Interleukin-17A blockade reduces albuminuria and kidney injury in an accelerated model of diabetic nephropathy

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Diabetic nephropathy (DN) is one of the most common complications of diabetes, and currently the first end-stage renal disease worldwide. New strategies to treat DN using agents that target inflammatory pathways have attracted special interest. Recent pieces of evidences suggest a promising effect of IL-17A, the Th17 effector cytokine. Among experimental DN models, mouse strain BTBR ob/ob (leptin deficiency mutation) develops histological features similar to human DN, which means an opportunity to study mechanisms and novel therapies aimed at DN regression. We found that BTBR ob/ob mice presented renal activation of the factors controlling Th17 differentiation. The presence of IL-17A-expressing cells, mainly CD4$^+$ and $\gamma\delta$ lymphocytes, was associated with upregulation of proinflammatory factors, macrophage infiltration and the beginning of renal damage. To study IL-17A involvement in experimental DN pathogenesis, treatment with an IL-17A neutralizing antibody was carried out starting when the renal damage had already appeared. IL-17A blockade ameliorated renal dysfunction and disease progression in BTBR ob/ob mice. These beneficial effects correlated to podocyte number restoration and inhibition of NF-κB/ proinflammatory factors linked to a decrease in renal inflammatory-cell infiltration. These data demonstrate that IL-17A takes part in diabetes-mediated renal damage and could be a promising therapeutic target to improve DN.

Translational Statement

Some clinical trials blocking IL-17A have proven beneficial effects in chronic inflammatory diseases. Novel therapeutic options to prevent renal damage clinically are unmet for diabetic nephropathy (DN). Our preclinical data suggest that treatment with neutralizing IL-17A antibodies could be a therapeutic option for diabetic patients with albuminuria.

In recent decades, diabetes mellitus became one of the greatest public health care problems worldwide. DN is one of the most prevalent microvascular complications in patients with diabetes[1,2] and currently is more prevalent than end-stage renal failure worldwide.[3] DN affects approximately 20% to 30% of patients with type 1 or type 2 diabetes. Knowledge about underlying pathophysiological processes leading to DN has evolved a great deal in recent years, with both genetic and environmental factors interacting to result in complex pathophysiological events.[4]

Although DN is considered a nonimmune disease, emerging evidence suggests that both systemic and local inflammatory mechanisms play an important role in its pathogenesis and progression through the regulation of cell adhesion molecules, growth factors, chemokines, and proinflammatory cytokines.[1,5–7]

The Th17 effector cytokine interleukin (IL)-17A has become an important therapeutic target for a wide variety of diseases.[8,9] Recent studies suggest that IL-17A blockade is a promising tool for chronic human inflammatory diseases, with special relevance in ankylosing spondylitis, chronic plaque psoriasis, and psoriatic arthritis.[10–13] Recent studies also have suggested that IL-17A is involved in acute and chronic kidney disease.[14]

IL-17A is a cytokine with pleiotropic functions. It coordinates tissue inflammation by inducing expression of proinflammatory cytokines, chemokines, and matrix metalloproteases, which mediate both tissue infiltration and destruction.[15] However, experimental data evaluating the direct effects of IL-17A modulation in the diabetic kidney are controversial.[16,17]

The lack of an experimental model to replicate the key features of human DN has hampered efforts to investigate novel therapeutic treatments for this disease. Among the different preclinical models of DN available, the leptin-deficient BTBR ob/ob mouse recently has been described as a robust and progressive animal model that uniformly develops human DN features. The model largely agrees with the criteria proposed by the Animal Models of Diabetic Complications Consortium[18] and therefore is a promising tool to test novel treatments for this disease. The aim of the current study was to explore whether the cytokine IL-17A could be involved in the pathogenesis of DN by using a neutralizing antibody against IL-17A in the experimental model of BTBR ob/ob mice.

RESULTS

Activation of the Th17 immune response in the kidney of BTBR ob/ob mice was associated with the onset of renal damage

Previous studies of the BTBR ob/ob animal model have described the presence of monocyte/macrophage infiltrate in the glomerulus at approximately 12 weeks.[18] In light of these findings, we extensively characterized the inflammatory process in this model of DN until 20 weeks, comparing BTBR ob/ob animals, consisting of diabetic obese animals (referred to here as "diabetic mice") and a nondiabetic, nonobese control, that is, BTBR wild-type (WT) mice (referred to here as "control mice").

