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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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Disclosures
None.

Keywords

Inflammation; Atherosclerosis; Macrophage

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 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 CVPPath 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 μ g/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 $\pm$ 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 Neovascularization

Serial sections of human atherosclerotic plaques with evidence of neovascularization 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 from six different post-mortem cases (Fig. 1C–D).

3.2. Hemoglobin:Haptoglobin differentiation alters macrophage production of INF- β /TNF- α in response to toll-like receptor 4 (TLR4) activation

Human monocytes were differentiated in hemoglobin:haptoglobin complex (HH) enriched media for 7 days which we have previously shown recapitulates the CD163 + macrophages phenotype found in areas of intraplaque hemorrhage and neovascularization [11]. Control and HH differentiated macrophages were exposed to the Toll-like receptor 4 (TLR4) activator, lipopolysaccharide (LPS, 25 ng/ml) and media analyzed by ELISA 4 h later. Expression of INF- β and TNF- α in response to LPS was suppressed in HH differentiated macrophages compared with control macrophages (Fig. 2A–B). We have previously shown that HH differentiated macrophages demonstrate lower intracellular free iron level which is partly due to upregulation of the iron transporter ferroportin-1 (FPN) [11]. In other contexts, lower macrophage intracellular iron levels have been linked to alterations in TLR4 signaling [17]. Because of this, we examined whether the reduction seen in HH macrophage cytokine responses after LPS treatment could be altered by pretreatment of these cells with hepcidin, a hepatic hormone whose major role is to degrade ferroportin-1 (FPN) and thus increase intracellular iron levels. The cytokine responses to LPS of HH differentiated cells after hepcidin treatment was similar to control cells (Fig 2A–B), suggesting intracellular free iron levels play a critical role in modulating TLR4 signaling within HH differentiated macrophages [11,18]. To more clearly mimic the milieu of the atherosclerotic plaque we also used the non-traditional TLR4 activator oxidized low density lipoprotein (oxLDL). Cytokine expression was decreased in HH differentiated macrophages compared with control macrophages when stimulated with oxLDL, (Fig. 2C) [8]. Control macrophage response to oxLDL was suppressed when cells were treated with a TLR4 blocking antibody, indicating macrophage cytokine expression of both TNF- α and INF- β in response to oxLDL is likely TLR4 dependent (Fig. 2C).

In addition to its effects on the degradation of FPN, hepcidin itself has transcriptional activity through activation of the transcription factor Stat3. A previous study showed

hepcidin modulated LPS-induced transcriptional responses in both cultured macrophages and *in vivo* mouse models as demonstrated by suppression of IL-6 and TNF- α [19]. This is consistent with the suppressive effects of hepcidin on LPS responses in control macrophages shown in Fig. 2A and B. To examine whether hepcidin's effect on TLR4 signaling in HH macrophages is mediated through FPN (versus direct transcription of target genes) we used an siRNA against FPN. Suppression of FPN in HH differentiated human macrophages compared with non-targeting siRNA increased both TNF- α and INF- β expression in response to LPS (Fig. 2D–E). Additionally peritoneal macrophages from mice with genetic deletion of FPN were similarly examined. In control (FPN floxed) mouse peritoneal macrophages expression of TNF- α in response to LPS was suppressed after hemoglobin (Hb)-treatment as compared to Hb-treated macrophages from FPN^{flox/flox}_Lys M Cre knockout mice (Fig. 2F). Collectively, these findings suggests that HH differentiated macrophages suppress TLR4-dependent cytokine expression through modulation of iron metabolism via hepcidin-ferroportin1 axis.

3.3. Hemoglobin:Haptoglobin Differentiated Macrophages Demonstrate Decreased TLR4 interaction with TRIF/MyD88 and trafficking to lipid rafts

The initiation of downstream signaling pathways by TLR4 activation requires trafficking of TLR4 to lipid rafts serving as a platform for receptor-mediated signal transduction [20,21]. In control differentiated human macrophages the distribution of FITC-cholera toxin B that stains the GM1 ganglioside component of lipid rafts (GM1) [22] was largely distributed in the plasma membrane while TLR4 localized to both the membrane and cytosol compartments (Fig. 3A). After TLR4 activation with LPS, both TLR4 and GM1 were predominately co-localized within the membrane compared to the cytosol suggesting increased TLR4 trafficking to lipid rafts. In HH differentiated human macrophages, LPS-induced TLR4 trafficking to the membrane was reduced compared to control macrophage after TLR4 activation with LPS (Fig. 3A). This effect was rescued when cells were treated with hepcidin (Hep) (i.e. HH + LPS + Hep group) with similar response to LPS as control macrophages.

