CD36 Regulates Oxidative Stress and Inflammation in Hypercholesterolemic CKD

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ABSTRACT
Scavenger receptors play a central role in atherosclerosis by processing oxidized lipoproteins and mediating their cellular effects. Recent studies suggested that the atherogenic state correlates with progression of chronic kidney disease (CKD); therefore, scavenger receptors are candidate mediators of renal fibrogenesis. Here, we investigated the role of CD36, a class B scavenger receptor, in a hypercholesterolemic model of CKD. We placed CD36-deficient mice and wild-type male mice on a high-fat Western diet for 7 to 8 wk and then performed either sham or unilateral ureteral obstruction surgery. CD36-deficient mice developed significantly less fibrosis compared with wild-type mice at days 3, 7, and 14 after obstruction. Compared with wild-type mice, CD36-deficient mice had significantly more interstitial macrophages at 7 d but not at 14 d. CD36-deficient mice exhibited reduced levels of activated NF-κB and oxidative stress (assessed by measuring fatty acid–derived hydroxyoctadecadienoic acid and protein carbonyl content) and decreased accumulation of interstitial myofibroblasts compared with wild-type mice. These data suggest that CD36 is a key modulator of proinflammatory and oxidative pathways that promote fibrogenesis in CKD.


The prevalence of chronic kidney disease (CKD) is increasing at an alarming rate, with the largest increases seen in the moderate to advanced stages.¹ ² Furthermore, CKD is a major risk factor for cardiovascular morbidity and mortality.³ Patients with CKD not only have a more atherogenic lipid profile but also have evidence of chronic inflammation and oxidative stress.⁴ ⁵ The combination of these factors likely accounts for the accelerated atherosclerotic disease that develops in patients with advanced CKD. Several epidemiologic studies suggested that this highly atherogenic state is correlated with more rapid progression of kidney fibrosis.⁶ ⁷ ⁸ Other studies further suggested that specific lipoproteins are linked to an increased risk for renal dysfunction and progression of CKD.⁹ ¹⁰ Studies of hypercholesterolemic animal models support the view that oxidized lipoproteins are directly involved in the pathogenetic pathways that trigger inflammation and oxidative stress leading to cardiovascular disease. Much less is known about receptor-dependent pathways that perpetuate renal parenchymal inflammation and fibrosis within the dyslipidemic environment of CKD. In particular, there is a paucity of information on the renal pattern of expression and function of specific scavenger receptors that figure prominently in atherogenesis.

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Scavenger receptors comprise a family of nine classes of structurally similar receptors that share oxidized lipoproteins as their primary ligand. CD36 is a class B transmembrane scavenger receptor that is known to be expressed by multiple cell types, such as macrophages, microvascular endothelial cells, platelets, adipocytes, and epithelial cells. CD36 has been proposed to be a major endocytic receptor for oxidized lipoproteins and accounts for 50 to 60% of the uptake of oxidized LDLs (oxLDLs) by macrophages. Binding of oxLDLs to CD36 may also activate intracellular signaling pathways that lead to proinflammatory chemokine and cytokine production. Peripheral blood monocyte CD36 levels are significantly higher in patients on dialysis than in normal individuals. In a recent study, we demonstrated that kidneys of mice with diet-induced hypercholesterolemia developed worse fibrosis after unilateral ureteral obstruction (UUO) than did normcholesterolemic mice. This difference was associated with higher renal oxidized lipoprotein levels, activation of inflammatory pathways, and a striking change in the intensity and cell-specific pattern of CD36 expression. CD36 and other scavenger receptors were predominantly expressed in tubular epithelial cells and interstitial macrophages with specific modulation of their expression pattern during chronic renal injury by obstruction. To gain further insight into the mechanisms that modulate fibrogenic pathways during hypercholesterolemia, we designed this study to investigate the hypothesis that CD36 promotes renal fibrosis by activating proinflammatory and oxidative pathways. We compared the severity of renal fibrosis induced by UUO in CD36 wild-type (CD36+/+) and deficient (CD36−/−) hypercholesterolemic mice.

