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ADRP/ADFP and Mal1 expression are increased in macrophages treated with TLR agonists

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ABSTRACT

Activation of macrophages by TLR agonists enhances foam cell formation, but the underlying mechanisms are not understood. We examined the effects of TLR agonists on ADRP/ADFP, a protein associated with forming lipid droplets, and Mal1 a fatty acid-binding protein, in two mouse macrophage cell lines and human monocytes. Low doses of LPS, a TLR4 agonist increased both mRNA and protein levels of ADRP/ADFP and Mal1 in RAW 264.7 macrophages. Following pretreatment with Intralipid, fatty acids, or acetyl-LDL to increase triglyceride or cholesterol ester storage, LPS treatment still increased ADRP/ADFP and Mal1 mRNA levels. LPS also induced ADRP/ADFP and Mal1 in J774 macrophages and ADRP/ADFP in human monocytes. Zymosan, a fungal product that activates TLR2, poly-I:C, a viral mimetic that activates TLR3, and imiquimod, a TLR7 agonist, also increased ADRP/ADFP. Zymosan, but not poly-I:C or imiquimod, induced Mal1. In contrast, neither gene was induced by TNFα, IL-1β, IL-6, or interferon-γ. Thus TLR agonists induce ADRP/ADFP and Mal1, which likely contributes to macrophage triglyceride and cholesterol ester storage leading to foam cell formation.

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1. Introduction

Atherosclerosis is now recognized to be an inflammatory disorder [1]. Moreover, the risk of atherosclerosis has been shown to increase with concurrent infection [2–5]. Toll-like receptors (TLRs) mediate immune responses triggered by a number of microbial pathogens, such as Gram negative bacteria (via TLR4), viruses (TLR3), and fungi and Gram positive bacteria (TLR2) [6,7]. These receptors are abundantly expressed in macrophages. Both exogenous and endogenous TLR ligands have been proposed to stimulate inflammatory cascades contributing to atheroma formation [8]. Individuals with a TLR4 polymorphism that attenuates receptor signaling have a decreased risk of atherosclerosis, thus establishing a link between TLRs and the development of atherosclerosis [9]. Additionally, studies in humans with periodontitis have suggested that the pro-atherogenic changes in these subjects may be mediated by increases in serum lipopolysaccharide (LPS) during the infection [5,10]. Putative endogenous TLR ligands, such as heat shock proteins, fatty acids, and oxidized LDL, have also been implicated in the pathogenesis of atherosclerosis [11–14].

In addition to playing a key role in innate immunity, macrophages also accumulate triglyceride and cholesterol esters and become foam cells, a characteristic finding in atherosclerotic lesions [1]. LPS stimulation of macrophages greatly enhances cholesteryl ester and triglyceride accumulation [15,16]. This phenomenon is also evident *in vivo* given the increase in atheroma size seen in LPS-treated apolipoprotein E-deficient mice and cholesterol fed rabbits [17,18].

The increased cholesterol ester and triglyceride content of macrophages is stored in cytoplasmic droplets that contain proteins including adipocyte differentiation-related protein (ADRP, also known as ADFP or adipophilin) [19]. ADRP/ADFP is a member of the PAT (perilipin–adipophilin–TIP47) protein family that is involved in the transport [20] and storage of neutral lipids in multiple cell types [21–23]. ADRP/ADFP is expressed in cholesterol ester loaded macrophages where it is found on the droplet surface. ADRP/ADFP expression is increased in the cholesterol ester enriched macrophages of atherosclerotic lesions [24]. In addition, RNA interference-based depletion of ADRP/ADFP mRNA decreases cholesterol ester accumulation in macrophages whereas ADRP/ADFP overexpression increases macrophage cholesterol

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ester storage and reduces apolipoprotein AI mediated cholesterol efflux [25].

Uptake of fatty acids by macrophages is facilitated by fatty acid-binding proteins (FABPs) [26]. We have recently reported that several different TLR agonists increase in the expression of adipocyte fatty acid-binding protein (aP2) paralleling triglyceride accumulation in murine macrophages [27]. Keratinocyte fatty acid-binding protein (Mal1, or FABP5) has a high degree of homology to aP2 and is expressed in macrophages and adipocytes among other tissues [28–30]. In adipocytes from aP2-deficient mice, there is a compensatory increase in Mal1 expression [31]. Combined deficiency of aP2 and Mal1 is associated with significant reductions in atherosclerotic lesion size in apoE-deficient mice [32]. Thus, deficiency of FABPs likely decreases foam cell formation, suggesting an important role for these proteins in the uptake and storage of triglycerides and cholesterol esters.

Given the evidence supporting a role for ADRP/ADFP and Mal1 in facilitating triglyceride and cholesterol ester accumulation in macrophages we hypothesized that LPS and other TLR ligands, which have been previously shown to increase macrophage triglyceride and cholesterol ester accumulation, would increase the expression of ADRP/ADFP and Mal1 as part of a coordinated change in macrophage physiology.

