analysis
- Stop Solution
- 96-well NFκB assay plate
- Plate sealer

8 or 12 channel multichannel pipet
Disposable reagent reservoirs (Baxter, 5082-128)
Orbital shaker
Microplate reader

Things to do before starting

- Read kit instruction manual.

Kit protocol

1. Prepare amount of Complete Lysis Buffer required for assay.
   - Combine 5 µL of 1M DTT, 10 µL Protease Inhibitor Cocktail per mL of Lysis Buffer AM2 (see Quick Chart in instruction manual).
   - Add Protease Inhibitor Cocktail immediately prior to use.
2. Prepare amount of Complete Binding Buffer required for assay.
   - Combine 2 µL of DTT and 10 µL of Herring Sperm DNA per mL of Binding Buffer AM3 (see Quick Chart in instruction manual).
   - Discard after use.
3. Prepare 500 uL of 1X Wash Buffer.
   - Prior to use, incubate at 50℃ for 2 min. and mix.

- Store for up to 1 week at 4℃

4. Prepare amount of 1X Antibody Binding Buffer required for assay.
    - Bring Antibody Binding Buffer AM2 to RT and vortex for 1 min before use.
    - For every 10 mL of 1X Antibody Binding Buffer required, dilute 1 mL 10X Antibody Binding Buffer AM2 with 9 mL distilled water (see Quick Chart in instruction manual).
    - Mix gently to avoid foaming.
    - Discard after use.
5. Dilute nuclear extract samples with Complete Lysis Buffer. Use 2 to 20 µg of nuclear extract diluted in Complete Lysis Buffer per well, total volume 20 µL. (My dilutions contained 2 µg nuclear extract per 20µL.)
6. Add 30 µL Complete Binding Buffer to each well to be used.
7. Add 20 µL of sample diluted in Complete Lysis Buffer per well.
8. Positive control wells: Add 2.5 µg of Jurkat Nuclear Extract (1 µL extract in 19 µL of Complete Lysis Buffer per well).
9. Blank wells: Add 20 µL Complete Lysis Buffer per well.
10. Seal plate with adhesive cover. Incubate 1 h at RT on orbital shaker (100 rpm). Keep any unused wells covered during remaining steps in order to preserve those wells for future assays. Any unused strips can be placed in original foil bag, sealed with tape and stored at 4℃.
11. Wash each well 3 times: Fill each well completely with 1X Wash Buffer. For each wash, flick plate over a large bin to empty wells, then, tap inverted plate on clean paper towels until no liquid comes out.
12. Add 100 µL of diluted NFκB antibody (1:1000 dilution in 1X Antibody Binding Buffer) to each well being used, including Blank wells.
13. Cover the plate and incubate for 1 h at RT without agitation.
14. Wash wells 3 times as described in step 11.
15. Add 100 µL of diluted HRP-conjugated Antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
16. Cover plate and incubate for 1 h at RT without agitation.
    - During this incubation step, place Development Solution at RT. (Developing Solution should be warmed to RT before use.) Protect from light.
17. Wash wells 3 times as described in step 11.
18. Add 100 µL Developing Solution to all wells being used.
19. Incubate 30 s to 5 min at RT protected from direct light. Read the Certificate of Analysis supplied with kit for optimal development time for specific kit lot. Monitor blue color development in sample wells. End incubation when medium to dark blue. Do not overdevelop.
20. Add 100 µL Stop Solution. In presence of acid, blue turns yellow.
21. Read absorbance on plate reader within 5 min at 450 nm with an optimal reference wavelength of 655 nm. Blank plate reader according to manufacturer's instructions using Blank wells.

16. Detection of cell surface expression of TLR4, TLR4-MD2 complex and CD14 by Flow Cytometry

Materials/Equipment

Monoclonal Anti-mouse TLR4-APC (R&D Systems, FAB2759A)
Rat IgG_{2A} Isotype Control-*APC* (R&D Systems, IC006A)
Purified Rat Anti-mouse CD16/CD32 (FcγIII/II), mouse BD Fc Block™ (BD Pharmingen, 553141)
Anti-mouse TLR4/MD-2 Complex APC (eBioscience, 17-9924)
IgG_{2A} K Isotype Control PE (EBioscience, 12-4321)
Anti-mouse CD14 PE (eBioscience, 12-0141)
Propidium iodide (PI) 2 mg/mL in PBS
Flow Cytometry Staining Buffer (1X) (R&D Systems, FC001)
FITC conjugate Lipopolysacharide from Escherichia *coli* 0111:B4 (Sigma F3665)
35 mm cell culture dishes
Culture media (DMEM containing 10% FBS, 100 U/mL Streptomycin/Penecillin)
Accuri® C6 Flow Cytometer
CFlow® software

Things to do before starting

- Read package inserts of all antibodies and buffers.
- Read Instrument manual for Accuri® C6 Flow Cytometer.
- Read CFlow® User Guide
- Prepare 2 mg/mL PI in PBS (store wrapped in foil ≤1 month at 4°C)
- Consult experienced operator of flow cytometer and CFlow.
- Lights off: Protect samples from light once working with fluorescent markers.