RESULTS

Attenuated Renal Injury in Hypercholesterolemic CD36−/− Mice after UUO

Metabolic Effects of Diet-Induced Hypercholesterolemia. After 7 to 8 wk on a high-fat Western diet, both CD36+/+ and CD36−/− mice developed significant hypercholesterolemia (Table 1). The mean serum cholesterol levels were similar between the CD36+/+ and CD36−/− mice at all time points, with the exception of day 3, although this did not affect intrarenal oxidized lipoprotein levels at this time point. On the day of UUO surgery, the two groups of male mice (10 to 11 wk)

Table 1. Mean cholesterol levels on high-fat diet at killing

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Surgery</th>
<th>CD36+/+ (mg/dl)</th>
<th>CD36−/− (mg/dl)</th>
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<tr>
<td></td>
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<td>(n = 6 to 8)</td>
<td>(n = 6 to 8)</td>
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<tr>
<td>Day 14</td>
<td>Sham</td>
<td>188 ± 17</td>
<td>180 ± 4</td>
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<tr>
<td>Day 3</td>
<td>UUO</td>
<td>140 ± 6a</td>
<td>215 ± 6</td>
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<tr>
<td>Day 7</td>
<td>UUO</td>
<td>193 ± 29</td>
<td>196 ± 10</td>
</tr>
<tr>
<td>Day 14</td>
<td>UUO</td>
<td>191 ± 15</td>
<td>172 ± 11</td>
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aMean cholesterol levels on standard chow diet from previous experiments were 80 to 102 mg/dl. bP < 0.01, CD36+/+ versus CD36−/−.

were matched for mean body weight. The average body weights were similar between CD36+/+ and CD36−/− mice at all time points, with the exception of day 7 (23.8 ± 0.5 versus 25.7 ± 0.6 g; n = 6 to 10 per group; P = 0.04).

Fibrosis Severity.

To investigate whether CD36 modulated the severity of renal fibrosis induced by UUO in mice with diet-induced hypercholesterolemia, we measured total collagen levels as hydroxyproline content per wet weight of kidney tissue. Fibrosis severity was significantly reduced by 19 to 29% in obstructed kidneys from hypercholesterolemic CD36−/− mice compared with CD36+/+ mice at each time point (n = 6 to 10 per group; P < 0.01; Figure 1).

Tubular Epithelial Cell Damage.

A limitation of the UUO model is the inability to measure functional consequences of the structural damage as a result of contralateral kidney compensation. Loss of expression of E-cadherin has been used as a surrogate marker of tubular cell damage and atrophy. Tubular integrity was significantly preserved with a 2.9-fold higher level of E-cadherin protein in obstructed kidneys at day 14 in the CD36−/− group compared with CD36+/+ group (n = 5 to 6 per group; P = 0.002; Figure 2A). Although E-cadherin can be expressed by macrophages, by immunostaining, the predominant expression in the kidney during UUO is by tubular epithelial cells (Figure 2B).

Biphasic Interstitial Macrophage Response in CD36−/− Mice

Macrophage Infiltration.

We observed a 68% increase in F4/80+ interstitial area at day 7 in CD36−/− mice compared with CD36+/+ mice despite significantly less fibrosis (n = 5 per group; F = 38, P = 0.0003; Figure 3). By day 14 after UUO, the pattern of macrophage infiltration had changed, now showing a NS decrease in the F4/80+ interstitial area in CD36−/− mice compared with...
mRNA levels, a key chemokine in macrophage migration, at any of the time points investigated (Figure 4B). Among the chemokines examined, CCL5 and CXCL10 had the highest steady-state mRNA levels at the time points investigated. There was a 140% increase in steady-state CCL5 mRNA levels at day 7 after UUO in hypercholesterolemic CD36−/− mice compared with CD36+/+ mice (n = 5 to 6 per group; P = 0.02; Figure 4C). Steady-state CXCL10 mRNA levels were significantly increased at days 7 and 14 with the largest increase (153%) at day 7 in CD36−/− mice compared with CD36+/+ mice (n = 5 to 6 per group; P < 0.01; Figure 4D). CXCL2, a marker of activated macrophages, was significantly decreased by 33% in CD36−/− mice compared with CD36+/+ mice at day 14 after UUO (n = 5 to 6 per group; P = 0.008; Figure 4, A and E).

### Attenuation of Proinflammatory Pathways in CD36−/− Mice

To investigate whether the proinflammatory pathways that are activated in obstructed kidneys are modulated by CD36, we examined levels of activated NF-κB at days 3 and 7, representing time points of early and established inflammation, respectively. Nuclear translocation of NF-κB was initially evaluated indirectly by Western blot analysis of phosphorylated IkB-α and total IkB-α. Phosphorylated IkB-α-to-total IkB-α ratios were significantly decreased by 56% at day 7 in obstructed kidneys of hypercholesterolemic CD36−/− mice compared with CD36+/+ mice; a NS 30% decrease was observed at day 3 (CD36+/+ versus CD36−/−; day 3, 0.70 ± 0.20 versus 0.50 ± 0.06 [P = 0.36]; day 7, 2.10 ± 0.40 versus 0.90 ± 0.20 [P = 0.02]; n = 6 per group; Figure 4, A and B). To confirm the