Table 1
Primer sequences used for QPCR.

m ADRP/ADFP	GGAGTGGAAGAGAAGCATCG CAACACAGTGGGACTCATGG
m Mal1	TGTGTGTACTGGCCATCGTT GTTGACGGCATAGCCAGAAT
m S3-12	GCTGACACCAAAACCCTTGT ACCACACTCCTCCACTGACC
m TIP47	AAACAGGGTGTGGACCAGAG GGCTTAGCTGGGTCCTTTTC
h ADRP/ADFP	TCAGCTCCATTCTACTGTTCACC CCTGAATTTTCTGATTGGCACT
h Mal1	ACCCTGGGAGAGAAGTTTGAA ATCCCACTCCTGATGCTGAA
h aP2	CCTTTAAAAATACTGAGATTTCCTTCA GGACACCCCCATCTAAGGTT
h FABP1	TGATCCAAAACGAATTCACG TCACCTTCCAACTGAACCACT
h FABP3	AGCAGATGACAGGAAGGTCAA TCTGCAGGTGAACAAGTTTCC
h/m 36B4	GCGACCTGGAAGTCCAACTAC ATCTGCTGCATCTGCTTGG

FABP1—liver fatty acid-binding protein. FABP3—heart fatty acid binding protein. m—mouse. h—human.

2. Methods

2.1. Materials

LPS from *Escherichia coli* strain O55:B5 was purchased from Difco (Detroit, MI) and diluted in sterile normal saline to the desired concentration. Dulbecco's Modified Eagle's Medium (DMEM) was from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Human serum albumin (HSA) was obtained from Bayer (Elkhart, IN). Intralipid (IL), a 10% emulsion of soybean oil, was from Fresenius Kabi Clayton (Clayton, NC). Tri Reagent, oleic acid–albumin, thalidomide, and protease inhibitor cocktail were from Sigma (St. Louis, MO). Zymosan, polyinosine:polycytidylic acid (poly-I:C), imiquimod, LY294002, SB20350, and PD98059 were from InvivoGen (San Diego, CA). FITC-conjugated mouse monoclonal antibody against ADRP/ADFP and guinea pig anti-mouse ADRP/ADFP antiserum were from RDI (Concord, MA). Goat anti-mouse Mal1 IgG and mouse TNF-α, IL-1β, and IL-6 were purchased from R&D Systems (Minneapolis, MN). Mouse interferon-γ was a gift from Genentech (South San Francisco, CA). Acetylated low density lipoprotein (AcLDL) was from Intracel (Frederick, MD).

2.2. Cell culture

RAW 264.7 and J774, two murine macrophage cell lines, were from American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% serum and incubated at 37 °C in 5% CO_2. When confluent, cells were washed with serum-free medium once and then treated in medium with 2.5% HSA for indicated times (4–24 h) prior to RNA isolation, protein isolation and staining. For inhibitor studies, cells were treated with LY294002 (100 μM), SB203580 (20 μM), or PD98059 (100 μM) in the presence or absence of LPS (100 ng/ml) for 16 h. For thalidomide treatment, cells were pre-incubated with 500 μg/ml thalidomide for 1 h before addition of LPS (100 ng/ml) for 16 h. For lipid loading, cells were co-incubated with LPS at 100 ng/ml and Intralipid at 150 μg triglyceride/ml, oleic acid (OA) complexed to BSA at 0.3 mM, or acetylated LDL (AcLDL) at 100 μg/ml for 16 h. For treatment with cytokines, cells were treated with TNF alpha, IL-1 beta, IL-6, and interferon-γ at 10 ng/ml in serum-free medium for 16 h. Human peripheral blood monocytic cells (PBMCs) were isolated utilizing Hypaque-Ficoll Plus (Amersham Bioscience, Pittsburgh, PA) from whole blood provided by normal healthy volunteers. PBMCs were cultured (10^7 cells/well) for 2 h in 12-well plates at 37 °C in a CO_2 incubator with RPMI 1640 and 10% heat-inactivated FBS. Nonadherent cells were removed and the medium was replaced with serum-free medium containing LPS at 1 μg/ml. Volunteers provided written informed consent under protocols that were approved by the UCSF Committee on Human Research.

2.3. RNA isolation and quantitative PCR

Total RNA was isolated from using Tri Reagent. First strand cDNA was synthesized from 1 μg of total RNA with the iScript™cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reversed transcribed total RNA, 450 nM forward and reverse primers, and 10 μl of 2× LightCycler 480 SYBR Green I Master in a final volume of 20 μl in 96-well plates using Mx3000P™ Real-time PCR System (Stratagene, La Jolla, CA). Quantification was performed by the comparative C_T method with 36B4 used for normalization. QPCR primers are listed in Table 1.

2.4. Immunostaining

RAW cells were seeded on dual chamber glass slides and grown to confluence prior to treatment with LPS at 100 ng/ml. After fixation with 2% formaldehyde, the cells were rinsed twice with PBS (pH 7.4). Cells were permeabilized and nonspecific binding was blocked by incubating with 3 mg/ml goat serum in PBS with 0.1% (w/v) saponin and 0.75 mg/ml glycine for 2 h at room temperature. Slides were then rinsed twice in PBS with 0.1% (w/v) saponin. For detection of ADRP/ADFP, the cells were incubated overnight at 4 °C in the dark with a monoclonal mouse anti-ADFP-FITC-conjugated antibody (Research Diagnostics, Inc., Concord, MA) at 20 μg/ml. The cells were subsequently rinsed three times with PBS containing 0.1% (w/v) saponin before microscopy. All staining was observed with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).