Low-Dose IL-17 Therapy Prevents and Reverses Diabetic Nephropathy, Metabolic Syndrome, and Associated Organ Fibrosis

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ABSTRACT
Diabetes is the leading cause of kidney failure, accounting for >45% of new cases of dialysis. Diabetic nephropathy is characterized by inflammation, fibrosis, and oxidant stress, pathologic features that are shared by many other chronic inflammatory diseases. The cytokine IL-17A was initially implicated as a mediator of chronic inflammatory diseases, but recent studies dispute these findings and suggest that IL-17A can favorably modulate inflammation. Here, we examined the role of IL-17A in diabetic nephropathy. We observed that IL-17A levels in plasma and urine were reduced in patients with advanced diabetic nephropathy. Type 1 diabetic mice that are genetically deficient in IL-17A developed more severe nephropathy, whereas administration of low-dose IL-17A prevented diabetic nephropathy in models of type 1 and type 2 diabetes. Moreover, IL-17A administration effectively treated, prevented, and reversed established nephropathy in genetic models of diabetes. Protective effects were also observed after administration of IL-17F but not IL-17C or IL-17E. Notably, tubular epithelial cell-specific overexpression of IL-17A was sufficient to suppress diabetic nephropathy. Mechanistically, IL-17A administration suppressed phosphorylation of signal transducer and activator of transcription 3, a central mediator of fibrosis, upregulated anti-inflammatory microglia/macrophage WAP domain protein in an AMP-activated protein kinase–dependent manner and favorably modulated renal oxidative stress and AMP-activated protein kinase activation. Administration of recombinant microglia/macrophage WAP domain protein suppressed diabetes-induced albuminuria and enhanced M2 marker expression. These observations suggest that the beneficial effects of IL-17 are isoform-specific and identify low-dose IL-17A administration as a promising therapeutic approach in diabetic kidney disease.


CKDs such as diabetic nephropathy are a serious public health problem. In the United States, >20 million adults have CKD, and 20%–40% of patients with diabetes develop nephropathy. The financial burden of diabetes is estimated to be $245 billion per year in the United States alone, and the cost for diabetic nephropathy is estimated to be >$20 billion per year. While intensive insulin therapy and control of hypertension can delay the onset of diabetic nephropathy,1–3 these therapies cannot reverse CKD once it has developed. Recently, feeding mice a strictly ketogenic diet (5% carbohydrate, 8% protein, 87% fat) reversed a mild form of nephropathy.4 Although implementing such an extreme diet...
in humans is practically impossible, these data suggest that diabetic CKD could potentially be reversible if key molecular pathways are identified and selectively modulated.

Inflammation plays a key role in the pathogenesis of diabetic CKD. The extent of disease is in part determined by the complex interplay between various cytokines in the IL family, which can both mediate and modulate inflammation. IL-17A is a member of the IL-17 family, which includes six structurally related isoforms: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A is produced by multiple cell types, including CD4+ αβ T cells, γδ T cells, natural killer cells, and neutrophils. IL-17A acts on a variety of cells through its ubiquitous receptors, IL-17RA and IL-17RC, and it can mediate neutrophil recruitment and migration through the induction of granulopoiesis and the production of neutrophil chemokines, including lipopolysaccharide-induced CXC chemokine, cytokine-induced neutrophil chemoattractant, and macrophage inflammatory protein-2.

IL-17A was originally implicated in several disease processes, such as encephalomyelitis; psoriasis; atherosclerosis; hypertension; viral myocarditis; dilated cardiomyopathy; and ischemia-reperfusion injury of brain, kidney, and intestine. Subsequent studies, however, have disputed a pathogenic role for IL-17 in many of these diseases, and provided data to the contrary showing that IL-17 can favorably modulate inflammation. Moreover, an IL-17A neutralizing antibody therapy developed to treat arthritis exacerbated the disease process, further pointing toward a protective role of this cytokine in certain disease states.

The capacity of cytokines such as IL-17A to mediate or modulate inflammation may vary depending on the level and duration of cytokine stimulation. In addition, responses may vary in accordance with the particular IL-17 isoform, receptor, and downstream signaling pathways. Here, we examined the effects of chronic, low-level IL-17A administration on the pathogenesis of diabetic CKD. We found that under these conditions, IL-17A protects against diabetes-induced kidney damage by suppressing podocyte and tubular epithelial injury. Furthermore, infusion of IL-17A reversed the diabetes-induced nephropathy and associated proteinuria in both type 1 and type 2 diabetic mouse models. In addition, IL-17A reduced plasma lipid levels and arterial lipid deposition. IL-17F administration also favorably modulated diabetic nephropathy, whereas IL-17C and IL-17E were ineffective. Dissecting out the mechanisms of these protective effects of IL-17A could lead to the development of novel therapy for the prevention or reversal of diabetic nephropathy.

RESULTS

IL-17 Expression Is Increased in Early-Stage but Is Downregulated at Late-Stage Diabetic Kidney Disease

A recent study among patients with type 2 diabetes showed that circulating levels of IFN-γ were elevated in both nephropathic and non-nephropathic diabetic patients, whereas IL-17A levels were elevated in non-nephropathic patients and downregulated in nephropathic patients. To test the association between urinary IL-17A excretion and nephropathy development, we quantified the levels of IL-17A in urine of diabetic mice and humans. Urinary excretion of IL-17A was increased after 12 weeks of streptozotocin (STZ)-induced diabetes in mice. In humans with diabetes and microalbuminuria, urinary IL-17A excretion was increased compared with control and diabetic patients without proteinuria. However, in patients with macroalbuminuria, urinary IL-17A excretion was reduced (Supplemental Figure 1), suggesting that downregulation of IL-17A may exacerbate proteinuria.

Genetic Deletion of IL-17A Gene Exacerbates Diabetic Nephropathy

To determine the role of endogenous IL-17A in diabetic nephropathy, wild-type (WT) and IL-17A knockout mice were made diabetic with STZ administration. Kidney function and albuminuria did not significantly differ in WT and IL-17A knockout mice without diabetes, and glucose levels were similar in diabetic mice of both genotypes (Figure 1, A–D). Diabetes induced a significant increase in albumin excretion, urine glucose, kidney weight-to-body weight ratio, and BUN in WT mice. These changes were further increased in IL-17A knockout mice with diabetes (Figure 1). In addition, tubular injury and interstitial fibrosis and mesangial expansion were more pronounced in IL-17A knockout mice than in WT mice. Interestingly, administration of IL-17A to diabetic IL-17A knockout mice suppressed albuminuria and mesangial expansion (Supplemental Figure 2). These data suggest that endogenous IL-17A protects against the development of diabetic nephropathy.