In the diabetic mice studied, periodic acid–Schiff staining showed that interstitial cellular infiltration can be initially perceived in the lesions at 8 weeks (Figure 1a) and increased progressively in older mice. At age 14 weeks, the kidneys of diabetic mice presented a significant increase in cellular infiltration compared with control subjects. To further characterize the cellular infiltration, immunohistochemistry studies using specific markers were performed at this time. Different immune cells, including monocytes/macrophages (F4/80$^+$ cells), T-lymphocytes (CD3$^+$, CD4$^+$, and γδ lymphocytes), and neutrophils (myeloperoxidase [MPO]) were observed in the diabetic kidneys, whereas few inflammatory cells were detected in control subject kidneys (Figure 1a).

Next, the renal expression of monocyte chemoattractant protein-1 (MCP-1), a key chemokine involved in the recruitment of inflammatory cells in the kidney,[19] was evaluated over the time. In diabetic mice, mcp-1 mRNA expression was significantly increased from week 10 until the end of the study (Figure 1b). In contrast, nondiabetic control kidneys exhibited no changes in mcp-1 gene levels.

To determine whether the inflammatory response was associated with kidney damage, we analyzed the gene expression of 2 two biomarkers of renal injury. Neutrophil gelatinase-associated lipocalin (NGAL) has been described as an early marker of kidney disease, and kidney injury molecule 1 (KIM-1) has been related to the transition from acute to chronic renal damage.[20,21] In diabetic mice, mRNA levels of ngal and kim-1 increased significantly at the eighth and 10th

week, respectively, compared with the control group (Figure 1c and d). These data confirm the importance of renal inflammation in this experimental model of DN, as described in other diabetic mice models and in patients with DN.

To investigate whether the Th17 immune response was involved in the pathogenesis of DN in BTBR ob/ob mice, we first evaluated the progression of renal levels of IL-17A, the effector cytokine of Th17 response. In the kidneys of diabetic mice, increased Il-17A mRNA expression was found as early as 10 weeks of age (Figure 2a) compared with the earliest time points and control mice and remained elevated thereafter. Moreover, in diabetic kidneys, elevated IL-17A protein levels also were observed relative to control subjects, both by Western blot analysis and enzyme-linked immunosorbent assay (Figure 2b, c, and e). Immunohistochemical staining was positive for IL-17A in interstitial areas of diabetic mice, whereas no IL-17A signal was observed in the kidneys of control mice (Figure 2c). The identification of IL-17A–expressing cells was performed by double immunostaining with antibodies against IL-17A and markers of inflammatory cells and confocal microscopy. We found out that CD4$^+$ and γδ lymphocytes produced IL-17A in murine diabetic kidneys (Figure 2d).

The process by which CD4$^+$ T lymphocytes differentiate into Th17 cells is regulated by the activation of the specific transcription factor signal transducer and activator of transcription 3 (STAT3) and the induction of retinoic acid–related orphan receptor γt (RORγt) expression.[22] In our study, diabetic mice presented elevated levels of ROR-γt and phosphorylated STAT3 (p-STAT3), the latter indicating activation of this transcription factor (Figure 2b and e). Changes in cytokine expression patterns can regulate Th differentiation, with IL-6 being the key driver of Th17 differentiation, whereas transforming growth factor (TGF)-β induces regulatory T cell polarization.[23,24] At 16 and 20 weeks, diabetic mice had increased il-6 and tgf-β mRNA levels compared with control mice (Figure 2f). Moreover, an increase in active TGF-β protein levels was noted in the kidneys of BTBR ob/ob mice compared with control subjects (data not shown) (Figure 2g).

Serum IL-17A levels are elevated in several inflammatory diseases, both in murine models and in patients with arthritis, multiple sclerosis, asthma, and atherosclerosis.[25–29] In diabetic mice, however, we observed that circulating IL-17A levels were not modified in any of the groups studied and remained at levels similar to those of control mice (data not shown).

Treatment with an anti–IL-17A neutralization antibody markedly reduced albuminuria in DN mice