CD36+/+ mice (9.7 ± 0.8 versus 11.9 ± 0.7%; n = 5 per group; F = 0.88, P = 0.4). To investigate whether the increase in F4/80+ cells at day 7 after UUO represented an increase in renal dendritic cells, we compared kidney CD68 and CD11b/c levels in CD36−/− mice with those of CD36+/+ mice. There was no difference in either the CD11b/c protein levels by Western blotting (CD36+/+ versus CD36−/− 0.15 ± 0.02 versus 0.19 ± 0.03; n = 6 per group; P = 0.3) or the steady-state CD68 mRNA levels measured by semiquantitative real-time qPCR (normalized to 18S, relative expression CD36−/− to CD36+/+ = 1.33; 95% confidence interval 0.50 to 5.02; P = 0.8; n = 5 per group). Differences in F4/80+ interstitial area between CD36+/+ and CD36−/− mice were NS at day 3 (4.0 ± 0.3 versus 3.0 ± 0.6%; n = 6 per group; P = 0.5).

### Chemokine Expression

To investigate the effect of CD36 expression on macrophage profile in obstructed kidneys, we compared renal chemokine expression levels in CD36+/+ and CD36−/− mice by RNAse protection assay. There was no difference in steady-state CCL2

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**Figure 2.** (A) Renal E-cadherin Western blot (top) illustrates the changes in E-cadherin protein levels (120 kD) on days 7 and 14 after UUO. E-cadherin expression was normalized to β-actin. The graph summarizes the results of single-band density measurements, expressed as β-actin-normalized E-cadherin band levels. Data are means ± SEM. †P < 0.01, CD36+/+ versus CD36−/−. (B) Representative E-cadherin immunohistochemistry photomicrographs demonstrate increased tubular localization of E-cadherin in CD36−/− mice 14 d after UUO. Magnification, ×400.

**Figure 3.** F4/80+ interstitial macrophage area is higher in CD36−/− mice 7 d after UUO despite less fibrosis. (Top) Representative F4/80 immunohistochemistry photomicrographs (400x) of CD36+/+ and CD36−/− mice at day 7 after UUO. (Bottom) Summary of the percentage F4/80+ interstitial area ± SEM. †P < 0.01, CD36+/+ versus CD36−/−.
Western blot findings, we performed electromobility shift assays (EMSAs) on nuclear extracts obtained from obstructed kidneys. These studies confirmed a 52% decrease in nuclear translocation of NF-κB heterodimers in CD36+/− mice at day 7 compared with CD36+/+ mice (n = 7 to 8 per group; P = 0.02; Figure 5C). We performed supershift assays to determine whether nuclear translocation of specific NF-κB heterodimers was altered. These studies demonstrated a significant decrease in nuclear translocation of the proinflammatory p50RelA (p50p65) heterodimer in CD36−/− mice compared with CD36+/+ mice (Figure 5D). Phosphorylated RelA immunostaining confirmed decreased p50RelA nuclear staining in both tubular and interstitial cells in CD36−/− obstructed kidneys (Figure 5, E and F).

**Attenuation of Oxidative Pathways in CD36−/− Mice**

**Accumulation of Oxidized Lipoproteins.**

We compared renal oxLDL levels in obstructed kidneys of hypercholesterolemic CD36+/+ and CD36−/− mice to determine whether they are modulated by CD36. Semiquantitative analysis of kidney sections stained for hypochlorous (HOCl)-modified LDL showed significantly decreased levels in the CD36−/− group at each time point investigated (n = 6 per group; P < 0.01; Figure 6). By immunostaining, less HOCl-modified LDL was detected within both renal tubules and the interstitium of CD36−/− obstructed kidneys (Figure 6).