In order to investigate the signaling defect seen in LPS-induced activation of TLR4 in HH differentiated macrophages, we investigated the interaction of TLR4 with its adaptor molecules, TRIF and MyD88, respectively. The interaction of TLR4 with both TRIF and MyD88 observed by immunoprecipitation (IP) was increased by LPS in control differentiated but not in HH differentiated macrophages (Fig. 3B) and partially rescued by Hep pre-treatment. No significant differences were seen in the total protein levels of TLR4, TRIF or MyD88 in all groups. These experiments suggest that the interaction of TLR4 with adaptor molecules TRIF and MyD88 is impaired within HH differentiated macrophages and not dependent on the overall expression of adaptor molecules. Moreover, they suggest that the location of TLR4 signaling defect is proximal to the interaction of TLR4 with its adaptor molecules consistent with inhibition of TLR4 lipid raft interactions.

To further confirm our results, we isolated lipid raft fractions from macrophages under various experimental conditions mentioned above with sucrose-gradient ultracentrifugation. (A flotillin antibody was used to identify the location of lipid rafts which are usually seen in

fractions 3–5). TLR4 was observed to be located within the lipid raft fraction in control differentiated macrophages upon LPS stimulation however this was not observed in HH differentiated macrophages after LPS stimulation (Fig. 3C). These observations suggest that TLR4 trafficking to lipid rafts occurs after LPS activation of TLR4 and that this trafficking is impaired in HH differentiated macrophages.

3.4. Pharmacologic Inhibition of Hepcidin in Mice Suppresses TLR4 response to LPS in peritoneal macrophages

To demonstrate the *in vivo* relevance of our findings we examined TLR4-lipid raft trafficking and cytokine responses to LPS in mice administered LDN 193189 (LDN), a bone morphogenetic protein (BMP) inhibitor and suppressor of hepcidin [14]. We have previously shown that LDN increases macrophage expression of the iron exporter ferroportin-1 (FPN), which reduces macrophage intracellular iron similar to HH differentiated macrophages [15]. In addition, we observed that lowered intracellular iron levels in HH macrophages can be reversed when treated with hepcidin (supplemental figure 1). Peritoneal macrophages were harvested via lavage from mice after either vehicle or LDN treatment (10 mg/kg intraperitoneally twice daily for 4 days) and subsequently exposed to LPS (100 ng/ml) *ex vivo* prior to analysis. Increases in TLR4-lipid raft trafficking, TNF- α and INF- β expression were seen in response to LPS in peritoneal macrophages from vehicle treated mice. Macrophages from LDN-treated mice had significantly less production of both cytokines and TLR-lipid raft trafficking (Fig. 4A–B). To demonstrate the critical role played by intracellular iron metabolism in modulating TLR4 signaling, we treated mice with hepcidin (25 ug intraperitoneally daily) [23] during days 3 and 4 of LDN treatment and examined macrophage responses to LPS. Increased cytokine expression and TLR4-lipid raft trafficking was observed in LDN treated peritoneal macrophages, emphasizing the role of macrophage iron metabolism in TLR4 signaling *in vivo*.

4. Discussion

It is well recognized that macrophage mediated inflammatory responses play an important role in atherosclerosis progression. How macrophage subtypes may influence this inflammatory milieu is less well understood and may explain the heterogeneous inflammatory response within advanced atherosclerotic plaques [24]. Here we examine a specific but important inflammatory pathway within a particular non-foam cell macrophages subtype characterized by increased hemoglobin:haptoglobin scavenger receptor (CD163) [25] expression and found in areas of intraplaque hemorrhage/neoangiogenesis. Previous mechanistic studies done by our lab demonstrate that by lowering intracellular iron in part by increasing free iron export via up-regulation of FPN, HH differentiated macrophages recapitulate the CD163 positive phenotype [11]. In this study, we observe altered TLR4 signaling resulting in reduced expression of the pro-inflammatory cytokines (i.e. TNF- α and INF- β) in response to TLR4 activators oxLDL and LPS in HH macrophages. Our data suggest this alteration is modulated by FPN, a key regulator macrophage intracellular iron [16]. These results indicate that intracellular iron metabolism plays an important role in TLR4-dependent cytokine expression. HH differentiation of human monocytes prevents the interaction of TLR4 with lipid rafts necessary for its signaling [20]. Given the important role

of TLR4 in mediating inflammatory signals in response to oxLDL [8], these mechanisms are likely responsible for reduced expression of these cytokines seen in CD163 positive macrophage in human atherosclerosis. These data enhance our understanding of inflammatory responses to hemorrhage by emphasizing how cellular iron homeostasis influences activation of this important pro-inflammatory pathway.