IL-17A and IL-17F but Not Other Isoforms Protect Kidney against Nephropathy

To determine the frequency and dose for optimal protection, we first examined the kinetics of IL-17A clearance after administering 10 ng of IL-17A per animal. IL-17A rapidly appeared in plasma, peaking at 1 hour after administration. However, urinary excretion was delayed, peaking at 24 hours after IL-17A administration and returning to baseline level by 48 hours (Supplemental Figure 3). On the basis of these kinetics, IL-17A was administered at 48-hour intervals in the subsequent dose-determination experiments. Our initial dose-response studies (Supplemental Table 1) had determined that the dose of 10 ng per animal was sufficient to protect against diabetic nephropathy. Therefore, we used this low dose of IL-17 in subsequent studies. To determine whether other isoforms of IL-17 also protect against diabetic nephropathy, we administered IL-17A, IL-17C, IL-17E, IL-17F (10 ng per animal) or vehicle to STZ-induced diabetic mice starting 1 week after induction of diabetes. Like IL-17A, IL-17F was effective in reducing albuminuria (Supplemental Table 1). In contrast, IL-17C and IL-17E had no significant effect, suggesting that protection against diabetic nephropathy is isoform-specific.
Low-Dose IL-17A Treats, Prevents, and Reverses Nephropathy in STZ Diabetic Mice

In treatment protocol (Figure 2A), IL-17A was administered (10 ng per animal every 48 hours, intraperitoneally) for 12 weeks after confirmation of hyperglycemia. To determine whether IL-17A can prevent diabetic nephropathy, IL-17A was administered only for the first 6 weeks of diabetes, and animals were monitored for another 6 weeks without...
treatment (Figure 2B). To determine whether IL-17A can effectively reverse diabetic nephropathy (early stage), treatment was initiated at 6 weeks after induction of diabetes and continued until the animals were euthanized (Figure 2C). With all three protocols, IL-17A administration resulted in a significant reduction in albuminuria, whereas blood and urine glucose levels were unaltered. These findings suggest a broad therapeutic window for IL-17A administration in diabetic nephropathy.
nephropathy. In fact, IL-17A works better in late stages of diabetic nephropathy.

Tissue and urine samples from the treatment group were further characterized to determine the IL-17A influence on inflammation, mesangial expansion, macrophage infiltration, interstitial fibrosis and stat3 activation. Diabetes led to increased kidney and glomerular hypertrophy (Figure 3, A and B) and inflammatory cytokine excretion (Figure 3, C–F) compared with controls, which was significantly suppressed with IL-17A treatment. IL-17A treatment also suppressed diabetes-induced stat3 phosphorylation, macrophage infiltration, and glomerular and interstitial fibrosis (Figure 3, G–I) as indicated by reduced collagen, fibronectin, and α-smooth muscle actin expression. STAT3 has been reported to mediate kidney fibrosis.27,28 Interestingly, most of the phosphorylated STAT3 appeared to be localized to tubular epithelial cells.

IL-17A Reverses Established Nephropathy in a Genetic Model of Type 1 Diabetes

We next tested the effects of IL-17A administration in Ins2Akita mutant mice, a model for type 1 insulin-dependent diabetes. By the age of 4 weeks, these mice develop hyperglycemia, and by the age of 18 weeks, they develop nephropathy as indicated by increased albuminuria (>1000 μg/24-hour urine) and polyuria. IL-17A or vehicle was administered to 18-week-old diabetic mice for 12 weeks to treat established nephropathy. As shown in Table 1, these mice are diabetic and exhibit increased kidney weight-to-body weight ratio compared with WT control mice. IL-17A administration also improved survival of diabetic mice (Table 1). Ins2Akita mice treated with vehicle developed marked albuminuria, polyuria, and glucosuria (Figure 4). IL-17A treatment strongly abrogated the albuminuria, diminishing the level to nearly that of nondiabetic controls (Figure 4B). A significant reduction in albuminuria was detected in these mice with 4–6 weeks of IL-17A administration. In contrast, IL-17A treatment did not affect plasma glucose or glucosuria in these mice (Figure 4C).

Consistent with massive albuminuria in vehicle-treated Ins2Akita mice, diabetic mice showed glomerular hypertrophy (Figure 4E), significant increase in arterial pressure (Figure 4F), and increased excretion of inflammatory cytokines and chemokines (Figure 4, G–I). IL-17A administration reduced these changes except BP. Interestingly, GFR was significantly reduced at 30 weeks in Ins2Akita mice and was restored with IL-17A treatment (Figure 4M). Kidney sections showed mesangial expansion (Figure 4J), glomerular sclerosis, interstitial fibrosis, tubular atrophy, and formation of proteinaceous casts (Figure 4K). These changes were minimal or absent in IL-17A–treated-Ins2Akita mice.

To determine whether IL-17A regulates the expression of proinflammatory and profibrotic genes in the kidneys of these mice, fibrotic gene expression in the kidneys was analyzed using PCR array as described in the Concise Methods section. Diabetes upregulated several proinflammatory and profibrotic genes, including α-smooth muscle actin; collagen I; connective tissue growth factor; decorin; endothelin-1; angiotensinogen; lysyl oxidase; latent TGF-β–binding proteins; matrix metalloproteinases 2, 3, 8, 9, and 14; tissue plasminogen activator; plasminogen; thrombospondin; and TNF-α. Expression of these genes was significantly reduced by IL-17A administration (Figure 4L).

IL-17A Treats Established Nephropathy in a Genetic Model of Type 2 Diabetes

To determine whether IL-17A is effective against type 2 diabetes–induced kidney complications in a genetic model, IL-17A or vehicle was administered to db/db mice or littermate WT control mice (Figure 5A). As expected, db/db mice exhibited increased body weight, hyperglycemia, polyuria, albuminuria, glucosuria, urinary cytokine excretion, and glomerular expansion compared with WT mice (Figure 5, B–I). IL-17A administration markedly improved albuminuria, polyuria, and glomerular expansion and suppressed urinary cytokine excretion but had no effect on kidney weight-to-body weight ratio, glucosuria, plasma glucose, or body weight (Figure 5, B–I). Consistent with improved kidney function, histologic changes (glomerular expansion and tubular proteinaceous casts) were also improved with IL-17A administration (Figure 5O). In addition, interstitial fibrosis significantly increased in db/db mice treated with vehicle but not with IL-17A (Figure 5N, Supplemental Figure 4).

Hyperlipidemia is a hallmark of type 2 diabetes and causes cardiovascular and kidney damage. To determine whether IL-17A alters the lipid profile in the circulation, various lipids were quantified. As shown in Figure 5, J–M, IL-17A significantly reduced triglycerides, VLDL/IDL cholesterol, and total cholesterol but did not alter HDL cholesterol levels. These data suggest that IL-17A administration improves not only kidney function but also metabolic dysfunction, which may have protective effects on cardiovascular complications in type 2 diabetes.

IL-17A Administration Suppressed Diabetes-Induced Oxidative Stress and Restored AMP Kinase Levels

Oxidative stress plays a key role in the pathogenesis of diabetes.29–32 We thus examined the effects of IL-17A administration on urinary excretion of thiobarbituric acid reactive substance (TBARS), a measure of oxidative stress. As shown in Figure 6, A–F, diabetes was associated with increased excretion of TBARS in urine, which was completely suppressed by IL-17A treatment, suggesting that IL-17A may exert direct or indirect effects on oxidative stress to protect kidneys from diabetes-induced kidney dysfunction.

