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Hepcidin-ferroportin axis controls toll-like receptor 4 dependent macrophage inflammatory responses in human atherosclerotic plaques

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ABSTRACT

Objectives: Toll-like Receptor 4 (TLR4) is implicated in modulating inflammatory cytokines though its role in atherosclerosis remains uncertain. We have recently described a non-foam cell macrophage phenotype driven by ingestion of hemoglobin:haptoglobin complexes (HH), via the scavenger receptor CD163, characterized by reduced inflammatory cytokine production. In this study, we examined the role of iron metabolism in modulating TLR4 signaling in these cells.
Methods and results: Areas in human atherosclerotic plaque with non-foam cell, CD163 positive macrophages demonstrated reduced expression of tumor necrosis factor alpha (TNF-α) and interferon-beta (INF-β) compared to foam cells. Human macrophages differentiated in hemoglobin:haptoglobin (HH) complexes expressed the CD163 positive non-foam cell phenotype and demonstrated significantly less TNF-α and INF-β compared to control macrophages when exposed to oxidized LDL (oxLDL) or lipopolysaccharide (LPS). LPS stimulated expression of TNF-α and INF-β could be restored in HH macrophages by pretreatment with hepcidin, an endogenous suppressor of ferroportin1 (FPN), or by genetic suppression of FPN in macrophages derived from myeloid specific FPN knockout mice. LPS stimulated control macrophages demonstrated increase in TLR4 trafficking to lipid rafts; this response was suppressed in HH macrophages but was restored upon pretreatment with hepcidin. Using a pharmacologic hepcidin suppressor, we observed a decrease in cytokine expression and TLR4-lipid raft trafficking in LPS-stimulated in a murine macrophage model.
Conclusion: TLR4 dependent macrophage signaling is controlled via hepcidin-ferroportin1 axis by influencing TLR4-lipid raft interactions. Pharmacologic manipulation of iron metabolism may represent a promising approach to limiting TLR4-mediated inflammatory responses.
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1. Introduction

Macrophage-mediated inflammatory signaling plays an important role in atherosclerosis progression [1]. Toll-like receptors (TLR) are pattern recognition receptors expressed on the surface of macrophages that activate pro-inflammatory signaling pathways in response to microbial pathogens or modified endogenous ligands [2]. There is evolving evidence for TLR4 signaling in promoting vascular disease with some data suggesting TLR4 deficiency confers vascular protection in mouse atherosclerosis models [3,4]. However, other experiments have shown TLR4 deficiency in animals

Abbreviations: FPN, ferroportin1; GM1, GM1 ganglioside; HH, hemoglobin:haptoglobin complex; Hep, hepcidin; INF-β, interferon beta; KO, knockout; LPS, lipopolysaccharide; Ox LDL, oxidized LDL; PCR, polymerase chain reaction; TIR, toll/interleukin-1 receptor; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

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does not confer these same effects, suggesting an alternative pathway may be involved [5,6]. Polymorphisms in TLR4 locus have been associated with variation in atherosclerosis risk in humans [7].

Upon activation, TLR4 signaling is propagated by recruitment of toll/interleukin-1 receptor (TIR) domain-containing adaptor molecules MyD88, TIRAP (also called MAL), TRIF and TRAM, that link to conserved signaling pathways activating interferon-regulated genes as well as other pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α) [2]. Uptake of oxidized lipids into macrophages is facilitated by a CD36/TLR4/TLR6 heterotrimer that initiates signals that are propagated by both the MyD88 and TRIF adaptors [8]. Of the downstream products of TLR4 signaling, data from advanced human atherosclerotic plaques and experimental animal models indicates an important role for both TNF-α and interferon β (INF-β) which promote macrophage infiltration and atherosclerosis progression [9,10]. Although macrophages within atherosclerotic lesions appear to be a major source of both cytokines, the relative contribution of different macrophage subtypes to the inflammatory milieu and the mechanisms regulating their expression in human atherosclerosis has not been previously explored.