Previous data from both experimental studies in mice and humans suggest an evolving role for TLR4 in vascular disease progression [4,7]. TLR4-null mice crossed onto the apolipoprotein E-null background show significant inhibition of atherosclerosis while polymorphisms in the TLR4 gene (Asp299Gly) have been associated with reduced atherosclerosis risk and lower levels of circulating pro-inflammatory cytokines. Although TLR4 is best known as a receptor for the bacterial endotoxin, LPS, more recent data suggest an important role for this receptor in promoting lipid mediated inflammatory responses through binding oxLDL [8]. Indeed, we demonstrate in human macrophages that oxLDL-mediated increases in both TNF- α and INF- β were inhibited by a blocking antibody against TLR4. OxLDL also had little effect upon cytokine production in HH differentiated cells *in vitro*. While foam cells in human atherosclerotic plaques demonstrated abundant staining for TNF- α and INF- β , little expression was detected within CD163 positive macrophages. While we previously reported that HH differentiated macrophages resist foam cell formation in response to lipid loading [11], these results also suggest that intracellular inflammatory pathways activated by oxLDL are also deactivated. Given the previously recognized pro-atherosclerotic role of both TNF- α and INF- β , these results further demonstrate the importance of iron homeostasis within macrophages in controlling inflammatory signaling.

In this study we used hepcidin to modulate intracellular iron levels in macrophages to demonstrate the effect of iron on TLR4 signaling. Complicating the interpretation of these studies is the fact that hepcidin has transcriptional activity (i.e. activator of the transcriptional factor Stat3) independent from its ability to degrade FPN [19]. Indeed, we demonstrate hepcidin treatment of control macrophages suppresses LPS-induced cytokine production (Fig. 2A–B). This effect is likely mediated through induction of the anti-inflammatory cytokine IL-10 by Stat3 [26].

To examine whether hepcidin's effect on TLR4 signaling in HH loaded macrophages is mediated through FPN (versus direct transcription of target genes), we directly knocked down FPN via siRNA (Fig. 2D–E) and conducted experiments in ferroportin KO mouse macrophages (Fig. 2F). Both experiments demonstrated that FPN plays a critical role in the suppression of LPS-induced cytokines in hemoglobin loaded macrophages. Our results demonstrate manipulation of the hepcidin-FPN axis in HH macrophages alters TLR4 trafficking to lipid rafts and inhibits inflammatory cytokine responses to LPS. Recently, Wang et al. demonstrated that hemochromatosis gene knockout mouse macrophages (which demonstrate lower intracellular iron due in part to upregulation of FPN) have intact Mal/MyD88 responses but impairment in TLR4/TRAM/TRIF pathway proximal to TRIF, which resulted in reduced LPS-induced cytokine expression of both TNF- α and INF- β [17]. Unlike Wang et al., we demonstrate defects in both arms of the TLR4 signaling pathway as indicated by lack of association of TLR4 with adaptor molecules MyD88 and TRIF in response to LPS in HH differentiated macrophages. The interaction of TLR4 with TRIF and

MyD88 could be increased by raising intracellular iron via hepcidin supplementation. Thus it appears that the major contributor to alterations in TLR-4 signaling in HH loaded macrophages involves the hepcidin-FPN axis.

How exactly does lowering intracellular iron inhibit TLR4 signaling? In the presence of hydrogen peroxide iron drives the generation of highly toxic hydroxyl radicals via the Fenton reaction. We have previously shown that HH differentiated macrophages demonstrate lower amounts of reactive oxygen species both in culture and in human atherosclerotic plaques [11]. An essential role for ROS in mediating TLR4 lipid raft interactions has been shown in mouse cell lines where treatment of cells with the anti-oxidant carbon monoxide (CO) inhibited both the interaction of TLR4 with TRIF and with MyD88²⁰. Stimulation of cells with hydrogen peroxide or with phorbol myristate acetate (PMA), a potent NADPH activator, recruited TLR4 to lipid rafts. In our data, pretreatment of HH cells with hepcidin, which increases macrophage ROS by altering iron metabolism, corrects TLR4 signaling as indicated by increased interaction of TLR4 with TRIF and MyD88¹¹. These findings were also corroborated using siRNA knockdown of FPN in LPS-stimulated HH differentiated macrophages which resulted in restoration of TLR4-dependent cytokine expression. Thus it appears that the major mechanism underlying all of these results is reduction in ROS but this requires further examination.