Downregulation of AMP kinase (AMPK), a key modulator of cellular energy balance and oxidative stress, is believed to contribute to the pathogenesis of diabetes and its complications.33,34 We thus examined the effect of IL-17A administration on AMPK in the kidneys and liver of diabetic animals. Diabetes significantly downregulated AMPK phosphorylation in both STZ and Ins2Akita diabetic mouse kidney (Figure 6G)
Figure 3. IL-17A treatment suppressed nephropathy and interstitial fibrosis. Mice with IL-17A treatment protocol (as shown in Figure 2A) where further characterized. (A) Kidney weight-to-body weight ratio. (B) Quantification of glomerular area. (C) Interferon gamma-induced protein 10 (IP-10) (D) TNF-α excretion in urine. (E) IL-6 excretion in urine. (F) Monocyte chemoattractant protein-1 excretion in urine. *P<0.05 versus other groups; **P<0.001 versus vehicle-treated diabetic group. n=8–10 in each group. (G) Representative kidney histology showing phospho-stat3, macrophage infiltration, collagen IV expression, and PAS (indicating tubular injury and mesangial...
and liver (Supplemental Figure 5A), which was restored to normal levels by IL-17A administration.

Consistent with in vivo data, maximum activation of AMPK was seen with IL-17A and IL-17F in human podocyte cells compared with other kinases (Supplemental Figure 4, B–G). In addition to AMPK activation, IL-17A and IL-17F also increased the activation of several other kinases, including protein kinase C, Akt, p38 mitogen-activated protein kinase, and ERK but suppressed the degradation of inhibitorκB (iκB). Interestingly, inhibition of ERK kinase significantly reduced IL-17A–induced AMPK activation, suggesting that ERK may be an upstream activator of AMPK (Supplemental Figure 5, H and I). In addition, we also saw an increase in iκBα proteins, suggesting that iκBα accumulation could essentially inhibit NFκB activation and inflammation.

IL-17A Administration Suppresses Podocyte and Tubular Epithelial Cell Injury

In kidneys, the IL-17A receptor is mainly expressed in podocytes and tubular epithelial cells (Figure 7A). To investigate whether IL-17A acts on these cells to suppress diabetic nephropathy, we quantified expression of the podocyte and the tubular epithelial injury markers nephrin, kidney injury molecule-1 (KIM-1), and semaphorin3A (sema3A). KIM-1 expression was undetectable in control kidneys but highly expressed in the proximal tubular epithelial cells of diabetic kidneys, and its expression was suppressed by IL-17A treatment (Figure 7A). In addition, Western blot analysis showed elevated levels of KIM-1 and sema3A (another podocyte and tubular injury marker) in the urine of diabetic animals compared with controls. Administration of IL-17A suppressed urinary excretion of both KIM-1 and sema3A (Figure 7B). Similarly, urinary excretion of the podocyte injury marker nephrin was markedly increased by diabetes in all three experimental models (STZ, Ins2Akita, and db/db) but was largely suppressed by concurrent treatment with IL-17A (Figure 7C).

However, the mRNA expression of podocin and nephrin is not altered in diabetes with or without IL-17A treatment (Supplemental Figure 6). Consistent with suppression of podocyte injury marker excretion by IL-17A administration, diabetes-induced podocyte loss was also suppressed and restored to control level by IL-17A administration (Figure 7, D and E), suggesting that IL-17A may prevent injury or regenerate podocytes in diabetes.

The Novel Activated Microglia/Macrophage-Restricted Whey Acidic Protein Domain Protein, a Counter-regulator of Proinflammatory Response, Is Highly Induced by IL-17A, Which Suppresses Diabetic Nephropathy

Microarray studies showed that a novel whey acidic protein (WAP) termed as activated microglia/macrophage WAP (AMWAP) domain protein gene was upregulated >100-fold in IL-17A–treated kidneys. Microarray observation was confirmed by real-time RT-PCR analysis (Figure 8A), immunohistochemistry, and Western blot analysis (Supplemental Figure 7). AMWAP was recently identified as a counter-regulator of the proinflammatory response in macrophages. However, its role in diabetes or any other disease has never been examined. To determine its regulation by IL-17A in podocyte, macrophage, and renal epithelial cells, cells were treated with different doses of IL-17A or IL-17F. IL-17A induced a dose-dependent increase in AMWAP expression in mouse podocytes, proximal tubular epithelial cells (TKPTS), and macrophages that depends on AMPK (Figure 8, B, C, G, and H). Interestingly, IL-17A treatment induced a large increase in IL-10 excretion in urine (Figure 8, E and F). Consistent with in vivo data, addition of recombinant AMWAP protein (custom synthesized from Selleckchem) to macrophages induced a large increase in anti-inflammatory M2 marker genes, such as IL-10 and arginase-1 (Figure 8, H–J) but completely suppressed LPS-induced IL-1β expression (Figure 8K). AMWAP treatment also induced IL-10

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Table 1. IL-17A treatment in Ins2Akita mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>DBA Control: Vehicle-Treated</th>
<th>Ins2Akita: Vehicle-Treated</th>
<th>Ins2Akita: IL-17A–Treated</th>
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<td>Glucose (mg/dl)</td>
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<td>609±23*</td>
<td>588±89*</td>
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<td>Body weight (g)</td>
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<td>23±1*</td>
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<td>Kidney weight (mg)</td>
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<td>570±74</td>
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<td>Kidney weight-to-body weight ratio(mg/g)</td>
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<td>25±1*</td>
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<tr>
<td>Serum creatinine (mg/dl)</td>
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<td>0.14±0.04</td>
<td>0.2±0.05</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>100</td>
<td>47*</td>
<td>87*</td>
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IL-17A was administered for 12 weeks starting at 18 weeks of age, and mice were euthanized at 30 weeks of age. Data are mean±SEM for n=16 per group.

*P<0.05 versus control.

bP<0.05 versus Ins2Akita vehicle treated.
expression in mouse podocytes (Figure 8L). These data suggest that AMWAP may mediate IL-17–induced IL-10 production in vivo.

To determine whether AMWAP has a protective role in diabetic nephropathy, recombinant AMWAP protein was administered to control and diabetic DBA/2J mice as described in the Concise Methods section (Figure 9A). As shown in Figure 9, B–D, administration of AMWAP had no effect on blood glucose level or body weight. However, AMWAP significantly reduced albuminuria and glomerulosclerosis (Figure 9, E–G). Because AMWAP is known to have anti-inflammatory activity by turning macrophages to the M2 phenotype, we analyzed M2 marker expression in vehicle- and AMWAP-treated control and diabetic mouse kidney. Vehicle-treated diabetic mouse kidney showed increased proinflammatory M1 marker (IL-6 and IL-1β) (Figure 9, H and I). Administration of AMWAP not only reduced expression of proinflammatory cytokines (IL-6 and IL-1β) but also increased expression of M2 markers, such as arginase-1 and IL-10 (Figure 8, J and K). In addition, the excretion of IL-10 was significantly increased in AMWAP-treated mouse urine (Figure 9, L and M). These data suggest that AMWAP protects the kidney against diabetic nephropathy, possibly through regulation of the macrophage phenotype and IL-10.

