Abnormal High-Density Lipoprotein Induces Endothelial Dysfunction via Activation of Toll-like Receptor-2

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SUMMARY

Endothelial injury and dysfunction (ED) represent a link between cardiovascular risk factors promoting hypertension and atherosclerosis, the leading cause of death in Western populations. High-density lipoprotein (HDL) is considered antiatherogenic and known to prevent ED. Using HDL from children and adults with chronic kidney dysfunction (HDLCKD), a population with high cardiovascular risk, we have demonstrated that HDLCKD in contrast to HDLHealthy promoted endothelial superoxide production, substantially reduced nitric oxide (NO) bioavailability, and subsequently increased arterial blood pressure (ABP). We have identified symmetric dimethylarginine (SDMA) in HDLCKD that causes transformation from physiological HDL into an abnormal lipoprotein inducing ED. Furthermore, we report that HDLCKD reduced endothelial NO availability via toll-like receptor-2 (TLR-2), leading to impaired endothelial repair, increased proinflammatory activation, and ABP. These data demonstrate how SDMA can modify the HDL particle to mimic a damage-associated molecular pattern that activates TLR-2 via a TLR-1- or TLR-6-coreceptor-independent pathway, linking abnormal HDL to innate immunity, ED, and hypertension.

INTRODUCTION

Atherosclerotic cardiovascular disease and hypertension are highly prevalent in Western populations and are the leading cause of death (Lloyd-Jones et al., 2010). Endothelial injury and dysfunction (ED) represent a common link of all cardiovascular risk factors acting upon the vascular system to promote development and progression of atherosclerosis and hypertension (Berenson et al., 1998; Landmesser et al., 2004; Libby et al., 2011; Ross, 1999). An important characteristic of ED is the dysbalance between reduced atheroprotective endothelial nitric oxide (NO) availability and an increased production of reactive oxygen species (ROS) (Landmesser et al., 2004), promoting vasoconstriction, inflammatory activation, and impaired endothelial repair (Kubes et al., 1991; Pober and Sessa, 2007).

Interestingly, lipids and lipoproteins critical in atherogenesis are also involved in immunological processes of the innate and adaptive immune response (Hansson and Hermansson, 2011). Arterial endothelial cells express signaling pattern-recognition receptors of the innate immune system, such as toll-like receptor-2 (TLR-2) and TLR-4 (Hansson and Hermansson, 2011). Innate immunity is mainly based on recognition of pathogens by pattern recognition receptors. Among these receptors, TLRs have been studied extensively. TLRs recognize a broad variety of exogenous pathogen-associated molecular pattern (PAMP) and endogenous danger-associated molecular pattern (DAMP) (Kawai and Akira, 2011). TLRs have been suggested to play a crucial role in the pathogenesis of atherosclerotic diseases (Mullick et al., 2005) (Edfeldt et al., 2002; Favre et al., 2007; Mullick et al., 2008). Recent evidence suggests that known atherogenic lipids, i.e., oxidized phospholipids and oxidized low-density lipoprotein (LDL), can trigger a TLR-2 dependent response and apoptosis in macrophages (Seimon et al., 2010). However, the mode of activation of endothelial TLRs in atherosclerosis and their relation to endothelial dysfunction remains to be characterized.

Based on a rather rigid picture of atherosclerosis, for a long time LDL was thought to promote the development of ED and
high-density lipoprotein (HDL) was thought to prevent the development of ED and atherosclerosis. However, recent evidence suggests that endothelial effects of HDL can be highly heterogeneous under several disease conditions (Charakida et al., 2009; Sorrentino et al., 2010). Furthermore, in contrast to observational trials revealing a protective effect of high HDL plasma concentrations, a recent study documented that genetic mechanisms raising HDL cholesterol plasma concentrations are not associated with a lower risk for myocardial infarction (Voight et al., 2012).

Focusing on HDL, we sought to determine whether modification of HDL may alter its endothelial effects by modulation of the innate immune system. Therefore, we isolated HDL from patients with chronic kidney dysfunction (CKD) as a population with particularly high risk for cardiovascular events and mortality and a high prevalence of ED and hypertension (Go et al., 2004; Klag et al., 1996). In marked contrast to previous reports, we observed that HDL\(^{\text{CKD}}\) not only lost its endothelial-protective properties, but rather became a noxious particle that induced endothelial dysfunction and increased arterial blood pressure. We observed that symmetric-dimethylarginine (SDMA) in the HDL fraction was responsible for adverse vascular actions of this “toxic” HDL. Moreover, we could demonstrate that such a modified HDL induced an endothelial TLR-2 response via a TLR-1- and TLR-6-coreceptor and NF-κB-independent pathway resulting in an enhanced ROS and suppressed endothelial NO bioavailability.

**RESULTS**

**Participants**

To examine the endothelial effects of HDL in a cardiovascular “high-risk” population, we isolated HDL from adults (n = 45) and children (n = 22) with different degrees of chronic kidney dysfunction and age- and gender-matched healthy control subjects (n = 15 adults and n = 10 children; see Tables S1 and S2 available online).