**Oxidative Stress.**

Because oxidized lipoprotein accumulation was diminished, the degree of oxidative stress was compared in CD36+/+ and CD36−/− mice. Lipid, protein, and DNA may be targets of oxidation by free radical species such as reactive oxygen during chronic renal injury. The most commonly used indicator of generalized protein oxidation is protein carbonyl content. Lipids contain polyunsaturated fatty acid moieties that are susceptible to peroxidation. Linoleates are the major polyunsaturated lipids in vivo and are oxidized by free radicals into hydroperoxy octadecadienoates. Total hydroxyoctadecadienoic acid (HODE) levels account for the majority of linoleate oxidation products. We measured kidney HODE levels and protein carbonyl content in CD36+/+ and CD36−/− mice at day 7, representing a key UUO time point when both inflammation and oxLDL generation were significantly attenuated. HODE levels were significantly increased after UUO in mice of both genotypes compared with genotype-matched sham kidneys; on UUO day 7, HODE levels were 41% lower in the CD36−/− kidneys (CD36+/+ versus CD36−/−, HODE pmol/mg protein: sham, 134 ± 10 versus 132 ± 13 [P = 0.9]; UUO, 409 ± 31 versus 241 ± 15 [P = 0.0002]; n = 7 to 8 per group). Protein oxidation as measured by protein carbonyl content was also reduced by 41% in obstructed kidneys from CD36−/− mice compared with CD36+/+ mice at day 7 (CD36+/+ versus CD36−/−, protein carbonyl pmol/mg protein: sham, 103 ± 4 versus 101 ± 4 [P = 0.8]; UUO, 275 ± 9 versus 163 ± 5 [P = 0.0001]; n = 7 to 8/group). Renal protein and lipid oxidation measures showed a strong positive correlation, evidence of an overall reduction in the degree of oxidative stress in obstructed kidneys of hypercholesterolemic CD36−/− mice (n = 29; r² = 0.8374; F 139.1, df = 27; P < 0.01; Figure 7).

**Inflammation Attenuation in CD36−/− Mice Modulates Fibrogenic Pathways**

**TGF-β Activation.**

We performed studies to determine whether the CD36-associated changes in inflammatory and oxidative pathways resulted in changes in the activity of the profibrotic growth factor TGF-β. We examined phosphorylated Smad2 and total Smad2 levels in obstructed kidneys of CD36+/+ and CD36−/− mice by Western blotting at each time point. There was a NS trend toward lower ratio of phosphorylated Smad2 to total Smad2 levels at days 3 and 7 (n = 6 per group; P = 0.07; Figure 8). By immunostaining, there was a reduction in nuclear expression of phosphorylated Smad2 in both tubular and interstitial cells in CD36−/− mice (data not shown). There was no difference in the ratio of phosphorylated Smad2 to total Smad2 between genotypes at day 14 after UUO (CD36+/+ versus CD36−/−, day 14, 0.07 ± 0.01 versus 0.08 ± 0.01; P = 0.6; n = 5 per group).

**Interstitial Myofibroblast Numbers.**

Because fibrosis severity was significantly attenuated, we performed studies to examine the effect of CD36 expression on the number of interstitial myofibroblasts. α-Smooth muscle actin (α-SMA)-positive myofibroblasts are considered the primary...
source of interstitial matrix proteins that accumulate during renal fibrosis. There was a >30% reduction in the mean number of interstitial α-SMA–positive myofibroblasts in obstructed kidneys of hypercholesterolemic CD36−/− mice at days 7 and 14 compared with CD36+/+ mice (n = 6 per group; P < 0.01; Figure 9).

CXCL10 Expression.
CXCL10 is a chemokine that has been reported to reduce fibroblast migration.24 Steady-state CXCL10 mRNA levels were significantly higher in UUO kidneys from CD36−/− mice at days 7 and 14 compared with CD36+/+ mice (n = 6 per group; P < 0.01; Figure 9). By immunostaining, CXCL10 protein was identified in both tubular and interstitial cells.

Attenuated Fibrosis in CD36−/− Mice Is Independent of Diet
To investigate whether the decrease in fibrosis severity in CD36−/− mice was dependent on hypercholesterolemia, we measured total collagen levels in CD36−/− and CD36+/+ mice on a control diet. As seen in hypercholesterolemic mice, fibrosis severity was significantly reduced in obstructed kidneys from CD36−/− mice by 16 and 13% compared with CD36+/+ mice on a control diet at days 7 and 14 after UUO, respectively (n = 6 to 8 per group; P < 0.01; Figure 11).

DISCUSSION
Dyslipidemia not only is a risk factor for CKD progression but also independently can lead to renal dysfunction.6,9 We recently demonstrated that hypercholesterolemia exacerbates renal fibrosis in association with increased oxLDL deposition and more severe inflammation.18 We now document in this study in vivo profibrotic effects of the CD36 scavenger receptor in a model of progressive renal disease associated with hypercholesterolemia. The genetic deficiency of CD36 resulted in a >20% reduction in renal fibrosis severity compared with CD36+/+ mice at each time point investigated after UUO. Although measurement of renal functional differences is not possible in the UUO model, the major structural consequence of interstitial fibrosis that determines functional decline is tubular damage and destruction.25 Tubular injury was significantly less in hypercholesterolemic CD36−/− mice 14 d after UUO. The results of this study further suggest three important features of the pathologic mechanisms that contribute to CKD. First, CD36 expression by interstitial macrophages determines an activated macrophage phenotype that is profibrotic. Second, CD36 contributes to the profibrotic response to chronic renal injury via activation of proinflammatory pathways and generation of oxidative stress. Third, downregulation of proinflammatory and oxidative pathways as a consequence of genetic CD36 deficiency limits myofibroblast accumulation and fibrosis within the obstructed kidney.