Cell culture preparation

This method was adapted from Wang [3]

1. Plate 0.75×10^6 cells per 35mm dish in 2 mL of culture media. Incubate 2 h.
2. Add MA, DHA (100 µM final concentration) or BSA to cells without changing culture media. Incubate 24 h.
3. Add ultra-pure LPS (100 ng/mL final concentration) to each dish except no-LPS control groups, without changing culture media. Incubate for 10, 30, 60, 180, or 360 min.
4. Collect cells.
    - Remove culture media and wash cells twice with Staining Buffer. Remove liquid.

- Add 1 mL of Staining Buffer and scrape cells into this volume. Pipet up and down to distribute cells evenly.
- Take out 200 µL for cell counting.
- Transfer 1 x 10^6 cells into 1.5 mL microcentrifuge tube.
- Centrifuge 125 x *g* for 5 min at 4˚C. Discard supernatant.

Sample preparation

1. Block FcγIII/II receptors*
    - Dilute 0.5 mg/mL BD Fc Block™ 5-fold to create 0.1 mg/mL solution (Ex. 22 µL of stock + 1078 µL staining buffer). Resuspend cell pellet with 100 µL of diluted Fc Block.
    - Incubate for 5 min at 4˚C.
2. Primary antibody: Add 0.5 µg primary antibody for TLR4, TLR4-MD2 or CD14 directly to cells. Incubate 1 h at RT in dark).
3. Wash cells: Add 1.4 µL Staining Buffer to cells. Centrifuge 125 x g for 5 min at 4˚C. Remove supernatant. Resuspend cells in 200 µL Staining Buffer.
4. Continue to section 18 "Flow Cytometry: Data collection and analysis." Run samples in flow cytometer immediately. Keep samples on ice between runs.

Notes

* BD Fc Block™ blocks non-antigen-specific binding of immunoglobulins to CD16/CD32 (FcγIII/II) receptors and possibly CD64 (FcγI) receptors.

17. Detection of FITC-binding to cell surface by Flow Cytometry

Materials/Equipment

FITC conjugate Lipopolysacharide from Escherichia *coli* 0111:B4 (Sigma F3665)
2% fetal bovine serum (FBS) in PBS (washing solution)
Propidium iodide (PI) 2 mg/mL in PBS
35 mm cell culture dishes
Culture media (DMEM containing 10% FBS, 1% Streptomycin/Penecillin)
Water bath
Accuri® C6 Flow Cytometer
CFlow® software

Things to do before starting

- Prepare 2% FBS in PBS
- Prepare 2 mg/mL PI in PBS (store wrapped in foil ≤1 month at 4°C)
- Read Instrument manual for Accuri® C6 Flow Cytometer.
- Read CFlow® User Guide
- Get tutorial from an experienced operator of the flow cytometer and analysis software.
- Set water bath to 37°C.
- Lights off once you begin working with FITC products.

Cell culture and sample preparation

1. Plate 0.75 x 10^6 cells per 35mm dish in 2 mL of culture media. Incubate 2 h.
2. Add MA, DHA (100 µM final concentration) or BSA to cells without changing culture media. Incubate 24 h.
3. Remove and discard 1 mL culture media from dish. Scrape and resuspend cells in remaining volume of culture media (1 mL). Transfer to 1.5 mL microcentrifuge tube. Add 1 µL of 1µg/µL FITC-LPS (1 µg/mL final concentration) to cells.* Incubate 1 h at 37°C in water bath with agitation, and invert tube 2 times every 10 min. (Samples do no need to be submerged in water.)
4. Centrifuge cells at 125 x *g* for 5 min at 4°C. Discard supernatant.
5. Resuspend cells in 200 µL of 2% FBS in PBS.
6. Continue to section 18 "Flow Cytometry: Data collection and analysis." Run samples in flow cytometer immediately. Keep samples on ice between runs.