**HDL from Patients with Chronic Kidney Disease Induces Endothelial Dysfunction and Increases Arterial Blood Pressure**

We analyzed NO production in human aortic endothelial cells (HAEC) incubated with HDL isolated from adult patients with chronic kidney dysfunction (HDL\(^{\text{CKD}}\)) and from corresponding healthy subjects (HDL\(^{\text{Healthy}}\)) by electron spin resonance (ESR) spectroscopy. HDL\(^{\text{Healthy}}\) stimulated endothelial NO production, but in marked contrast, HDL\(^{\text{CKD}}\) strongly inhibited NO production in HAECs (Figure 1A). Notably, HDL from patients with mildly reduced kidney function already substantially inhibited endothelial NO release (Figure 1A). Both effects were observed to be dose-dependent (Figure 1B).

In a next step, we examined whether chronic kidney disease per se and not concomitant diseases are responsible for these adverse effects of HDL on endothelial NO production. For this purpose, we isolated HDL from children with an impaired kidney function and measured its effects on the endothelial NO production. Importantly, HDL\(^{\text{CKD}}\) from children substantially inhibited endothelial NO production, whereas HDL\(^{\text{Healthy}}\) from children induced endothelial NO production (Figure 1C).

A reduced endothelial NO production has been shown to increase ABP (Huang et al., 1995). To address the effects of HDL on ABP, we injected HDL\(^{\text{Healthy}}\) and HDL\(^{\text{CKD}}\) into C57BL/6J mice and measured ABP before and 90 min after HDL injection. HDL\(^{\text{Healthy}}\) decreased systolic ABP (–12 ± 2 mmHg), whereas HDL\(^{\text{CKD}}\) significantly increased ABP in vivo (+9 ± 2 mmHg, Figure 1D). These effects were completely abolished in Nos3\(^{-/-}\) mice (Figure 1E) pointing to endothelial NO synthase (eNOS) mediating the different effects of HDL on ABP.

It is well established that superoxide radicals can directly react with NO, reducing its bioavailability and inducing hypertension (Landmesser et al., 2002). We therefore analyzed the effect of HDL on endothelial cell superoxide production in HAECs and observed that HDL\(^{\text{CKD}}\) strongly induced endothelial superoxide production. In contrast, treatment with HDL\(^{\text{Healthy}}\) did not change basal endothelial superoxide release (Figures 1F and 1G).

**SDMA but Not ADMA Is Associated to HDL and Inhibits Endothelial NO Production**

We hypothesized that ADMA, an endogenous inhibitor of eNOS that accumulates in the plasma in different clinical disease conditions (e.g., chronic kidney disease [Fliser et al., 2005]), may be involved in this process. We fractionated plasma from healthy subjects and patients with reduced kidney function and measured ADMA in the HDL fraction using a mass spectrometry approach (ESI-MS/MS). However, although ADMA serum concentrations were elevated in patients with chronic kidney diseases (Figures S1A and S2–S4), we could not detect significant amounts of ADMA in the HDL fraction. Instead, we found its structural isomer SDMA in the HDL fraction in a substantial amount, but only in samples obtained from patients with reduced kidney function (Figure 2A).

To address whether SDMA converts physiological HDL into “noxious” HDL, we supplemented HDL and LDL from healthy donors with SDMA or ADMA and measured subsequently the effects on endothelial NO production. Interestingly, only HDL supplemented with SDMA inhibited endothelial NO production, whereas neither supplementation of HDL with ADMA nor supplementation of LDL with ADMA and SDMA caused any significant changes of the lipoproteins on endothelial NO release. The amount of SDMA in the HDL fraction after supplementation was comparable to that observed in HDL\(^{\text{CKD}}\). Notably, SDMA alone, in the absence of HDL, did not significantly change endothelial NO production (Figure 2B). Supplementation of HDL (HDL\(^{\text{SDMA}}\)) with increasing concentrations of SDMA dose-dependently suppressed endothelial cell NO production (Figure 2C). Similar to the effect of HDL from patients with reduced kidney function, HDL\(^{\text{SDMA}}\) induced basal endothelial superoxide production (Figure 2D).

Furthermore, we supplemented reconstituted HDL (rHDL) consisting of Apo-A1:POPC:cholesterol in a molar ratio of 1:100:10 with and without SDMA and measured its effect on endothelial cell NO production. Supplementation of HDL with SDMA reduced endothelial NO production (Figure 2E). Moreover, after supplementation with SDMA, apolipoprotein A1 (Apo-A1) inhibited endothelial NO production, whereas Apo-A1 without SDMA did not significantly affect endothelial NO production (Figure 2E). This indicates that SDMA may associate with Apo-A1, the major apolipoprotein of HDL.
Figure 1. HDL from Patients with Kidney Dysfunction Induces Endothelial Dysfunction and Hypertension

(A) NO production in HAEC determined by ESR spectroscopy after incubation with HDL (50 µg/ml, 1 hr) from adult patients with different degrees of kidney dysfunction and healthy control subjects (n = 15 per group).

(B) NO production in HAEC determined by ESR spectroscopy after incubation with different concentrations of HDL as indicated (1 hr).

(C) NO production in HAEC determined by ESR spectroscopy after incubation with HDL (50 µg/ml, 1 hr) from children with kidney dysfunction and healthy children as control.

(D) Systolic ABP in WT mice 90 min after intravenous (i.v.) injection of HDL (15 mg/kg, n = 5–6 per group).

(E) Systolic ABP in Nos3−/− (eNOS) mice 90 min after i.v. injection of HDL (50 mg/kg).