BTBR ob/ob mice develop albuminuria and renal lesions, and thus they share some of the characteristics of human patients with DN.[18] To investigate the role of endogenous IL-17A in the pathogenesis of DN, we used a neutralizing antibody against IL-17A in BTBR ob/ob animals (referred to here as "anti-IL-17A–treated diabetic mice"). This antibody was
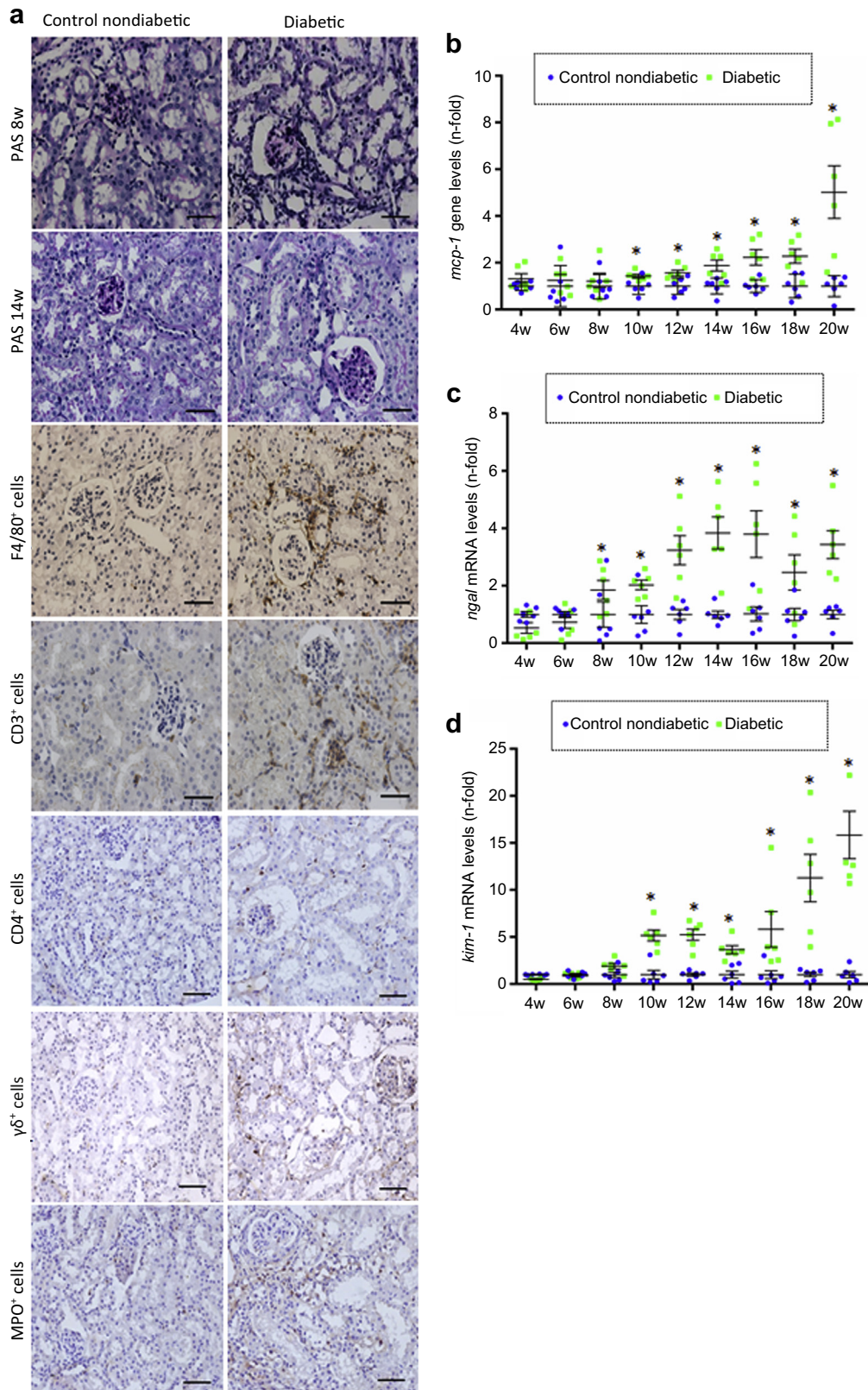
Figure 1 | Evolution of renal damage and inflammation in the model of experimental diabetes. BTBR ob/ob mice, diabetic and obese (called diabetic mice) and their corresponding controls (BTBR wild-type [WT] littermate mice) were studied from 4 to 20 weeks of age. Evaluation of renal lesions and characterization of inflammatory cell infiltration. To evaluate renal structure, mice were studied at different time points (8 and 14 weeks), and kidney sections were stained with periodic acid–Schiff (PAS). Inflammatory cell infiltration was characterized at 14 weeks by immunohistochemistry using specific antibodies for monocyte/macrophages (F4/80), (continued)

administered using osmotic minipumps, which release a continuous and controlled dose of 50 mg/kg/d of anti–IL-17A. This dose was similar to those described in other experimental models of tissue damage.[23,30,31]

Because our goal was to block IL-17A as a therapeutic regime, BTBR ob/ob mice were treated with IL-17A neutralizing antibody in the 15th week of life when they developed renal damage classified as DN (as shown by morphologic lesions, Figure 1a) and when renal IL-17A mRNA and protein levels were already elevated (Figure 2a and b). Then the mice were randomly distributed in 2 groups, one receiving IL-17A neutralization treatment (referred to here as "anti-IL-17A–treated mice") and the other treated with its corresponding isotype IgG (referred to here as "IgG-treated diabetic mice"), as described in the Methods section. Other control groups studied were untreated BTBR ob/ob mice and BTBR WT nonobese, nondiabetic mice.