**Epithelial Cell–Specific Overexpression of IL-17A or IL-17F Is Sufficient to Suppress Diabetic Nephropathy in Mice**

We have created IL-17A and IL-17F overexpressing mice using the epithelial cell–specific E-cadherin promoter. Epithelial cell–specific activity of this promoter is well characterized. We obtained three positive lines for each gene (Supplemental Figure 7). Characterization of IL-17A and IL-17F expression in plasma, kidney, and urine is shown in Supplemental Figure 8.

**Figure 4.** IL-17A administration treats established nephropathy in a genetic model of type 1 diabetes. (A) IL-17A administration protocol in Ins2Akita mutant mice. (B) Albumin excretion rate (AER). (C) Glucose excretion in urine. (D) BUN. (E) Glomerular area. (F) Mean arterial BP measured by radio telemetry. (G) Inducible protein-10 (IP-10) excretion in urine. (H) IL-6 excretion in urine. (I) Monocyte chemoattractant protein-1 excretion in urine. (J) Mesangial expansion expressed as percentage of glomerular area. (K) Masson trichrome staining for interstitial fibrosis and glomerulosclerosis. MMP, matrix metalloprotease. Scale bar=100 μM. (L) Quantification of profibrotic gene expression by real-time PCR. *P<0.01 versus other groups; #P<0.05 versus vehicle-treated Ins2Akita mice. (M) GFR was determined as described in the Concise Methods section. *P<0.05 versus other groups. n=12–16.
All three lines for IL-17A excrete large amounts of IL-17A in urine, whereas only lines 2 and 3 express small amounts of IL-17A in serum. In the case of the IL-17F transgenic line, all three lines excrete very low levels of IL-17F in urine, and none was found in serum. Localization studies showed that both IL-17A and IL-17F expression was localized in tubular epithelial cells (Supplemental Figure 8, E and J). Both IL-17A and IL-17F transgenic mice show normal kidney function and morphologic characteristics under basal conditions. Similarly, other organs in these mice show normal histologic features. Because FVB/N background strains are relatively resistant to diabetic nephropathy, these transgenic mice were crossed with DBA/2J mice, and F1 offspring were used for diabetic nephropathy studies.

Induction of diabetes causes albuminuria (Figure 10, C and D), kidney hypertrophy (Figure 10E), increase in glomerular area (Figure 10H), mesangial expansion (Figure 10I), and histologic changes (Figure 10, J–M) in WT mice. These changes were largely suppressed in transgenic animals, which do not have IL-17A in their circulation (line 1) (Figure 10, C–M).
Blood glucose level is elevated in both groups (Figure 10B). Interestingly, overexpression of IL-17A in epithelial cells upregulated AMWAP (Figure 10N), mannose receptor (Figure 10O), and IL-10 (Figure 10P) in both control and diabetic mice, which was associated with suppression of oxidative stress (Figure 10Q).

Similarly, induction of diabetes in line 2 (Figure 11), which showed IL-17A expression in both kidney and circulation (Figure 11, F and G), suppressed kidney hypertrophy (C), albuminuria (Figure 11, D and E), mesangial expansion (Figure 11H), increase in glomerular area (Figure 11I) and histologic changes (Figure 11, J–M), and ultrastructural changes (such as podocyte foot process effacement and basement membrane thickness) (Figure 11, N–Q) compared with WT diabetic mice. Blood glucose level is elevated in both groups (Figure 11B). Protection of kidney was associated with upregulation of AMWAP (Figure 11R), mannose receptor (Figure 11S), IL-10 (Figure 11T), and suppression of oxidative stress (Figure 11U) in control and diabetic IL-17A transgenic mice compared with WT diabetic mice. These data suggest that epithelial cell-specific overexpression is sufficient to suppress diabetic nephropathy.

As shown in Supplemental Figure 8, IL-17F excretion was detected only in IL-17F transgenic mice; this was increased further after induction of diabetes. Induction of diabetes causes albuminuria (Supplemental Figure 8D), polyuria (Supplemental Figure 8E), kidney hypertrophy (Supplemental Figure 8F), increase in glomerular area and mesangial expansion as determined in periodic acid-Schiff (PAS)–stained sections (Supplemental Figure 8, G and H) in WT mice. IL-17F overexpression in tubular epithelial cells significantly suppressed and F) mice. Urine samples are from the same animal in Figures 2, 4, and 5. *P<0.005 versus control; # P<0.05 versus vehicle-treated diabetic mice. n=10–15. Diabetes-induced down-regulation of AMPK phosphorylation in the kidney was restored to control levels with IL-17A treatment. *P<0.001 versus control; # P<0.001 versus vehicle-treated db/db mice. n=4–6.

Figure 6. IL-17A suppresses oxidative stress and restored diabetes-induced down-regulation of phospho AMPK levels. Oxidative stress was assessed by TBARS excretion in urine. Diabetes induced a large increase in TBARS excretion in urine, which was suppressed with IL-17A treatment in STZ (A and B), Ins2Akita (C and D), and db/db (E and F) mice. Urine samples are from the same animal in Figures 2, 4, and 5. *P<0.005 versus control; # P<0.05 versus vehicle-treated diabetic mice. n=10–15. Diabetes-induced down-regulation of AMPK phosphorylation in the kidney was restored to control levels with IL-17A treatment. *P<0.001 versus control; # P<0.001 versus vehicle-treated db/db mice. n=4–6.
Figure 7. IL-17 receptor A (IL-17RA), podocyte, and tubular injury marker expression in kidney and urine. (A) Immunohistochemical localization of IL-17RA and KIM-1 in control and diabetic kidney. IL-17RA expression is mostly seen in proximal tubular epithelial cells. Diabetes increased staining intensity, but the pattern is not changed. IL-17A administration did not alter IL-17A expression. KIM-1 expression is absent in control kidney and expression of KIM-1 is increased in diabetic mice kidney. IL-17A treatment suppressed KIM-1 expression in diabetic kidney. Scale bar=100 μM. (B) Western blot analysis of KIM-1 and sema3A excretion in urine. (C) Quantification of podocyte injury marker nephrin excretion in urine. STZ diabetic mice (12 weeks), Ins2Akita mice (30 weeks) and db/db mice (20 weeks) treated with vehicle or IL-17A and excretion of nephrin was quantified by ELISA as described in the Concise Methods. IL-17A administration significantly reduced the excretion of nephrin in urine. *P<0.001 versus other groups. *P<0.001 versus vehicle-treated diabetic mice. (D and E) Quantification of podocytes in glomerular section that are immunostained with Wilms tumor-1 antibody (D) and positive cells in 20 glomeruli in each kidney was counted then averaged (E). Scale bar=100 μM. *P<0.05 versus other group. n=4–6.
Figure 8. Regulation of AMWAP and IL-10 by IL-17A in different cells. (A) Administration of IL-17A induced a large increase in AMWAP expression in Ins2Akita kidney analyzed by real-time PCR. *P<0.001 versus WT control; **P<0.01 versus vehicle treated Ins2Akita mice. (B) RT-PCR analysis of AMWAP and IL-10 expression in mouse podocyte. *P<0.01 versus vehicle treated control. (C) RT-PCR analysis of AMWAP expression in TKPTS (mouse proximal tubular epithelial cells). *P<0.01 versus 0 hour. (D) AMPK inhibitor suppressed IL-17A–induced AMWAP expression in TKPTS cells. **P<0.001 versus other groups. (E) IL-17A administration enhanced IL-10 excretion in urine of Ins2Akita mice. *P<0.001 versus WT control; **P<0.01 versus vehicle-treated Ins2Akita mice. (F) IL-17A administration enhanced IL-10 excretion in diabetic mouse urine at 12 weeks after STZ administration. *P<0.01 versus vehicle treated nondiabetic control;
diabetes-induced albuminuria (Supplemental Figure 8D), mesangial expansion and glomerular hypertrophy (Supplemental Figure 8, I and J). Blood glucose level was elevated in both groups (Supplemental Figure 8C).