(F) Superoxide production in HAEC determined by ESR spectroscopy after incubation with HDL (50 µg/ml, 1 hr).

(G) ESR spectra of endothelial superoxide production after stimulation with HDL.

All data are presented as mean ± SEM and are representative for at least three independent experiments. See also Tables S1 and S2.
Figure 2. HDL Supplemented with SDMA Induces Endothelial Dysfunction and Hypertension

(A) Amounts of ADMA and SDMA associated to HDL from healthy subjects and patients with kidney dysfunction determined by ESI-MS/MS.

(B) HDL supplemented with SDMA induces endothelial dysfunction and hypertension.

(C) Graph showing the effect of HDL supplemented with SDMA on endothelial nitric oxide (NO) production.

(D) Graph showing the effect of HDL supplemented with SDMA on endothelial superoxide production.

(E) Graph showing the effect of rHDL, HDL, ApoA1, and ApoA1 supplemented with SDMA on endothelial NO production.

(F) Western blot analysis showing the phosphorylation of eNOS at Ser473 and Thr495 in HDL supplemented with SDMA.

(G) Graph showing the effect of HDL supplemented with SDMA on p-Akt (Ser473) and p-Akt (Thr175).

(H) Graph showing the effect of HDL supplemented with SDMA on p-eNOS (Ser1177) and p-eNOS (Thr495).

(I) Graph showing the effect of HDL supplemented with SDMA on endothelial superoxide production.

(J) Graph showing the effect of HDL supplemented with SDMA on endothelial NOS expression.

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HDL from healthy subjects is known to stimulate endothelial NO production by Akt kinase-dependent eNOS phosphorylation via activation of endothelial scavenger receptor B-1 (SR-BI) or sphingosine-1 phosphate receptors (Acton et al., 1996; Nofer et al., 2004). Here, we found—in contrast to HDL\textsuperscript{Healthy}—that HDL\textsubscript{CKD} and HDL\textsubscript{SDMA} reduced phosphorylation of Akt (Ser473), leading to a reduced eNOS-activating phosphorylation (Ser1177) and an enhanced eNOS-inhibiting phosphorylation (Thr495) (Figure 2F; Figures S1E–S1G). Therefore, we hypothesized that such modified HDL changes its affinity to these endothelial receptors mediating the protective endothelial effects of HDL\textsuperscript{Healthy}.

Interestingly, binding of HDL\textsuperscript{Healthy} and HDL\textsubscript{SDMA} to endothelial cells did not differ significantly (Figure 2G). However, the interaction of HDL\textsubscript{SDMA} at 37°C (i.e., cell association) was significantly lower compared to HDL\textsuperscript{Healthy}, indicating that SDMA changes the association of HDL with endothelial cells (Figure S1B). To further examine the binding affinity of HDL\textsubscript{SDMA} to the HDL receptor SR-BI, we performed additional binding studies using HEK293 cells transfected with hSR-BI or with an empty vector (control). Here, binding of HDL\textsuperscript{Healthy} and HDL\textsubscript{SDMA} to HEK cells transfected with hSR-BI did not differ significantly (Figure S1C).

We repeated the experiment using rHDL supplemented with and without SDMA. Also here, we did not observe a difference in the binding capacity of HDL\textsuperscript{Healthy} and HDL\textsubscript{SDMA} to SR-BI (Figure S1D). Collectively, supplementation of 125I-labeled HDL with SDMA reduced the association, but not the specific binding to SR-BI or endothelial cells.

**HDL Supplemented with SDMA Activates Endothelial Toll-like Receptor 2**

TLR-2 and TLR-4 represent receptors of the innate immune system involved in recognition of pathogen-associated lipoproteins (Takeuchi and Akira, 2010). Both TLR-2 and TLR-4 are highly expressed on HEAEs (Figures SSA and SSB). We measured endothelial NO production in HEAEs stimulated with HDL\textsuperscript{Healthy}, HDL\textsubscript{CKD}, or HDL\textsubscript{SDMA} in the presence or absence of specific neutralizing antibodies against TLR-2 and TLR-4. We observed that blocking of TLR-2 but not TLR-4 restored the effects of HDL\textsubscript{CKD} and HDL\textsubscript{SDMA} on endothelial NO production (Figure 3A). Importantly, the neutralizing antibodies did not affect NO production in the presence of HDL\textsuperscript{Healthy}. Consistently, injection of HDL\textsubscript{CKD} into Tlr2\textsuperscript{−/−} mice, but not in Tlr4\textsuperscript{−/−} mice, failed to increase ABP (Figure 3B).

To examine whether TLR-2 on circulating mononuclear cells contributes to the effect of HDL\textsubscript{CKD} on ABP, we analyzed wild-type (WT) and Tlr2\textsuperscript{−/−} bone-marrow chimeric mice, which were sublethally irradiated and transplanted with bone marrow from WT or Tlr2\textsuperscript{−/−} mice (Figure 3C). We observed an increased ABP in HDL\textsubscript{CKD}-treated mice with intact TLR-2 expression on vascular cells but lack on bone-marrow-derived circulating mononuclear cells. These data suggest that TLR-2 on the endothelium but not on circulating mononuclear cells mediates the adverse effects of HDL\textsubscript{CKD} on ABP. Of note, the response of ABP did not differ between WT and Tlr2\textsuperscript{−/−} mice after injection of the eNOS inhibitor L-NAME (Figure 3D).