It has long been known that macrophages are phenotypi-
cally and functionally heterogeneous, potentially able to promote inflammation and fibrosis or to prevent it via scavenging activities. In most studies, the number of renal interstitial macrophages has correlated closely with fibrosis severity. One no-

Figure 6. Kidney oxLDL levels are reduced in CD36+/− mice after UUO. (Top) Representative HOCl-LDL/oxLDL immunohistochemical photomicrographs in CD36+/+ and CD36−/− mice at day 14 after UUO. (Bottom) Summary of the quantification results obtained by computer-assisted image analysis expressed as the percentage of tubulointerstitial area staining positive for HOCl-LDL. Data are means ± SEM. †P < 0.01, CD36+/+ versus CD36−/−. Magnification, ×400.

Figure 7. Reduction in total oxidative stress in obstructed kidneys of CD36−/− mice after UUO. Protein oxidation was measured as total protein carbonyl content by ELISA. Lipid peroxidation was measured as HODE by RP-HPLC. The levels of HODE and protein carbonyl content were reduced at day 7 after UUO in CD36+/+ and CD36−/− mice. Linear regression demonstrated a strong positive correlation between HODE levels and protein carbonyl content and indicated much lower levels in CD36−/− mice 7 d after UUO (r² = 0.8374; F = 139.1, P < 0.01). •, CD36+/+ sham-operated (n = 7); ▲, CD36−/− sham-operated (n = 7); ■, CD36+/+ day 7 UUO (n = 7); ○, CD36−/− day 7 UUO (n = 8).

Figure 8. Reduction in fibrosis severity after UUO in CD36−/− mice is independent of TGF-β activation. (Top) Representative Western blot of phosphorylated Smad2 (pSmad2) and total Smad2 for days 3 and 7 after UUO in CD36+/+ and CD36−/− mice. (Bottom) Summary of the results of band density measurements for pSmad2 normalized to total Smad2. Data are means ± SEM. P > 0.05, CD36+/+ versus CD36−/−.

Figure 9. Myofibroblast accumulation is attenuated in CD36−/− mice after UUO. (Top) Representative α-SMA immunohistochemical photomicrographs in CD36+/+ and CD36−/− mice 14 d after UUO. (Bottom) Summary of the quantification by computer-assisted image analysis, expressed as the percentage of tubulointerstitial area staining positive for α-SMA. Data are as means ± SEM. †P < 0.01, CD36+/+ versus CD36−/−. Magnification, ×400.
leads to renal fibrosis is characterized by the generation of proinflammatory mediators. NF-κB transcription factors are key regulators of inflammation and immunity that control expression of important immunoregulatory genes. In this study, there was a >50% reduction in nuclear translocation of NF-κB heterodimers in hypercholesterolemic CD36−/− mice compared with CD36+/+ mice. Studies suggested that RelA (p65) is a key regulator in the requisite inflammatory phase of progressive renal fibrosis. By supershift assay and immunostaining, the decrease in NF-κB nuclear translocation in both tubular and interstitial cells after UUO in CD36−/− mice was largely attributable to a reduction in the proinflammatory p50RelA heterodimer. Whether this decrease is related to a reduction in a RelA-dependent signaling pathway, increased ubiquitination, or proteasomal degradation of RelA is not clear and deserves further investigation.

This study also detected a significant reduction in both HODE and carbonyl protein formation in hypercholesterolemic CD36−/− mice compared with CD36+/+ mice 7 d after UUO. Generation of lipid peroxides such as HODE and carbonyl proteins are established markers of oxidative stress and reflect the balance between pro-oxidant and antioxidant mechanisms within the tissue. There was a strong positive correlation between the presence of CD36 and the level of oxidative stress within obstructed kidneys. In an experimental stroke model, Cho et al. demonstrated that the degree of ischemia was reduced in CD36−/− mice and was associated with less reactive oxygen species (ROS) production in the ischemic area; however, the exact mechanism by which CD36 regulates ROS production is not known. There is increasing evidence that ROS and other reactive species may also be directly involved in redox signaling pathways that promote inflammation and apoptosis.