Consistent with reversal of plasma lipid levels in db/db mice with IL-17A administration, transgenic overexpression of IL-17A and IL-17F also suppressed the diabetes-induced increase in triglyceride levels compared with WT (Supplemental Table 2). Interestingly, IL-17A/F overexpression not only suppressed the diabetes-induced increase but also further downregulated from baseline compared with nondiabetic transgenic animal controls. HDL cholesterol level is significantly downregulated in WT mice in response to diabetes, which was restored in transgenic animals (Supplemental Table 2). No changes were seen for LDL/VLDL and total cholesterol levels among WT and transgenic animals with or without diabetes except in the diabetic line 2, which showed a significant decrease in LDL/VLDL concentration. This finding suggests that the presence of IL-17A in serum could be the underlying reason for this decrease.

**DISCUSSION**

The IL-17 family of proteins has been implicated as positive regulators of inflammation and fibrosis in diseases such as arthritis and psoriasis by promoting the induction of cytokines and extracellular matrix proteins. Although much focus has been placed on the detrimental role of IL-17A in disease states, recent studies in animals and humans suggest that IL-17A can also inhibit fibrosis and reduce tissue injury. In addition, administration of IL-17A suppressed atherosclerosis, while deficiency of the IL-17A gene accelerated the development of unstable atherosclerotic plaque formation in apolipoprotein E knockout mice. However, the role of IL-17A in CKD is unknown. Here, we provide novel data suggesting a protective role for both endogenous and exogenous IL-17A and IL-17F in the pathogenesis of diabetic nephropathy.

We observed that the absence of IL-17A expression accelerates the development of nephropathy and increases disease severity in STZ-induced diabetes. Conversely, administration of low-dose recombinant IL-17A prevented and reversed interstitial fibrosis, loss of podocytes, tubular epithelial atrophy, and albuminuria in mice with STZ-induced diabetes, Ins2Akita mice, and db/db mice, supporting the notion that IL-17A is broadly protective against diabetic kidney disease. Similarly, administration of IL-17F suppressed the development of nephropathy, whereas equivalent doses of IL-17C and IL-17E did not, suggesting isoform-specific renoprotective effects.

Our dose response studies suggest that 10 ng per animal (which translates into 0.4 μg/kg body wt or 0.028 mg or 28 μg/70 kg for humans) is sufficient for reversing nephropathy. Animal studies suggest that even at a dose of 200 μg/kg body wt (which translates to 14,000 μg [14 mg]/70 kg in humans) does not have major immune stimulatory effects. Therefore, the dose we used is easily achievable in humans. Moreover, our animal studies also suggest that IL-17 is cleared rapidly from the circulation but takes much longer to appear in urine. Therefore, concerns of chronic immune stimulation are not there. In addition, it is also possible that a dose much lower than 10 ng per animal may be effective, but this needs to be tested. Both IL-17A and IL-17F form homodimers and heterodimers and are known to bind to the same receptor (IL-17RA) to mediate their biologic activities. However, the IL-17A homodimer has more affinity for IL-17RA than IL-17RC, whereas IL-17F homodimer has more affinity toward IL-17RC. IL-17RA is ubiquitously expressed, whereas IL-17RC expression is mostly found in nonhematopoietic tissues. Because IL-17A and IL-17F, but not IL-17C or IL-17E, conferred renoprotection, these findings suggest that the beneficial effects of IL-17 in diabetic nephropathy are mediated via IL-17RA and IL-17C. However, we did not examine the effects of IL-17B or IL-17D, so further studies are required to establish this with certainty. Interestingly, a low dose of IL-17A did not increase neutrophil counts in mice treated with low-dose IL-17A (Supplemental Figure 10) and G-CSF levels in plasma and urine were actually decreased by IL-17A treatment in diabetic mice (Supplemental Figure 11). Therefore, neutrophilia may not occur at such a low dose. Also of note, IL-17RC is expressed mostly in nonimmune tissues but is still effective, suggesting that IL-17F may have less immune stimulatory effects compared with IL-17A and may have fewer adverse effects, if any.

The mechanism underlying IL-17–mediated protection against diabetic nephropathy may be mediated through multiple pathways, such as regulation of macrophage polarization through AMWAP, regulation of podocyte and epithelial survival and regeneration, suppression of inflammation by M2 macrophages, and suppression of metabolic syndrome through AMPK activation in multiple cells in and outside the kidney. This notion was supported by our data showing that IL-17A treatment upregulated AMWAP in podocyte,