To assess the interaction between HDL\textsubscript{SDMA} and TLR-2, we transfected endothelial cells with a plasmid encoding for human TLR-2 without its intracellular Toll/Interleukin-1 receptor (TIR)-domain (TLR2-A5TIR-HA) and incubated them with fluorescent-labeled HDL. As a control, we used a plasmid encoding human TLR-5 (TLR2-5TIR-HA), which is also expressed on the cell surface (Ramos et al., 2004) (Figures S5C and S5D). Overexpression of TLR-2 increased the interaction of endothelial cells with HDL\textsubscript{SDMA}, whereas overexpression of TLR-5 did not change the interaction with HDL and endothelial cells (Figure 3E; Figure S5E).

**Modified HDL Does Not Activate NF-κB-Dependent TLR-2 Signaling**

Next, we asked whether activation of TLR-2 in the absence of HDL\textsubscript{CKD} or HDL\textsubscript{SDMA} is sufficient to inhibit endothelial NO production. Pam3CSK4 and FSL-1 are synthetic lipopeptides, known to bind to TLR-2 (Fujita et al., 2003; Jin et al., 2007). Treatment of HAECs with Pam3CSK4 or FSL-1 inhibited endothelial NO production. This effect was completely abolished in the presence of anti-TLR-2 neutralizing antibodies (Figure 4A). In contrast, TLR-4 agonist LPS increased endothelial NO production in a TLR-4 dependent manner (Figure 4A). In vivo, a single-dose of Pam3CSK4 was sufficient to significantly increase ABP in WT, but not in Tlr2\textsuperscript{−/−} mice (Figure 4B).

Recognition of bacterial lipoproteins usually requires formation of heterodimer complexes of TLR-2 with TLR-1 or TLR-6, respectively (Alexopoulos et al., 2002; Fujita et al., 2003; Sandor et al., 2003). Pam3CSK4 is recognized by the TLR-1-TLR-2 and FSL-1 by the TLR-2-TLR-6 heterodimer resulting in NF-κB pathway activation and proinflammatory cytokine release (Beutler, 2004). We used human embryonic kidney (HEK)-Blue TLR-2 reporter cells to address whether modified HDL activates the NF-κB pathway. Surprisingly, in contrast to Pam3CSK4 and FSL-1, both HDL\textsubscript{CKD} and HDL\textsubscript{SDMA} failed to activate NF-κB pathway in the reporter cells under basal conditions (Figure 4C). Pam3CSK4 induced release of cytokines in bone-marrow-derived macrophages from WT, but not from Tlr2\textsuperscript{−/−} mice, and in human peripheral blood mononuclear cells (PBMC; Table S3). In contrast, production of these cytokines was not stimulated

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*(B) Effect of SDMA alone (4 μM), healthy HDL (50 μg/ml) or LDL (100 μg/ml) supplemented with or without SDMA or ADMA (4 μM equivalent to 0.5 μmol/g lipoprotein) on endothelial NO production.

*(C) Effect of HDL\textsuperscript{Healthy} supplemented with different concentrations of SDMA (0.5 μmol/g HDL protein) on endothelial NO production.

*(D) Effect of HDL (50 μg/ml, 1 hr) supplemented with SDMA (0.5 μmol/g HDL protein) on endothelial superoxide production.

*(E) Effect of HDL (50 μg/ml) or Apo-A1 (25 μg/ml) supplemented with SDMA (0.5 μmol/g protein) on endothelial NO production.

*(F) Phosphorylation of Akt at Ser473, eNOS activating phosphorylation at Ser1177 and eNOS inhibiting phosphorylation at Thr495 determined by immunoblot analysis in HAEC incubated with HDL (50 μg/ml) for 10 min as indicated.

*(G) Quantification of Akt phosphorylation at Ser473 in HAEC incubated with HDL (50 μg/ml) for 10 min.

*(H) Quantification of eNOS phosphorylation at Ser1177 in HAEC incubated with HDL (50 μg/ml) for 10 min.

*(I) Quantification of eNOS phosphorylation at Thr495 in HAEC incubated with HDL (50 μg/ml) for 10 min.

*(J) Effect of SDMA-supplementation on endothelial binding of [125]HDL.

All data are presented as mean ± SEM and are representative for at least three independent experiments. See also Figure S1.*
Figure 3. Modified HDL Activates TLR-2 to Induce Endothelial Dysfunction and Hypertension

(A) Effect of TLR-2 and TLR-4 inhibition using a blocking antibody (10 μg/ml, each) or an isotype control antibody (10 μg/ml) on endothelial NO production after incubation with HDL Healthy, HDL CKD, and HDL SDMA (0.5 μmol/g SDMA, 50 μg/ml HDL).

(B) Δ ABP in Tlr2−/− and Tlr4−/− mice 90 min after i.v. injection of HDL Healthy and HDL CKD (15 mg/kg HDL).

(D) Δ ABP 90 min after i.v. injection of HDL Healthy and HDL CKD (15 mg/kg HDL) in WT, Tlr2−/− mice 6 weeks after bone-marrow transplantation from WT and Tlr2−/− mice as indicated.