To demonstrate the importance of these mechanisms in macrophage behavior *in vivo*, we treated mice with a pharmacologic inhibitor of hepcidin to increase expression of the iron exporter FPN. We previously reported that LDN decreases macrophage ROS, in part, by lowering intracellular free iron through increased expression of the iron exporter FPN [15]. We now demonstrate that macrophages from LDN treated animals demonstrate significant inhibition of TLR4 signaling as indicated by significantly reduced expression of TNF- α and INF- β in response to LPS. Inhibition of TLR4 signaling was accompanied by inhibited trafficking to lipid rafts. We previously reported that Apo E^{-/-} mice treated for 10 weeks with LDN demonstrated significant reductions in atherosclerosis [15]. The results presented here also suggest part of the salutary effects of LDN on atherosclerosis may be mediated through inhibition of TLR4 mediated inflammatory cytokine responses.

In conclusion, we have characterized TLR4 signaling within a novel phenotype of non-foam cell macrophage and determined the mechanisms by which this signaling pathway is inhibited. Iron driven modulation of TLR4 signaling impairs trafficking of TLR4 to lipid rafts and reduces pro-inflammatory cytokine expression. These data enhance our understanding of inflammatory pathways within distinct macrophages subtypes within atherosclerotic plaques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2015.06.025>.

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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Fig. 1. Cytokine Expression is Lower in CD163 Positive Macrophages Within Human Atherosclerotic Plaques. (A) Human coronary fibroatheroma with eccentric stenosis collected at autopsy from sudden death victim. Note region of interest (ROI) with red box on the edge of the fibroatheroma. (B) Oil red O (ORO) staining localizing foam cells (right of dotted line) and CD163 area (left of dotted line). Below images shows dual immunofluorescent staining for CD68 (red) and CD163 (green) in the same ROI distinguishing foam cells from CD163 + macrophages. The blue signal represents the DAPI nuclear stain. Scale bar(s) = 100 μm (C) Dual immunofluorescence labeling for CD68 (red) and cytokine (i.e. TNFα or INF-β, both green) with yellow representing areas of merged red and green signal. Note the relative low expression of INF-β and TNF-α in the area of CD163 predominant macrophages. (D) Bar plots depicting semi-quantitative analysis of

inflammatory markers in lesional macrophages. Each bar represents an analysis of mean fluorescence intensity in Arbitrary Fluorescence Units (AFU) based on 90 individual cells from six independent cases. The expression of inflammatory markers were significantly lower in CD163 positive compared with foam cell macrophages ($*p < 0.05$ vs. CD163). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Hemoglobin:Haptoglobin Differentiation Alters Human and Mouse Macrophage Production of IFN-β/TNF-α in Response to Toll-like Receptor 4 (TLR4) Activation (A–B) TNF-α and INF-β expression were measured by ELISA in cell culture supernatants of monocytes differentiated in either Hemoglobin:Haptoglobin (HH) enriched media (HH, 1 mg/ml for 1 week) or media alone (control) with and without exposure to lipopolysaccharide (LPS, 25 ng/ml for 4 h prior to analysis). For hepcidin experiments, differentiated human macrophages were exposed to human hepcidin (Hep, 700 nmol/L for 24 h) on day 6 prior to

LPS treatment on day 7 (* $p < 0.05$ compared with cont, ** $p < 0.05$ compared with LPS and † $p < 0.05$ compared with HH + Hep + LPS). (C) TNF- α and IFN- β expression were measured by ELISA in cell culture supernatants in 7-day HH differentiated human macrophages with and without exposure to oxidized LDL (oxLDL, 30 μ g/ml for 4 h prior to analysis) with pre-treatment with either a control IgG antibody (Cont. Ab) or a TLR4 blocking antibody (TLR4 Ab) prior to oxLDL exposure (bars under line $p < 0.05$ v. oxLDL and oxLDL + cont Ab, $n = 4$ wells). (D–E) TNF- α and IFN- β mRNA expression was measured by real time quantitative polymerase chain reaction in HH or control media differentiated human macrophages transfected with non-specific or FPN siRNA with and without exposure to lipopolysaccharide (LPS, 25 ng/ml for 4 h prior to analysis). Inset shows western blot for human macrophages transfected with siRNA for FPN or non-targeting siRNA (control) (* $p < 0.05$ compared with cont, ** $p < 0.05$ compared with LPS and † $p < 0.05$ compared with HH + siFPN + LPS with $n = 3$ –5 groups for each experiment). (F) TNF- α expression was measured by ELISA in cell culture supernatants of peritoneal macrophages from FPN knockout mice (flox/flox LysM-Cre) and control mice (flox). Macrophages were differentiated in mouse hemoglobin enriched media (hemoglobin 0.1 mg/mL, for 24–48 h) or media alone followed by exposure to LPS (100 ng/mL) for 4 h prior to analysis; $n = 6$ wells, $p < 0.05$).