Notes

* *Cells did not stain using 100 ng/mL of FITC-LPS, but stained with 1 µg/mL. Using 5 ug/mL did not increase level of staining, so 1 µg/mL was used.*

18. Flow Cytometry: Data collection and analysis

Materials/Equipment

Propidium iodide (PI) 2 mg/mL in PBS
Accuri® C6 Flow Cytometer
CFlow® software
Samples
Bucket of ice (for samples)

Things to do before starting

- If using instrument for the first time, notify the flow cytometer manager about your desire to use instrument.
- Reserve flow cytometer and/or computer in advance. Notify manager about using instrument after hours (nights/weekends).
- Read Instrument manual for Accuri® C6 Flow Cytometer.
- Read CFlow® User Guide
- Get tutorial from an experienced operator of the flow cytometer and analysis software.
- Prepare 2 mg/mL PI in PBS (store wrapped in foil ≤1 month at 4°C)
- Lights off.

Clean the SIP of Accuri® C6 Flow Cytometer (see CFlow User Guide, "Collecting Sample Data")

1. Open new CFlow workspace: There are 3 workspaces organized by tabs, Collect, Analyze and Statistics.
2. In the Collect workspace, click on the first empty well in the 96-well grid.
3. Place empty 12x75 mm tube on the SIP.
4. Click "Backflush"
5. After backflush, place fresh tube with 2 mL of filtered, de-ionized water on SIP. *Solutions for cleaning/maintaining SIP and instrument are kept next to instrument.*
6. Disable Run "Unlimited" check box in Instrument Control Panel (the area below 96-well grid).
7. Enable "Time" check box next to "Min" and "Sec" fields in Instrument Control Panel and type in run time of two minutes.
8. Select "Fast" in Fluidics section of Instrument Control Panel.
9. Click "RUN" to rinse out SIP.
10. Once run is finished, click on "Delete Sample Data" to delete data collected during rinse.
11. Remove the tube from the SIP.

Collect sample data (see CFlow User Guide, "Collecting Sample Data")

1. Set fluidics rate in Fluidics section of Instrument Control Panel.
    - Click on "Slow," "Medium," or "Fast."
    - **NOTE:** It is recommended to start data collection on slow and observe the data rate. You can then adjust the setting to medium or fast, if necessary.
2. Set threshold in Threshold section of Instrument Control Panel
3. Set run limits on Run Limits section of Instrument Control Panel.
4. Run Sample: Resuspend cells in sample tube and place tube on SIP. Select an empty data well in 96-well grid. Enter sample name in text box above 96-well grid. Be sure traffic light is green, then click "RUN" to start sample collection.
5. To add another sample to the file, remove previous sample tube from SIP, wipe outside of SIP with Kimwipe to minimize carryover between samples. Resuspend new sample and place tube on SIP, and click "RUN." NOTE: You do not need to perform a backflush between samples. Click on a new data well in 96-well sample grid. If you select an empty well, any plots and gates you created earlier are still displayed, but they do not contain any data.
6. PI staining of dead cells
    - After running sample, add 2.5 µL PI to sample. Mix well.
    - Click on a new cell and click "RUN."

Ending a Data Collection Session

When you finish collecting samples, rinse out the SIP to ensure cells or other particles are not left in the SIP. If you plan to collect more samples later in the day, perform the steps described in this section after running the last sample.

1. Place tube with 2 mL of filtered, de-ionized water on SIP and advance to any empty data well.
2. Set time limit for 2 min.
3. Click "RUN."
4. Place tube with 2 mL of decontamination solution (#KR-200) on SIP.
5. Select an empty data well.
6. Set time limit for 2 min. and fluidics speed to Fast.
7. Click "RUN."
8. Once run is finished, remove tube of decontamination solution from SIP.
9. Place tube with 2 mL of filtered, de-ionized water on SIP and advance to any empty data well.
10. Set time limit for 2 min and click "RUN."
11. When run is finished, leave tube on SIP.

Create plots (see CFlow User Guide, "Collecting Sample Data")

1. Click "Density Plot," "Dot Plot" or "Histogram Plot" in an empty plot corral.

2. Configure plot specifications as needed (CFlow displays an FSC-A vs. SSC-A plot, or FSC-A, for histogram by default.) Click "Plot Spec Tool." In the Set Plot Specs dialog box, do the following for each axis:
    - Select the parameter you want to view in the parameter drop-down list. For histogram, set x-axis to FL4-H for anti-TLR4 APE, anti-TLR4-MD2 APE, and FITC-LPS. Set x-axis to FL2-H for CD14 PE and propidium iodide (PI.)
    - Select "Linear" or "Log" to specify how data are displayed.
    - Type minimum and maximum channels to set channel range to view.
    - Enable or disable "Hide First Decade" check box to indicate whether you want CFlow to display the first decade of channels in plot.
    - Click "OK."