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by HDL^{CKD} and HDL^{SDMA}. Notably, neither Pam3CSK4 nor HDL did affect cytokine release in HAEC as determined by ELISA (Table S3) or intracellular cytokine staining (Figures S6C and S6E). Furthermore, we used neutralizing antibodies against TLR-1, TLR-2, and TLR-6 to block NF-κB activation in the HEK-Blue TLR-2 reporter cells stimulated with Pam3CSK4 and FSL-1. As expected, blocking of either TLR-1 or TLR-6 and TLR-2 almost completely abolished Pam3CSK4 or FSL-1 induced NF-κB activation (Figure 4D).

**Activation of Endothelial TLR-2 Inhibits eNOS Activating Pathways and Stimulates NADPH Oxidase**

Next, we assessed the molecular mechanisms on how TLR-2 activation affects endothelial superoxide and nitric oxide production. At first, we analyzed the effect of the TLR-2 agonist Pam3CSK4 on endothelial Akt and eNOS phosphorylation. Comparable to HDL^{CKD} and HDL^{SDMA}, Pam3CSK4 reduced Akt phosphorylation at Ser473 and thereby induced eNOS-inhibiting phosphorylation at Thr495 (Figure 6A). In addition, it has been shown that TLR-2 activation induces NADPH oxidase to produce ROS in monocytes and macrophages (Beaulieu et al., 2011); however, its role in endothelial cells remained unclear. By measuring endothelial superoxide production after preincubation with a specific chemical inhibitor of NADPH oxidase (VAS-2870), we demonstrated a pivotal role for NADPH oxidase in TLR-2 induced endothelial superoxide production. By using L-NAME, an eNOS inhibitor, we ruled out eNOS uncoupling as an immediate source of TLR-2-mediated superoxide production (Figure 6B). Notably, inhibition of NADPH oxidase almost completely prevented the effect of HDL^{CKD}, HDL^{SDMA}, and Pam3CSK4 on endothelial nitric oxide production (Figure 6C). TLR-2 activation is known to induce phosphorylation of c-Jun N-terminal kinase (JNK), and we have recently shown that activation of JNK in endothelial cells induces endothelial superoxide production by increasing NADPH oxidase activity (Cabanski et al., 2008; Osto et al., 2008; Shi et al., 2011). Here, we observed that Pam3CSK4 induces JNK phosphorylation in HAEC (Figure 6D; Figure S7A). Accordingly, SP600125, a JNK inhibitor, abolished the effect of HDL^{CKD}, HDL^{SDMA}, and Pam3CSK4 on endothelial superoxide and nitric oxide production (Figures 6E and 6F).

**HDL^{CKD} and HDL^{SDMA} Impair Endothelial Repair and Promote Inflammatory Activation**

We and others have previously demonstrated that reduced NO bioavailability along with enhanced superoxide production may also impair other endothelial functions, such as endothelial repair after injury and its anti-inflammatory capacity (Kubes et al., 1991; Sorrentino et al., 2007). To elucidate whether modified HDL also affects these endothelial properties by inhibiting endothelial NO production, we finally assessed the effect of modified HDL on endothelial repair and inflammation.

In an endothelial wound healing assay, HDL^{Healthy} strongly stimulated endothelial cell migration, whereas HDL^{CKD} inhibited endothelial migration (Figures S8A and S8B). In line with these in vitro observations, HDL^{CKD} and HDL^{SDMA} inhibited endothelial repair in vivo in a carotid artery injury model (Figures 7A and 7B). This was in marked contrast to HDL^{Healthy}, which promoted endothelial repair. In Tlr2^{−/−} mice, we observed that HDL^{CKD} regained its capacity to promote endothelial repair (Figure 7C). These findings underscore the important role of TLR-2 to mediate adverse endothelial effects of HDL^{CKD} and HDL^{SDMA}.

Furthermore, we observed that HDL^{CKD} increased endothelial proinflammatory activation as determined by a higher amount of adhering mononuclear cells to a TNF-α-stimulated endothelial monolayer, which was in contrast to HDL^{Healthy} (Figures 7D and 7E). Accordingly, HDL^{CKD} and HDL^{SDMA} increased endothelial expression of vascular cell adhesion molecule-1 (VCAM-1), whereas HDL^{Healthy} significantly reduced VCAM-1 expression on endothelial cells (Figure 7F). Next, we analyzed the effect of NO on TNF-α induced VCAM-1 expression in the presence of HDL. Inhibition of eNOS through L-NAME reduced the anti-inflammatory effects of HDL^{Healthy} by enhancing endothelial VCAM-1 expression, whereas supplementation of NO using a solid NO-donor, diethylenetriamine-nitric oxide (DetaNO), restored the anti-inflammatory function of the endothelium by reducing endothelial VCAM-1 expression (Figure 7F). Taken together, these results clearly suggest that a reduced endothelial NO bioavailability induced by HDL^{CKD} and HDL^{SDMA} represents a major mechanism explaining adverse endothelial effects of modified HDL.

**DISCUSSION**

Our results demonstrate that HDL from children and adult patients with CKD substantially inhibits endothelial NO production and thereby increases ABP. We have identified (D) Δ ABP in WT and Tlr2^{−/−} mice 30 min after i.v. injection of L-NAME (17 mg/kg).
(E) Interaction of Atto-488-labeled HDL (20 μg/ml, 30 min) with endothelial cells transfected with a plasmid containing hTLR2-ΔTIR-HA or hTLR5-ΔTIR-HA. Mean fluorescence was recorded using flow cytometry. All data are presented as mean ± SEM and are representative for at least three independent experiments. See also Figure S2.
accumulation of SDMA in HDL from subjects with even mild CKD as the culprit converting HDL into a noxious particle. Furthermore, we have shown that this abnormal HDL activates endothelial TLR-2 via a TLR-1- and TLR-6-coreceptor-independent alternative pathway impairing endothelial repair and enhancing endothelial proinflammatory activation.