We recently described a unique macrophage phenotype within areas of intraplaque hemorrhage and neoangiogenesis in postmortem human atherosclerotic plaques [11]. These macrophages are characterized by resistance to foam cell formation both *in vivo* and *in vitro*, increased expression of the hemoglobin:haptoglobin scavenger receptor, CD163, and reductions in local TNF-α and reactive oxygen species (ROS) content [11]. The aim of this study was to examine the expression of TLR4-dependent cytokines TNF-α and INF-β within CD163 positive macrophages compared with foam cell areas within human atherosclerotic plaques and investigate the mechanisms likely responsible for the differential cytokine expression seen in these macrophage subtypes.

2. Methods

2.1. Human tissue specimens

Human atherosclerotic plaques were selected from the CVPath Institute Sudden Coronary Death registry (CVPI SCDr) [12]. All plaques were identified according to a modified AHA classification [13]. See supplemental section for more details.

2.2. Cell culture, immunoblotting, immunoprecipitation and reagents

Human monocytes collected from healthy volunteers (Astarte Biologics, Redmond, WA) were differentiated over 1 week into macrophages in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% human serum (Invitrogen). In some experiments hemoglobin:haptoglobin (HH) complexes (Sigma–Aldrich, St. Louis, MO) were added to differentiation media. See supplemental section for more details.

2.3. Immunofluorescence and confocal microscopy

Human atherosclerotic plaques were incubated with primary antibodies, including those against CD163, CD68, CD31, CD34, TNF-**α** and INF-**β.** Differentiated human and mouse peritoneal macrophages were incubated with anti-TLR4 antibody and anti-cholera toxin antibody for lipid rafts. Fluorescent images were obtained by a laser scanning confocal microscopy. See supplemental section for more details.

2.4. Macrophage transfection, RNA isolation and real time quantitative PCR

Human macrophages were transfected with siRNA (Invitrogen) for ferroportin-1 (FPN), RNA isolation and real time quantitative polymerase chain reaction (PCR) was performed as previously described [11]. See supplemental section for more details.

2.5. Sucrose-gradient lipid raft fraction isolation

Differentiated human macrophages were lysed and 12 subfractions were isolated for immunoblotting for TLR4 and flotillin-1. See supplemental section for more details.

2.6. Animals and experimental protocols

The Institutional Animal Care and Use Committee (IACUC) at Emory University approved all animal protocols and protocols were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J male mice 12–14 weeks old (Jackson Laboratories, Bar Harbor, ME) were used.

2.7. In vivo pharmacologic hepcidin suppression

LDN-193189 (4-[6-(4-piperazin-1-ylphenyl) pyrazolo[1,5-a] pyrimidin-3-yl]quinoline), a bone morphogenetic inhibitor and hepcidin suppressor, or vehicle solution was given twice daily intraperitoneally to experimental animals for 4 days [14]. Hepcidin was given in addition daily at days 3 and 4 in accordance to Emory University IACUC and previously published protocol [15]. Animal underwent peritoneal lavage to isolate peritoneal macrophages. See supplemental section for more details.

2.8. FPN knockout mice cell culture

Floxed FPN (FPN $^{flox/flox}$) mice [16] (obtained from Nancy Andrews, Duke University) and FPN $^{flox/flox}$_Lys M Cre mice underwent peritoneal lavage, and peritoneal macrophages were cultured in media (10% fetal bovine serum, 1% penicillin streptomycin in RPMI medium 1640) with or without mouse hemoglobin (100 μg/mL) for 24 h prior to lipopolysaccharide (LPS, 100 ng/mL) treatment. Cells were treated with LPS for 4 h before supernatant was collected. See supplemental section for more details.

2.9. Statistical analysis

Data are expressed as mean ± SD. For comparisons between groups for continuous variables, a Student's t-test or 1-way ANOVA test was performed using JMP software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Interferon-β (INF-β) and Tumor Necrosis Factor-α (TNF-α) Are Differentially Expressed Within Macrophages in Advanced Human Atherosclerotic Plaques with Neoangiogenesis

Serial sections of human atherosclerotic plaques with evidence of neoangiogenesis were stained with antibodies against CD68 (macrophages) and CD163 as well as Oil Red O (ORO) to delineate areas of foam cells versus CD163 + macrophages as previously described (Fig. 1A–B) [11]. Using dual immunofluorescence, both TNF-α and INF-β expression were significantly higher in CD68 positive/CD163 negative foam cells compared with CD163/CD68 positive macrophages in the sampled advanced human atherosclerotic plaques

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