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aP<0.001 versus vehicle-treated diabetic control. IL-17A (50 ng/ml) (G) and IL-17F (50 ng/ml) (H) treatment induced AMWAP expression in macrophages within hours, whereas IL-10 expression takes 72 hours. *P<0.001 versus 0 hour. (I) Recombinant AMWAP treatment induced IL-10 and arginase-1 expression in macrophages. **P<0.001 versus vehicle-treated. (J) Recombinant AMWAP treatment induced IL-10 protein expression in macrophages. IL-10 protein in culture supernatant was quantified by ELISA. **P<0.01 versus vehicle-treated. (K) AMWAP suppressed LPS-induced IL-1β expression in macrophages. P<0.001 versus other groups. (L) Recombinant AMWAP treatment induced IL-10 expression in mouse podocyte. *P<0.001 versus vehicle treated. n=6–10.
macrophage, and epithelial cells. Although AMWAP expression was suggested to be restricted to microglial cells and macrophages, our studies show that AMWAP expression is inducible in podocytes and epithelial cells as well. Expression is observed in microglial cells upon stimulation with multiple toll-like receptor ligands and IFN-γ. AMWAP overexpression reduces the proinflammatory cytokines IL-6 and IL-1β and concomitantly increases expression of the alternative activation markers arginase 1 and CD206. Consistent with these data, our in vitro studies suggest that IL-17A regulates AMWAP expression in an AMPK-dependent manner. Moreover, AMWAP increased the level of M2 markers, such as IL-10 and arginase-1, in macrophages but suppressed the M1 markers IL-6 and IL-1β. Administration of recombinant AMWAP protected the kidney against diabetic nephropathy and enhanced IL-10 expression and excretion, suggesting that AMWAP could be a downstream mediator of IL-17A protective activity through regulation of the macrophage phenotype and IL-10 expression. The regulation of IL-17 in different cells in diabetes is unknown. Moreover, the source of IL-17 that may regulate macrophage function in diabetes is also not clear. T cells and neutrophils are major sources of IL-17A and IL-17F. Therefore, it is possible that both T cells and neutrophils may have crosstalk with macrophages within the kidney in mediating its polarization state. Future studies with targeted deletion of IL-17A in these cells will enable us to answer these questions.

The interesting unexpected finding is the effects of IL-17A on blood lipid levels. IL-17A treatments dramatically reduced VLDL cholesterol, triglyceride, and LDL cholesterol levels but did not alter HDL cholesterol level. Similarly, transgenic overexpression also reduced the diabetes-induced increase in triglyceride levels and suppressed the diabetes-induced decrease in HDL cholesterol compared with WT mice. The mechanism underlying this correction of hyperlipidemia is not clear. However, we show that AMPK is highly induced in both liver and kidney. AMPK is a critical regulator of lipid homeostasis. In addition, microarray data from the kidney show that the diabetes induced the expression of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase 2, which was suppressed with IL-17A administration, suggesting it may suppress HMG-CoA synthesis. Therefore, IL-17A reduced metabolic dysfunction through both

Figure 9. Recombinant AMWAP administration suppressed diabetic nephropathy in mice. Six-week-old DBA2/J mice were made diabetic with STZ. (A) Four weeks after confirmation of hyperglycemia, mice received vehicle or recombinant AMWAP for another 4 weeks (10 ng per animal per day).Administration of recombinant AMWAP suppressed diabetes-induced albuminuria (E and F), glomerulosclerosis (G), but not blood glucose level (B), kidney weight (C), or body weight (D). AER, albumin excretion rate. Scale bar=100 μM. *P<0.001 versus control; †P<0.01 versus vehicle-treated diabetic animals. (F) AMWAP suppressed M1 polarization markers, such as IL-6 (H) and IL-1β (I) but increased M2 marker arginase-1 (J), IL-10 (K) expression in kidney and excretion in urine (L and M). *P<0.001 versus control; †P<0.01 versus vehicle-treated diabetic animals. n=8–10.
increased utilization of lipid and suppression of synthesis. This observation has clinical ramifications because HMG-CoA reductase inhibitors, such as statins, do not work in all patients. Many patients have severe adverse effects and only a very moderate reduction in hyperlipidemia. In addition, IL-17A treatments also reduced lipid accumulation in and around the arteries as well as lipid droplets in liver (Supplemental Figure 4B), suggesting that it could improve vascular function and suppresses atherosclerosis-associated with type 2 diabetes.
Figure 11. Epithelial cell–specific overexpression of IL-17A is sufficient to suppress diabetic nephropathy. Data from IL-17A transgenic Line 2. (A) IL-17A transgenic mice were crossed with nephropathy prone strain DBA/2J. Six-week-old WT and IL-17A–positive F1 mice were given a single dose of STZ (150 mg/kg body wt). Mice were euthanized 8 weeks after STZ administration and albuminuria was quantified. (B) Blood glucose level at 8 weeks of diabetes. (C) Kidney hypertrophy was calculated as ratio of kidney weight and body weight (KW/BW). (D) Albumin excretion rate (AER) expressed as μg/24-hour urine. (E) AER expressed as μg/mg of creatinine. (F) Serum IL-17A levels. (G) Urine IL-17A levels. (H) Mesangial index. (I) Glomerular area. (J–M) PAS-hematoxylin–stained kidney section. Scale
Reduction of blood glucose levels by IL-17A, however, was not detected in any of our animal models, suggesting that the renoprotective effects of IL-17A are independent of glycemic control. In addition, we showed no significant changes in BP in response to IL-17A administration in Ins2Akita diabetic mice, which are mildly hypertensive. Consistent with our data, a recent study also demonstrated that IL-17A knockout mice showed exacerbated renal injury without altering BP in a model of deoxycorticosterone acetate salt/angiotensin II–induced hypertension. Collectively, these data support the notion that IL-17A protects against diabetic nephropathy via intrinsic actions on the kidneys. Our studies in IL-17A transgenic mice suggest that epithelial specific overexpression is sufficient for prevention of diabetes-induced albuminuria and glomerular sclerosis. Consistent with exogenous administration, overexpression in epithelial cells also increased AMWAP expression in the kidney, which was associated with an increase in M2 markers, such as IL-10 and mannose receptor; this finding suggests possible regulation of macrophage phenotype by epithelial cell derived factors, such as AMWAP.

In summary, our findings suggest that both endogenous and exogenous IL-17A protect against nephropathy in models of type 1 and type 2 diabetes. Importantly, administration of IL-17A was also effective in treating established nephropathy in our animal models. Protective effects were also observed following administration of IL-17F but not IL-17C or IL-17E, suggesting that the beneficial effects may be linked to activation of IL-17RA/C. Mechanistically, IL-17A administration suppressed phosphorylation of STAT3, a central mediator of fibrosis, and favorably modulated renal oxidative stress and AMPK activation and induced a novel anti-inflammatory protein, AMWAP. Interestingly, epithelial cell–specific overexpression is sufficient to suppress diabetes-induced albuminuria and glomerular changes. The possible mechanism of IL-17–mediated protection against diabetic nephropathy is shown in Supplemental Figure 12). These preclinical studies in mice, plus observations in humans showing reduced plasma and urinary IL-17A levels in patients with advanced diabetic nephropathy, suggest that low-dose IL-17A/F is a promising approach to treating and reversing diabetic kidney disease.

**CONCISE METHODS**

**Ethics Statement**  
All studies were approved by the appropriate institutional animal review board (Institutional Animal Care and Use Committee, Georgia Regents University; approval ID 07–0044). All animals used in these studies were obtained from The Jackson Laboratory (Bar Harbor, ME) or bred in house at our animal facility and housed with free access to food and water under 12-hour/12-hour light/dark cycle (lights on at 7:00 a.m.). Human study protocol was approved by the local ethics committee at University Medical Center Groningen and the institutional review board at Georgia Regents University. This is a post hoc study using urine samples collected from a previously published study. IL-17A in urine was quantified in 87 of 92 diabetic samples published earlier.