We have described the effects of HDL from patients with CKD as a population with a particularly high cardiovascular morbidity and mortality (Go et al., 2004; Van Biesen et al., 2007). In healthy individuals, endothelial NO production stimulated by HDL serves as an important stimulus to preserve endothelial function (Bisoeudial et al., 2003; Landmesser et al., 2004; Nofer et al., 1020).
Immunity

HDL Promotes Endothelial Dysfunction via TLR-2

A

Δ Endothelial NO production (% of buffer-treated cells)

<table>
<thead>
<tr>
<th></th>
<th>Untreated cells</th>
<th>Pam3CSK4</th>
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<th>HDL CKD V°</th>
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<td>Anti-TLR1</td>
<td>+</td>
<td>+</td>
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<td>Anti-TLR6</td>
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B

Δ Systolic Blood Pressure (mmHg)

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<tr>
<td>HDL Healthy</td>
<td>P&lt;0.05</td>
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<tr>
<td>HDL CKD V°</td>
<td>P&lt;0.05</td>
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C

Endothelial superoxide production (nmol/mg/250,000 cells)

<table>
<thead>
<tr>
<th></th>
<th>Untreated cells</th>
<th>Pam3CSK4</th>
<th>FSL-1</th>
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<td>Anti-TLR6</td>
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<td>Isotype control</td>
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2004; Spiker et al., 2002; Yuhanna et al., 2001). By contrast, in the present study, we found that HDL from patients with CKD almost completely suppressed endothelial NO production in a dose-dependent manner. Importantly, this adverse effect of HDL\textsuperscript{CKD} was already present with HDL from patients with an early stage of CKD and children with CKD. Moreover, infusion of HDL\textsuperscript{CKD} into mice significantly increased ABP. This observation is of overriding importance, because hypertension is a strong promoter of cardiovascular disease and frequently observed in patients with CKD (Peterson et al., 1995). In contrast to previous reports describing “dysfunctional HDL,” we have demonstrated here that HDL\textsuperscript{CKD} not only loses its vasoprotective properties but rather changes into a noxious particle strongly promoting endothelial dysfunction and hypertension.

ADMA, a methylation product of L-arginine residues, is an endogenous eNOS inhibitor increased in patients with CKD (Böger et al., 1998; Fiser et al., 2005; Kielsch et al., 2002; Zoccali et al., 2001). By using a modified mass-spectrometry approach, we ruled out the presence of ADMA in the HDL fraction. Surprisingly, we detected substantial amounts of SDMA, an isomer of ADMA, in HDL\textsuperscript{CKD}, but not in HDL\textsuperscript{Healthy}. We and others have previously demonstrated that SDMA serum concentrations are elevated in several clinical conditions such as CKD and pulmonary arterial hypertension (Kielsch et al., 2002; Pullamsetti et al., 2005; Schepers et al., 2011). Although SDMA is thought to be functionally inactive, several studies highlighted its predictive value for cardiovascular events (Bode-Böger et al., 2006; Meinitzer et al., 2011). Interestingly, supplementation of HDL with SDMA in concentrations as measured in patients with CKD resulted in deleterious effects of HDL\textsuperscript{SDMA} on endothelial NO production and endothelial repair by inhibiting Akt-dependent eNOS phosphorylation. Because supplementation of LDL with SDMA did not change its effect on endothelial NO production, we hypothesized that SDMA may be associated to the protein part of the HDL complex. Indeed, we observed that HDL and Apo-A1 supplemented with SDMA reduced endothelial NO production, which indicates that SDMA may associate to Apo-A1. These findings indicate that the presence of SDMA in the HDL particle may represent a mechanism leading to deterioration of HDL’s vascular effects and its transformation into a proatherogenic particle.

HDL\textsuperscript{Healthy} exerts its endothelial-atheroprotective effects by binding to the endothelial scavenger receptor SR-BI or S1P3 receptors as well as by activating ATP binding cassette transporter-1 (ABCG-1) (Acton et al., 1996; Assanasen et al., 2005; Nofer et al., 2004; Terasaka et al., 2008; Yuhanna et al., 2001). Our data indicate that supplementation with SDMA does not change the affinity of HDL to the receptor SR-BI. Therefore, we focused on receptors, which are associated with abnormal endothelial activation. TLR-2 and TLR-4 are both expressed on endothelial cells (Edfeldt et al., 2002; Grote et al., 2010) and known to be activated by microbial lipoprotein patterns (Takeuchi and Akira, 2010). Indeed, activation of TLR1-TLR2, TLR2-TLR6, or TLR4 by microbial lipoproteins initiates defense mechanisms of the innate immunity against infectious pathogens (Brightbill et al., 1999; Roux et al., 2011). Interestingly, it has been shown that TLR-2 may also play an important role in development of atherosclerosis (Mullick et al., 2005, 2008). Moreover, TLR-2 deficiency has been demonstrated to ameliorate ED after coronary endothelial injury (Favre et al., 2007). However, the underlying molecular mechanisms remained widely unknown.