Fig. 3. Hemoglobin:Haptoglobin Differentiated Macrophages Demonstrate Decreased TLR4 Interaction with TRIF/MyD88 and Trafficking to Lipid Rafts (A) Human monocytes were allowed to differentiate into macrophages in hemoglobin:haptoglobin (HH, 1 mg/ml, equimolar, for 1 week) or media alone (cont.) with and without exposure to lipopolysaccharide (LPS, 25 ng/ml for 1 h prior to analysis). Dual immunofluorescence was conducted using an antibody against TLR4 (red) and FITC-labeled cholera toxin B (GM1, green) to label GM1 ganglioside component of lipid rafts. For hepcidin group, macrophages

were treated with human hepcidin (Hep, 700 nmol/L for 24 h) on day 6 and for LPS group exposed to LPS on day 7 (n = 4 experiments/mice per group with representative examples shown). Bar plots depicting analysis of relative florescent intensity of GM1 and TLR4 staining in the cell membrane compared to the cytosol (M:C) based on > 15 individual cells from four high powered fields. The relative expression of GM1 and TLR4 in the membrane compared with the cytosol were significantly different in all individual groups except LPS and HH + LPS + Hep (* $p < 0.01$). (B) The interaction of TLR4 with its adaptor molecules, TRIF and MyD88, for the experimental conditions described for (A) with the addition of an HH differentiated macrophages treated with Hep (day 6) or Hep (day 6) + LPS (day 7) was examined using immunoprecipitation (IP) for TLR4 and immunoblotting for TRIF and MyD88. The blots show an increased interaction between TLR4 and TRIF/MyD88 in the LPS and HH + Hep + LPS groups but not control, HH, or HH + LPS groups. Overall expression of TLR4, TRIF and MyD88 was analyzed by immunoblotting (IB) and were shown to be similar from total cell lysates (representative examples shown, n = 3). (C) Cell lysates of HH macrophages with and without LPS treatment as described in (A) were fractionated by sucrose-gradient ultracentrifugation, followed by fractionation to 12 subfractions for immunoblotting with TLR4 and flotillin-1. (Flotillin-1 is enriched in lipid rafts and usually is seen in fractions 3–5 as shown in lower blot in all groups studied). TLR4 was identified using a TLR4 antibody. The upper blot demonstrates that TLR4 can be found in lipid raft fractions after LPS stimulation in control macrophages (in fractions 3–4) but not after LPS stimulation of HH macrophages (n = 3–4 for each group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Pharmacologic Inhibition of Hepcidin in Mice Suppresses TLR4 Responses to LPS in Peritoneal Macrophages. (A) Mouse peritoneal macrophages treated with vehicle (control) or LDN, a bone morphogenetic inhibitor and hepcidin suppressor (10 mg/kg IP twice daily for 4 days), or LDN + hepcidin (Hep, 25 ug IP daily on days 3 and 4) were subsequently exposed to LPS (100 ng/ml for 4 h). Dual immunofluorescence was conducted using an antibody against TLR4 (red) and FITC-labeled cholera toxin B (GM1, green) to label GM1 ganglioside component of lipid rafts. Representative examples shown with $n = 4$ mice/group. Bar plots depicting analysis of relative fluorescent intensity of GM1 and TLR4 staining in the cell membrane compared to the cytosol (M:C) based on > 15 individual cells from four high powered fields. The relative expression of GM1 and TLR4 in the membrane compared with the cytosol were significantly different in all groups except LPS and LDN + LPS + Hep ($*p < 0.01$). (B) TNF- α and INF- β expression were measured by ELISA in cell culture supernatants of mouse peritoneal macrophages ($n = 4$ mice/group for each experiment). TNF- α and INF- β expression in the LPS and LDN + LPS + Hep groups was significantly higher compared with all other groups ($*p < 0.01$). INF- β expression in LPS and LDN + LPS + Hep groups was also significantly different ($*p < 0.01$). (For

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