**IL-17 Quantification in Serum and Urine**  
IL-17A and IL-17F levels in serum and urine were quantified using ELISA kits (for human samples: R&D System, Minneapolis, MN; for mouse samples: eBioscience, San Diego, CA).

**Quantification of Nephrin and TBARS in Urine**  
Nephrin (ELISA) and TBARS (colorimetric assay) were quantified in urine using a kit from Exocell Inc. (Philadelphia, PA).

**STZ Diabetic Nephropathy**  
The induction protocol we followed was described by the Animal Models of Diabetic Complication Consortium. The Institutional Animal Care and Use Committee of the Georgia Regents University approved all of the protocols and procedures using animals (approval number 2011–0348). Eight-week-old WT mice (C57BL/6J) were given STZ (150 mg/kg body wt in citrate buffer) as a single dose. Citrate buffer was administered to control animals, which were otherwise subjected to the same treatment as the diabetic animals. One week after injection, blood glucose level was measured to confirm induction of diabetes. Animals were divided into four groups. For treatment group 1, IL-17A (10 ng per animal every 48 hours) or vehicle (0.1% BSA in PBS) was administered for 12 weeks. Group 2 received IL-17A (10 ng per animal every 48 hours) or vehicle for the first 6 weeks only; animals were euthanized at 12 weeks. Group 3 received IL-17A (10 ng per animal every 48 hours) or vehicle starting 6 weeks after confirmation of diabetes; animals were euthanized 12 weeks later. Blood glucose and serum creatinine levels were measured every 3 weeks after induction of diabetes. Twelve weeks after induction of diabetes, 24-hour urine was collected and animals were euthanized to collect kidney tissues and blood. To determine whether IL-17A prevents nephropathy in a dose-dependent manner, group 4 animals were administered vehicle or IL-17A at a dose of 10, 50, or 100 ng per animal every 48 hours. Animals were euthanized 12 weeks after diabetes induction, and urine albumin was quantified. Kidney tissue was processed for histopathologic analysis.

To determine the effects of other isoforms of IL-17, diabetic mice were administered IL-17C, IL-17E, or IL-17F at a dose of 10 ng per
animal every 48 hours for 12 weeks. Animals were euthanized 12 weeks after induction of diabetes, and urine albumin was quantified. Kidney tissue was processed for histopathologic analysis.

To determine the effects of AMWAP on diabetes-induced kidney injury and albuminuria, 6-week-old DBA/2 mice were made diabetic with a single dose of STZ (150 mg/kg body wt). Four weeks after confirmation of diabetes, AMWAP (10 ng/d) or vehicle was administered (intraperitoneally) for 4 weeks. Twenty-four-hour urine was collected, and animals were euthanized at the end of the treatment. Samples were processed for albuminuria quantification and molecular analysis.

**Akita Diabetic Nephropathy**

Male 4-week-old DBA/2 mice (WT or with the Akita mutation [JAX# 007562, D2.B6-In2Akita/Mahi]) were purchased from The Jackson Laboratory. At 18 weeks of age, blood and urine were collected to confirm diabetes and nephropathy. At that time, half of WT mice and half of the Akita mice were treated with recombinant mouse IL-17A (10 ng per animal every 48 hours, intraperitoneally). The remaining animals were treated with vehicle (0.1% BSA). Mice were maintained on the IL-17A or vehicle treatment for 12 weeks, at which time urine was collected to determine albumin excretion rate. Animals were then euthanized to collect plasma and kidney tissues, which were fresh-frozen for analysis of gene expression. Age-matched DBA/2 mice treated with vehicle served as a nondiabetic control group. Sixteen mice per group were used in this study.

**db/db Diabetic Nephropathy**

Male 10-week-old C57Bl/KsJ (WT or db/db; JAX#000642, BKS.Cg-Dock7m+/-Leprdb/J, homozygous for Lprdb) were purchased from The Jackson Laboratory. At 12 weeks, WT and db/db mice received vehicle (0.1% BSA in Dulbecco PBS) or IL-17A (10 ng per animal every 48 hours). Body weight and blood glucose were monitored throughout the study. After 10 weeks of treatment, 24-hour urine was collected, and animals were euthanized by administration of pentobarbital sodium (100 mg/kg body wt) followed by cervical dislocation. Kidneys were collected and fresh-frozen for gene expression analysis or fixed for renal histopathologic assessment.

**Induction of Diabetes in IL-17A Knockout Animals**

To determine the role of endogenous IL-17A in diabetic nephropathy, 8-week-old IL-17A knockout (Stock#016879, Il17atm1.1(icre)Stck/J) and WT mice (10 animals per group) were administered a single dose of STZ (150 mg/kg body wt). Twenty-four-hour urine was collected at 12 weeks after induction of diabetes, and animals were euthanized to harvest kidney tissues. Urine albumin and glucose were quantified. To determine whether IL-17A can suppress diabetic nephropathy in IL-17A knockout mice, IL-17A or vehicle was administered (10 ng per animal every 48 hours) for 4 weeks starting at 4 weeks after induction diabetes. Animals were euthanized at 8 weeks and tissue was processed for histopathologic evaluation.

**Measurement of BP**

At 28 weeks of age, Ins2Akita mice were instrumented with telemetry transmitters to record BP and heart rate (PA-C10; Data Sciences, Saint Paul, MN). Transmitters were implanted as described previously. After 7 days of recovery from surgery, BP data were recorded. IL-17A or vehicle treatment was continued during the recovery and recording periods. Two weeks after transmitter implantation, animals were euthanized to collect tissues and plasma for analysis. BP values were every 10 minutes for the duration of the study. Mean values were collected from 24-hour averages.

**Measurement of GFR**

GFR was measured in conscious mice using an insulin clearance kit (Biopal, Worcester, MA), and GFR calculator program (Biopal) was used to calculate the GFR.

**Measurement of Urinary Albumin Excretion Rate**

Excretion of urinary albumin was determined using albumin-to-creatinine ratio in 24-hour urine collections. Twenty-four-hour urine was collected using Nalgene Metabolic Cage System (Rochester, NY), which allows efficient separation of urine and feces from a single mouse. The concentration of albumin in urine was quantified by ELISA (Bethyl Laboratories, Inc., Montgomery, TX), and urine creatinine was determined using creatinine assay kit (Diazyme Laboratories, Poway, CA).

**Glomerular Morphometry**

At the time of euthanasia, kidneys were harvested for pathologic examination. One kidney was fresh-frozen for gene expression analysis, and the other was fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO). The formalin-fixed tissue was embedded in paraffin, and 4-µm sections were stained with periodic acid-Schiff stain (American Histolabs, Gaithersburg, MD). Glomerular sclerosis was quantified using a semi-automated image analysis technique with an examination of the total cortical area. To perform morphologic analyses, a total of 30 glomeruli were randomly selected from each kidney by moving the slide from the outer to the inner cortex in a random fashion to obtain nonoverlapping sample fields. Glomerular images were recorded using a CCD camera (Olympus DP72 color CCD camera; Olympus, Pittsburgh, PA) mounted on an Olympus light microscope. The glomerular tuft was traced, and the enclosed area was calculated using CellSens Standard software.