Notably, in the present study, the selective inhibition or genetic deficiency of TLR-2, but not TLR-4, almost completely abrogated the adverse effects of modified HDL on endothelial NO bioavailability, endothelial repair, and ABP. Recent evidence suggests that circulating cells of the lymphoid and myeloid compartment are crucially involved in the pathogenesis of hypertension (Harrison et al., 2011). Blood pressure measurements using WT and Tlr2\textsuperscript{−/−} bone marrow chimeras suggested that TLR-2 expression on endothelial cells, rather than circulating mononuclear cells, mediated the adverse effect of HDL\textsuperscript{CKD} on ABP. This indicates that the presence of SDMA transforms HDL toward a phenotype that activates this innate immune receptor on the endothelium.

It is well known that activation of TLR-1-TLR-2 and TLR-2-TLR-6 by their classical bacterial ligands induces the NF-\kappaB-dependent production of cytokines and other proinflammatory molecules in a variety of cell types (Alexopoulos et al., 2002; Beutler, 2004; Fujita et al., 2003; Sandor et al., 2003). Surprisingly, we observed that abnormal HDL did not induce TLR-1-TLR-2 or TLR-2-TLR-6-mediated NF-\kappaB activation and subsequent cytokine release in endothelial cells or PBMC. Notably, blockade of TLR-1 and/or TLR-6 did not affect endothelial NO production and superoxide production in response to classical TLR-2 ligands or HDL\textsuperscript{CKD} and HDL\textsuperscript{SDMA}. Thus, we were able to document that the effect of TLR-2 activation on endothelial NO production is NF-\kappaB independent.

It has recently been shown that TLR-2 activation can induce ROS production in monocytes and macrophages (Beaulieu et al., 2011; West et al., 2010, 2011). In the present study, we could demonstrate that endothelial activation of TLR-2 induced endothelial ROS production. Of note, TLR-2-induced ROS production was abolished after TLR-2 and not TLR-1 or TLR-6 blockade. This indicates that TLR-2 stimulates endothelial ROS production thereby inhibiting NO bioavailability via a TLR-1- and TLR-6-coreceptor-independent alternative pathway. Furthermore, we demonstrated that the TLR-2 induced NF-\kappaB-dependent cytokine release and the TLR-2 induced ROS production were two distinct pathways, which can be independently targeted by TLR-2 activating agents.

Taken together, the present studies provide several important insights. First, we showed that even mild CKD inverts HDL’s endothelial-protective properties. In fact, HDL\textsuperscript{CKD} induces
**Figure 6. Activation of Endothelial TLR-2 Inhibits eNOS Activating Pathways and Stimulates NADPH Oxidase via Phosphorylation of JNK**

(A) Phosphorylation of Akt at Ser473, eNOS activating phosphorylation at Ser1177 and eNOS inhibiting phosphorylation at Thr495 determined by immunoblot analysis in HAEC incubated with Pam3CSK4 (1 μg/ml) for 10 min.

(B) Endothelial superoxide production after preincubation with L-NAME (0.3 mM, 1 hr), a specific eNOS inhibitor, or VAS-2870 (10 μM, 1 hr), a specific NAPDH-oxidase inhibitor and subsequent stimulation with Pam3CSK4 (1 μg/ml; 1 hr).

(C) Endothelial nitric oxide production after stimulation with Pam3CSK4 (1 μg/ml) or HDL (50 μg/ml) with or without preincubation with VAS-2870 (10 μM, 1 hr).

(D) Time-dependent effect of TLR-2 activation with Pam3CSK4 (1 μg/ml) on endothelial SAPK-JNK phosphorylation (***p < 0.001 compared to basal).

(E) Endothelial superoxide production after incubation with Pam3CSK4 (1 μg/ml) or HDL (50 μg/ml) for 1 hr with or without preincubation with SP600125, a SAPK-JNK inhibitor (1 μM).

(F) Endothelial nitric oxide production after stimulation with Pam3CSK4 (1 μg/ml) or HDL (50 μg/ml) with or without preincubation with SP600125 (1 μM, 1 hr).

All data are presented as mean ± SEM and are representative for at least three independent experiments. See also Figure S5.
Figure 7. Modified HDL Induces Endothelial Inflammation and Suppresses Endothelial Repair
(A) Reendothelialized area at day 3 after carotid injury and injection of HDL Healthy, HDL CKD, or HDL SDMA (15 mg/kg) in nude mice. PBS treated animals serve as control.
(B) Representative photographs of carotid arteries after Evans Blue staining.
(C) Reendothelialized area at day 3 after carotid injury in Tlr2−/− mice with injection of HDL Healthy, HDL CKD, or HDL SDMA (15 mg/kg). PBS treated animals serve as control.
(D) Adhesion of mononuclear cells (MNC) to TNF-α (5 ng/ml) treated endothelial cells after incubation with HDL Healthy or HDL CKD (50 μg/ml). MNC are stained with DiI and HAEC with DAPI.