Table exception has been in models of renal injury induced in genetically engineered animals with impaired macrophage phagocytic activities. The findings in this study suggest that the CD36 scavenger receptor can be added to this list. We previously demonstrated that CD36+ cells infiltrated the interstitium 7 and 14 d after UUO; dual staining indicated that most of the CD36+ interstitial cells were F4/80+ macrophages. In this study, interstitial macrophages were significantly more numerous in CD36−/− mice at day 7 after UUO despite reduced fibrosis. The increased numbers of F4/80+ interstitial cells in CD36−/− mice could not be attributed to an increase in renal CD11b/c+ dendritic cells and likely represents a separate population of macrophages that will be characterized in future studies. Despite an increase in macrophage number, functionally these interstitial cells did not augment proinflammatory and oxidative pathways. CD36 expression has been reported to increase as monocytes differentiate into activated macrophages. In this study, the expression pattern of CXCL2 corresponded to the severity of fibrosis in CD36−/− mice. Recent studies suggested that CXCL2 may be an important marker of macrophage activation and is important in the generation of proinflammatory mediators. Taken together, these data suggest that CD36 expression by renal interstitial macrophages may serve as a marker for a profibrotic phenotype in chronic renal injury.

The initial phase of cellular activation and tissue injury that...
duced CD36 expression and apoptosis in cultured proximal tubular cells. In this study, the decrease in fibrosis severity was also seen in CD36−/− mice on a normal diet. These data are consistent with the view that CD36 not only is a receptor for oxidized ligands such as oxLDL but also CD36 propagates a state of oxidative stress and worsening fibrosis in chronically damaged kidneys.

Within the renal interstitium, the transition from inflammation to fibrosis is characterized by the appearance of interstitial myofibroblasts. Several studies have demonstrated a close association between inflammation intensity and severity of renal fibrosis.41–43 In this study, a reduction in proinflammatory and oxidative pathways in the obstructed kidneys of hypercholesterolemic CD36−/− mice resulted in a significant reduction in interstitial myofibroblast density and subsequent collagen deposition. These findings extend those of Duffield et al.41 and suggest that CD36-positive macrophages regulate interstitial myofibroblast accumulation, but the mechanisms by which this occurs remains unclear.

CXCL10 is an important chemokine expressed in the later phases of wound remodeling in the skin and serves to communicate key signals between dermal layers to end the regenerative phase and initiate the remodeling phase.44 In this study, renal tubular CXCL10 levels remained elevated at day 14 after UUO in CD36−/− mice compared with decreasing levels in CD36+/+ mice. Studies of in vivo models of pulmonary and renal fibrosis have suggested that the increased fibrosis reported with genetic deficiency or pharmacologic blockade of CXCL10 was due to greater myofibroblast accumulation.24,45 Evidence suggested that CXCL10 serves as an important inhibitor of fibroblast motility via suppression of m-calpain activation46,47 and may promote fibroblast/myofibroblast differentiation.44,48 Whether CD36 directly or indirectly modulates CXCL10 expression is unclear but suggests another potential mechanism that bridges CD36 activity and the degree of renal fibrosis.

The results of this study begin to elucidate the cellular pathways involved in hypercholesterolemic chronic renal injury. Similar to its role in atherogenesis, CD36 is a major receptor for oxLDL within the kidney, where it functions as a key modulator of inflammatory, oxidative, and fibrogenic pathways. Further studies are under investigation to determine the relative contribution of CD36+ tubules and CD36+ macrophages and their potential as therapeutic targets to prevent CKD progression.

**CONCISE METHODS**

**Experimental Design**

Breeding pairs of CD36−/− and CD36+/+ mice on C57BL/6 background were obtained from Dr. Maria Febbraio’s colony13 and were bred in our animal facility in Seattle. Male mice were fed a high-fat Western diet (15.8% total fat, 1.25% cholesterol with 0.5% sodium cholate [TD 88051; Harlan Teklad, Madison, WI])49 beginning after weaning at 3 to 4 wk of age. After a run-in period of 7 to 8 wk on the high-fat Western diet, animals were randomly assigned to one of four experimental groups: CD36+/+, CD36−/−, sham surgery, or UUO. Groups of male mice (n = 6 to 10 each) were killed at 3, 7, and 14 d after surgery. Groups of CD36+/+ and CD36−/− mice male mice (n = 6 to 8) were also placed on a standard chow diet with 0.5% sodium cholate for 7 to 8 wk after weaning, and UUO surgery was performed at 10 to 11 wk of age. For mice in the UUO group, the left ureter was exposed through a mid-abdominal incision and ligated using 4-0 silk. All surgeries were performed under general anesthesia with isoflurane. All procedures were performed in accordance with the guidelines established by National Research Council Guide for the Care and Use of Laboratory Animals and approval of our Institute Animal Care and Use Committee.