Set and apply gates (see CFlow User Guide, "Collecting Sample Data")

1. To create a gate on a density dot plot, click on a gating tool. Use mouse to draw region (labeled P1 for a polygonal gate, R1 for a rectilinear gate, or Q1 for a quadrant gate). CFlow automatically displays the percentage of cells within the region.
2. To create a vertical marker in a histogram plot. Click on "Vertical Marker Tool." Click cursor at point along x-axis where you want to place marker. CFlow automatically displays the percentage of cells to the left (V1-L) and right (V1-R) of the marker.
3. To create a horizontal marker in a histogram plot, click on "Horizontal Marker Tool." Click and drag cursor horizontally across area you want to gate. CFlow automatically displays percentage of cells within margins of marker (M1).
4. Apply gate, click on "GATE" at top of plot to which you want gate applied. Only polygon (P), rectilinear (R), and marker (M) gating regions automatically appear in Gating dialog box list of options. To view list of vertical markers or quadrant markers, enable associated check box(es) in the Change Gating dialog box.
5. Select gating icon associated with gate you want to use.
    - "Include" icon: to analyze events within region. You can choose more than one gate with "Include" to analyze events in either one *or* other Gate.
    - "Exclude" icon: to analyze events outside of region.
    - "Intersection icon: to analyze events within intersection of two or more regions (select this icon for each region you want to use).

6. Click "Apply." CFlow displays the type of gate that is applied next to "GATE" button in plot.

Set and apply gates on viable cells based on PI staining

PI staining can be done with APC and FITC fluorochromes, but for PE fluorochrome, need to use different stain, or do compensation between PE detector and PI detector because there is overlap in emission wavelength.

1. Open a histogram plot. Set x-axis to FL2-H (to see PI fluorescence).
2. Create a vertical marker that divides two populations of cells shown as two peaks. Left peak are viable cells, and right peak are dead cells.
3. Open a new histogram to view fluorescence associated with anti-TLR4-APC, or FITC-LPS, Set x-axis to FL4-H.
4. Click "GATE" button in plot. Choose "Include" for gate you created in step 38. Click "OK." Note: Anti-TLR4-APC staining of cells resulted in 2 peaks. When data was gated for live cells (steps 1-3), only the first peak (left) remained.

Analyze sample data (see CFlow User Guide, "Collecting Sample Data")

<u>Creating a new plot</u>

1. Click on "Analyze" tab.
2. Click on empty plot corral.
3. Click on one of the following icons under the Sample Grid:
    - Histogram
    - Dot
    - Density
    - Overlay Histogram
4. Click on sample well that contains the data you want to view.

<u>Creating an overlay histogram</u>

This type of plot enables you to compare multiple distributions from different sample treatments (and isotype control) at the same time.

1. Click on empty plot corral.
2. Click on Overlay Histogram Tool to open a blank single-parameter FSC-A plot.
3. Click on x-axis label (FSC-A) and select a different parameter in pop-up list if desired.
4. Click "GATE" and apply gate as appropriate.
5. Select data wells to be overlaid from 96-well grid.
6. Click Overlay Histogram Legend Tool to view legend for overlay histogram.

Viewing Statistics

Use this feature to create a customized master statistics table to view selected data across multiple samples. For example, include sample mean fluorescent intensity as one data item since this value is often reported and displayed in histograms or line graphs created in Excel or other graphing software.

1. Click on “Statistics” tab.
2. In Statistics Column Selector, enable check boxes under data items you want to view per plot. (CFlow automatically adds columns to the Master Statistics Table.)
3. In Sample Selector list, enable check box of each sample you want to view. (CFlow automatically adds rows of samples to the Master Statistics Table and displays sample data.)

Copying data into other applications

You can copy and paste plots and data from Master Statistics Table into most Microsoft Office compatible applications.

1. Use mouse to highlight fields you want to copy.
2. Press **Ctrl+C** to copy the data.
3. In Microsoft application (Excell, Powerpoint, Word) press **Ctrl+V to paste data.**

19. References

1. Folch, J., M. Lees, and G.H. Sloane Stanley, *A simple method for the isolation and purification of total lipides from animal tissues.* J Biol Chem, 1957. **226**(1): p. 497-509.
2. Morrison, W.R. and L.M. Smith, *Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride--Methanol.* J Lipid Res, 1964. **5**: p. 600-8.
3. Wang, J., et al., *Epigallocatechin-3-gallate inhibits expression of receptors for T cell regulatory cytokines and their downstream signaling in mouse CD4+ T cells.* J Nutr, 2012. **142**(3): p. 566-71.