To quantify mesangial extracellular matrix, 3-µm sections from paraformaldehyde-fixed, paraffin-embedded kidney slices were stained using PAS. Mesangial area was expressed quantitatively by calculating the percentage of the total glomerular area that was PAS positive. Fifty glomerular tufts per animal were chosen randomly for analysis.

**Transmission Electron Microscopy**

Animals were perfused with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH of 7.4 (PB). Kidneys were removed, cut into small pieces, and immersed in 2.5% glutaraldehyde containing 1% tannic acid in 0.1 M PB for 2 hours at 4°C. They were postfixed with 1% OsO4, dehydrated, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined under a Phillips CM10 electron microscope.
Assessing Kidney Function
Kidney function was assessed by measuring BUN (Bioassay Systems) and serum creatinine (Diazyme Laboratories).

Cytokine and Chemokine Measurements
Cytokines and chemokines in urine were measured using ELISA kits (eBioscience).

Flow Cytometry
To quantify the neutrophils in blood, 10 μl blood was stained with rat anti–mouse Gr-1 PE-cy5 and CD45-FITC and analyzed in BD FACS-Calibur at our university’s core facility. Data were then reanalyzed with Flow Logic V.1.2.1 software.

Quantification of mRNA by Real-Time RT-PCR
RNA was isolated from kidney, macrophage, and podocyte cell lines using TRIzol reagent (Life Technologies, Grand Island, NY). Real-time RT-PCR was performed in a 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Three micrograms total RNA was reverse transcribed in a reaction volume of 40 μl using an Omniscript RT kit and random primers. The product was diluted to a volume of 150 μl, and 5-μl aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen) and Mouse Inflammatory Cytokines & Receptors PCR Array (catalog # PAMM-011E-4; SABiosciences, Frederick, MD) or gene-specific primers. Data were analyzed using Web analysis tools from SABiosciences.

Western Blot Analysis
Protein extracted from kidneys, TKPTS cells, human podocytes,47 and mouse podocytes (CLS cell lines, Germany) were used for Western blot analysis as described previously.48,49 The membrane was probed with rabbit anti-phospho AMPK, rabbit anti–phospho p38 mitogen-activated protein kinase, rabbit anti-phospho ERK, rabbit anti-phospho PAN PKC, anti-α-tubulin (Cell Signaling Technologies), rabbit anti–total AMPK, rabbit anti–sera3A, goat anti–KIM-1, rabbit anti–fibronectin, α-smooth muscle actin, or rabbit anti-collagen I and collagen IV antibodies (Abcam, Inc., Cambridge, MA). Proteins were detected using enhanced chemiluminescence detection reagents (Thermo Fisher Scientific). Protein loading was normalized to β-actin or glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling Technologies).

To determine expression of the tubular injury marker KIM-1 and sera3A in urine, protein extracted from the volume of urine containing 1 μg creatinine was loaded onto the gel and transferred to a polyvinylidene difluoride membrane. The membrane was probed with goat anti–mouse KIM-1 (R&D Systems) and rabbit anti–mouse sera3A (Abcam, Inc.) antibodies. Proteins were detected using enhanced chemiluminescence detection reagents.

Histology and Immunostaining
Kidney tissue was fixed in buffered 10% formalin for 12 hours and then embedded in paraffin wax. For assessment of injury, 5-μm sections were stained with PAS, followed by hematoxylin. Acute tubular necrosis and apoptosis were quantified by determining the percentage of the tubules that showed epithelial cell necrosis, brush-border loss, cast formation, and apoptotic bodies in the cortex. Ten fields of × 40 magnification were examined and averaged. The individual scoring of the slides was blinded to the genotype of the animal.

Interstitial fibrosis was assessed semi-quantitatively on both Mason trichrome and collagen-stained paraffin sections at magnification of ×20. Interstitial fibrosis was quantified using computer-based morphometric analysis software (Analysis; Olympus) that allowed the formation of a binary image in which the stained area could be automatically calculated as percentage of the image area. Ten fields per specimen were randomly selected that covered nearly the whole piece of cortex. Scoring was performed blind on coded slides.

To determine podocyte number in a glomerular section, a kidney section was stained with rabbit anti–Wilsms tumor-1 (WT1) antibody (Santa Cruz Biotechnology). WT1-positive cells were counted in 20 glomeruli/section and then averaged and expressed as podocyte number per glomerular section.

To quantify leukocyte infiltration, sections were stained with rat anti-mouse neutrophil antibody or anti-mouse macrophage antibody (1:200 dilution; Abcam, Inc.) followed by goat anti-rat biotin conjugate. Color was developed after incubation with ABC reagent (Vector Laboratories). Stained sections were photographed and five 40× fields of neutrophils were examined for quantification of leukocytes. To determine KIM-1, IL-17R, and AMWAP expression, sections were stained with rabbit anti–goat TIM-1 antibody (1:100 dilution; R&D System) or rabbit anti–AMWAP peptide antibody (custom made from SydLabs.com) or rabbit anti–IL-17R antibody (LifeSpan Biosciences, Inc., Seattle, WA) followed by rabbit anti-goat biotin or goat anti–rabbit biotin conjugate. Color was developed after incubation with ABC reagent (Vector Laboratories). Stained sections were photographed using an Olympus inverted microscope with color CCD camera.

Generation of IL-17A and IL-17F Transgenic Mice
Epithelial cell–specific overexpression of mouse IL-17A and IL-17F was accomplished using the partial E-cadherin promoter.37 E-cadherin promoter (~178 to +17) with mouse IL-17A cDNA or IL-17F cDNA was synthesized (Integrated DNA Technologies, Inc.) and then cloned upstream of a poly A signal in pcDNA3.1 plasmid using Sphl and Xhol. Expression in epithelial cells was confirmed by transfecting into mouse proximal tubular epithelial cells (TKPTS). Plasmid DNA was cut with Bgl III and Dra III to release the construct, which was gel purified and used for microinjection. Microinjections were carried out by the Emory Transgenic core, and three founder lines for both the IL-17A and the IL-17F constructs in the FVB/N background were obtained. To determine whether IL-17A and IL-17F overexpression protects against diabetes-induced nephropathy and fibrosis, IL-17A and IL-17F transgenic mice were crossed with DBA/2J mice. F1 mice positive for the transgene were administered a single dose of STZ to induce diabetes. F1 mice negative for the transgene were used as littermate wild-type controls. Animals were euthanized at 8 weeks after induction of diabetes.

Statistical Analyses
All data are presented as mean ± SEM. Statistical analyses were performed using GraphPad instat 3. A t test was used for single
comparisons, and ANOVA followed by Bonferroni correction was used for multiple comparisons. *P*<0.05 indicates statistically significant differences.

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**DISCLOSURES**

None.

**REFERENCES**


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