(legend continued on next page)
endothelial dysfunction, impairs endothelial repair, and increases ABP. Second, we identified SDMA in HDL\textsubscript{CKD} as the culprit that modifies HDL to induce these deleterious endothelial effects. Moreover, we demonstrated that such modified HDL activates endothelial TLR-2 signaling resulting in enhanced ROS production and inhibition of endothelial NO bioavailability. Third, our data provide evidence that these effects were mediated by activation of TLR-2 via a TLR-1- or TLR-6-coreceptor-independent alternative pathway. These data illustrate as to how a small compound like SDMA can modify the HDL particle mimicking a damage-associated molecular pattern to activate TLR-2, thereby linking innate immunity, endothelial dysfunction, and arterial hypertension.

Pharmacological therapies to raise HDL cholesterol serum concentrations are currently in the focus of cardiovascular research. Although epidemiological studies indicated that higher HDL cholesterol serum concentrations are associated with a reduced risk for cardiovascular events (Miller et al., 1977), recent interventional trials using different approaches to raise HDL cholesterol serum concentrations failed to demonstrate a beneficial effect on CV mortality (Barter et al., 2007; Boden et al., 2011; Schwartz et al., 2012). The present study could provide an explanation for these findings to gain a more complex picture of lipoprotein biology. Our results indicate that the vascular effects of HDL\textsubscript{healthy} and HDL\textsubscript{CKD} are completely different. Therefore, it might be necessary to distinguish between quantitative measurements of HDL serum concentrations and qualitative examinations of HDL’s functionality. The present study suggests that simply raising HDL in serum does imply the restoration of HDL’s atheroprotective properties. Moreover, HDL serum concentrations per se do not provide adjuvant information on the biological function of HDL. Future research should reveal strategies to evade detrimental remodeling of HDL under disease conditions such as CKD.

**Experimental Procedures**

**Animals**

Wt (C57BL/6j), Tlr2\textsuperscript{−/−} (B6.129-Tlr2tm1Kir/J), Tlr4\textsuperscript{−/−} (B6.120ScN-Tlr4ips-del/JthJ), and eNOS\textsuperscript{−/−} (B6.129P2-Nos3tm1Unc/J) mice were obtained from The Jackson Laboratory, Tlr1\textsuperscript{−/−} and Tlr6\textsuperscript{−/−} mice from OrientalBioService, and CD1 (nu/nu) from Charles River. Mice were housed and maintained in the Saarland University Hospital Animal Facility. All animal studies were approved by the animal ethics committee of the Saarland University and the University of Saarland. Mice were obtained from The TLR-2 deficient mice. Nat. Med. 11(7), 754–768, April 18, 2013

**ESR Spectroscopy Analysis of NO Production in HAEC and BAEC**

The NO production was measured by ESR spectroscopy analysis with the use of the spin-trap colloid Fe\textsubscript{2}DETcO\textsubscript{3} as described previously (Kleschyov et al., 2000; Sorrentino et al., 2010). Cells were stimulated with the isolated lipoproteins or Pamp3CSK4, respectively, for 1 hr.

**ESR Spectroscopy Analysis of Superoxide Production in HAEC**

Endothelial superoxide was measured using ESR spectroscopy and the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroolidine (CMH) as described previously (Landmesser et al., 2002; Sorrentino et al., 2010). Cells were stimulated with the isolated lipoproteins or Pamp3CSK4, respectively, for 1 hr.

**Blood Pressure Measurements in Mice**

Systolic blood pressures were measured by a computerized tail-cuff system (Visitech Systems) as described previously (Landmesser et al., 2002). On each day of blood pressure determination, 20 measurements were obtained and averaged for each mouse.

**Bone-Marrow Transplantation**

Bone-marrow transplantation experiments were performed as described previously (Kania et al., 2009). In brief, 6- to 8-week-old WT or Tlr2\textsuperscript{−/−} mice were lethally irradiated with 2 × 6.5 Gy by using Gammatron (Co-60) system and reconstituted with 2 × 10\textsuperscript{6} donor bone-marrow cells from WT or Tlr2\textsuperscript{−/−} mice, respectively. After reconstitution, all mice received prophylactic antibiotics in the drinking water and were housed in a specific pathogen-free environment for 6 weeks.

**Determination of Methylarginines by Using HPLC-ESI-MS/MS**

Quantification of methylarginines (ADMA, SDMA) in serum samples as well as in HDL fractions was performed as described previously with some modifications (Di Gangi et al., 2010).

**Statistics**

All data are expressed as mean ± SEM. Statistical comparisons were made by one-way ANOVA or the nonparametric Kruskal-Wallis test. P value < 0.05 was considered statistically significant. Post hoc Tukey test or Bonferroni adjustment was performed for multiple comparisons. All analyses were performed with GraphPad Prism (Version 4.0, GraphPad Software).

**Supplemental Information**

Supplemental Information includes six figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.02.009.

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(E) Representative micrographs of high-power fields of six independent experiments obtained by fluorescence microscopy.

(F) Effect of HDL\textsubscript{healthy}, HDL\textsubscript{CKD}, or HDL\textsubscript{SDMA} (50 μg/ml) with or without coincubation with L-NAME (0.3 mM) and HDL\textsubscript{CKD} or HDL\textsubscript{SDMA} with or without coincubation with DetA(NO (1 mM) on TNF-α-induced (5 ng/ml) endothelial VCAM-1 expression.

All data are presented as mean ± SEM and are representative for at least three independent experiments. See also Figure S6.
HDL Promotes Endothelial Dysfunction via TLR-2


HDL Promotes Endothelial Dysfunction via TLR-2