Sham and UUO kidneys were harvested and processed for RNA and protein extraction and histologic studies as described previously.19 Frozen tissue samples were stored at −80°C. Serum cholesterol levels were measured in blood samples that were obtained at killing using the Total Cholesterol Kit (WAKO Chemicals USA, Richmond, VA).

**Genotyping**

Genotyping was performed by PCR using genomic DNA isolated from tails. PCR primer sequences were obtained from Dr. Febbraio, and genotyping was performed as described previously.13 Primers for the CD36+/+ allele are 5′-CAGCTCATACTTTGTTTATG-CATG and 3′-GTTACAATCAGTTTTGCTACGG (band size approximately 600 bp). The primers for the CD36−/− allele include the CD36+/+ 5′ primer and 3′-GGTTCTCTGGCTTTAGG-TATC (band size approximately 750 bp).

**Collagen Content**

Hydroxyproline content of kidney tissue (μg of hydroxyproline per mg of wet weight kidney section) was performed by acid hydrolysis of the tissue section using procedures established in our laboratory.19

**Histologic Examination**

Immunohistochemical staining was performed on sections of paraffin-embedded tissue or cryosections of snap-frozen tissue using procedures established in our laboratory with VECTASTAIN Elite ABC Kits (Vector Laboratories, Burlingame, CA) and AEC Substrate Chromogen K3464 (Dako Corp., Carpentaria, CA) or DAB (Sigma, St. Louis, MO) as the peroxidase substrate. Sections were blocked with Avidin/Biotin blocking kit (Vector Laboratories). Primary antibodies used were reactive with F4/80 (rat anti-mouse F4/80 monoclonal; AbD Serotec, Raleigh, NC), CD11b/c (rabbit anti-mouse CD11b/c; Abcam, Cambridge, MA), CXCL10 (rabbit anti-mouse CXCL10 polyclonal; PeproTech, Rocky Hill, NJ), IκB-α and phosphorylated IκB-α (rabbit anti-human IκBα polyclonal and rabbit anti-human phospho IκB-α polyclonal; Cell Signaling Technology, Danvers, MA), phosphorylated p65 (rabbit anti-mouse p65 polyclonal; Cell Signaling Technology), Smad2 (rabbit anti-human Smad2 monoclonal; Cell Signaling Technology), phosphorylated Smad2 (rabbit anti-human phospho Smad2 polyclonal; Upstate Biotechnology, Billerica, MA), and oxLDL (rabbit anti-human hydrochloric acid modified LDL polyclonal; Chemicon Int., Temecula, CA). Interstitial myofibroblasts were quantified by staining using peroxidase-conjugated
murine anti-human α-SMA 1A4 mAb (Dako). The 1A4 antibody was detected using the Enhanced Polymer One-Step Staining reagent (Dako) as described previously. Secondary antibodies were shown to be nonreactive with tissue sections stained without primary antibody. Semi-quantitative computer-assisted image analysis of tubulointerstitial proteins was performed on six randomly selected ×400 magnified images of slides from individual animals with Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Glomerular areas and space not occupied by tissue were subtracted in the analysis. Interstitial macrophage density was expressed as percentage of F4/80 + interstitial area on DAB-stained cryosections. The percentage of macrophage density was determined from results of five randomly selected ×400 magnified images of slides from individual animals with assistance from Image-Pro Plus software (Media Cybernetics). The investigator was blinded to the experimental groups at the time of analysis.

**RNAsse Protection Assay**

Total kidney RNA was extracted using the TRIZOL single-step reagent (Life Technologies BRL Life Technologies, Grand Island, NY). A mouse chemokine RNase protection assay (mCK-5c) was performed according to protocol (BD Biosciences Pharmingen, San Diego, CA). Mouse chemokine multiprobe templates were radiolabeled with [α-32P]UTP (Perkin Elmer Life Sciences, Boston, MA). Probe hybridization with 15 μg of total RNA was performed overnight at 56°C, and unbound probe or single-stranded RNA was digested with RNase. Hybridized RNA samples were separated by electrophoresis, and gels were dried and visualized with the Typhoon 9410 PhosphorImager (GE Healthcare, Pittsburgh, PA). Chemokine band intensities were normalized relative L32 band intensities because it was closer to chemokine band levels. There was difference in significance between normalizing between glyceraldehyde-3-phosphate dehydrogenase and L32.

**EMSA and Supershift Assay**

Kidney tissue nuclear extracts were prepared according to the “small-scale” method of Andrews and Faller. In brief, frozen tissue was homogenized on ice in Buffer A (0.5 M sucrose, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA + 0.5 mM dithiothreitol, and 0.5 mM PMSF) including Proteinase Inhibitor cocktail (Sigma-Aldrich). Homogenates were centrifuged and resuspended in Buffer C (10% glycerol, 20 mM HEPES, 420 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, and proteinase inhibitor cocktail). Nuclear extract protein concentrations were determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Complementary oligonucleotides were annealed by heating and cooling overnight: NF-κB1 (5’-AGCTTAGAGGACCTTGAGAGGAC-3’), NF-κB2 (5’-AGCTTTCCCTGTGAAAGGGTTCTTCTA-3’), AP1–1 (5’-GATCCATGACTTGAGGAAAACA-3’), and AP1–2 (5’-GATCCTTTGTGCATGAGGATG-3’). NF-κB or AP1 radiolabeled probes were prepared using Klenow enzyme and [α-32P]dATP (Perkin Elmer Life Sciences). Specific (unlabeled or cold probe) and non-specific (AP-1) competitors were included to determine probe specificity. Phorbol myristate acetate–stimulated and unstimulated Jurkat T cells were used as positive and negative controls, respectively. Supershift was achieved using polyclonal rabbit anti-human p65 and p50 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Gels were dried and visualized with the Typhoon 9410 PhosphorImager.

**Western Blotting**

Protein was isolated from homogenized frozen kidney, and Western blotting was performed as described previously. Bands were normalized against β-actin (anti-mouse β-actin; Sigma). The secondary antibodies were anti-rabbit and anti-mouse IR700Dye and IR800Dye (Rockland Immunochemicals, Gilbertsville, PA). Protein bands were visualized and quantified using the Odyssey (Li-Cor Biosciences, Lincoln, NE).

**Oxidative Stress**

Anesthetized mice were perfused through the left ventricle with cold antioxidant solution (100 μM diethylenetriaminepentaacetic acid dihydrate, 1 mM 2,6-bis(1,1-dimethylhexyl)-4-methylphenol, 10 mM 3-amino-1,2,4-triazole stock, 50 mM sodium phosphate (pH 7); Sigma-Aldrich) at the time of killing. Kidney tissue was immediately harvested, immersed in antioxidant buffer, and stored at −80°C until analysis.

Lipids were extracted from kidney tissue using modified Dole procedure. HODEs were quantified by reverse-phase hydrolysis C18 HPLC analysis of triphenylphosphate-reduced lipid extracts after base hydrolysis. The protein content of tissue pellets was determined by Bradford assay using BSA as standard.

The quantity of protein carbonyls in a protein sample were determined by derivatizing with dinitrophenylhydrazine (DNP) and measuring bound DNP immunologically using the Zentech PC Test ELA Kit (Biocell Corp., Auckland, New Zealand). Samples containing protein were reacted with DNP, and then the protein was nonspecifically adsorbed to an ELISA plate. Unconjugated DNP and nonprotein constituents are washed away. The adsorbed protein was probed with biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. Absorbances were related to standard curve for BSA containing increasing proportions of hypochlorous acid–oxidized protein.
formed using the Pfaffl algorithm with the REST analysis software version 1.9.9 (Corbett Research Pty. Ltd., Mortlake, NSW, Australia).

Statistical Analysis
All data are presented as means ± SE. A nested ANOVA was used for all semiquantitative computer-assisted image analysis. For image analysis data, the arithmetic mean of six randomly selected images of slides for each animal was used to calculate the reported mean of the group and the SE. A Pearson linear pairwise correlation was performed on lipid peroxidation and protein oxidation data. All other results were analyzed by unpaired t test. P < 0.05 was considered statistically significant.

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DISCLOSURES
None.

REFERENCES
4. Himmelfarb J: Linking oxidative stress and inflammation in kidney disease: Which is the chicken and which is the egg? *Semin Dial* 17: 449–454, 2004
26. Ma X, Bacci S, Mlynarski W, Gottardo L, Socci T, Menzagh C, Iori E,


44. Paigen B, Holmes PA, Mitchell D, Albee D: Comparison of atherosclerotic lesions and HDL- lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c, and C3H. *Atherosclerosis* 64: 215–221, 1987


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