First, to validate our model in relation to previous studies,[18] the onset of albuminuria was measured by using the albumin/creatinine ratio in spot urine samples from week 4 until the end of the study. The IgG-treated diabetic mice showed an increase in albuminuria as early 6 weeks of age, remaining elevated up to 20 weeks and reaching a difference of more than 10-fold compared with control mice of the same age (885 ± 112 μg/mg vs. 92 ± 26 μg/mg; $P < 0.05$ vs. control mice; Figure 3a). As expected, no significant changes were found between IgG-treated diabetic mice and untreated diabetic mice (data not shown). Interestingly, the albumin/creatinine ratio was correlated with changes in renal *ngal* mRNA expression in diabetic mice (Figure 1d).

The evolving effectiveness of IL-17A neutralization treatment on albuminuria values in diabetic mice was also examined (Figure 3b). Importantly, in response to IL-17A blockade, the albumin/creatinine ratio was significantly decreased compared with IgG-treated diabetic mice (359 ± 85 μg/mg vs. 885 ± 112 μg/mg, at 20 weeks, $P < 0.05$ vs. IgG-treated diabetic mice).

The percentage of albumin/creatinine ratio reduction in diabetic mice treated with the IL-17A antibody compared with IgG-treated animals was 41%, clearly showing that IL-17A neutralization ameliorates renal dysfunction in experimental DN (Figure 3b). Creatinine blood levels were stable across groups and remained in the normal range rate over the course of the diabetic disease (Figure 3c), as previously described.[32] Moreover, treatment with IL-17A–neutralizing antibody also markedly diminished renal gene expression of

kim-1 and *ngal* (Figure 3d). All these data suggest that IL-17A neutralization ameliorates renal damage in BTBR ob/ob mice.

As described in this model,[18] blood glucose levels were significantly increased in IgG-treated diabetic mice compared with control mice, showing a significant elevation at 6 weeks of age and progressing to values averaging 598 ± 2 mg/dl ($P < 0.05$ vs. control mice) at the end of the study, that is, at 20 weeks (Figure 3e). In response to IL-17A neutralization, a slight decrease in blood glucose levels was found compared with the IgG-treated diabetic group (540 ± 15 mg/dl vs. 599 ± 2 mg/dl at the end of the study; $P < 0.05$; Figure 3e) but still remained highly elevated compared with control mice. These slight changes in blood glucose levels could be explained by the impairment of systemic inflammation and subsequent improvement in insulin resistance.

Body weight also was affected by treatment. IgG-treated diabetic mice presented a significant increase in body weight compared with control mice at 20 weeks, as expected in this obese model. Interestingly, in response to anti–IL-17A antibody treatment, diabetic mice presented a significant reduction in body weight (Figure 3e). However, there were no differences detected in kidney weight among all the BTBR ob/ob mice groups, whereas this weight was significantly increased compared with control mice (Figure 3f).

Of note, the discrete decrease of both renal weight (6%) and blood glucose levels (11%) during anti–IL-17A treatment does not seem to justify the marked reduction in the proteinuria *per se*. Despite the slight reduction in glucose blood levels, the animals remained diabetic throughout the study.

IL-17A neutralization diminishes renal lesions in DN mice
As previously described, BTBR ob/ob diabetic mice present several renal changes similar to human DN,[18] as noted here in the IgG-treated diabetic mice (Figure 4). Changes in renal structure in response to IL-17A neutralization were studied at 20 weeks. The tissue damage score was calculated as described,[33] including the degree of glomerular sclerosis, increased mesangial matrix, hyalinosis, tubular casts, acute tubular damage, and tubular atrophy, as well as the presence of interstitial inflammatory cells and interstitial fibrosis. Histopathologic analysis of IgG-treated diabetic mice showed a significantly higher kidney damage score compared with control mice, decreasing significantly in the anti-IL-17A–treated mice group (Figure 4a).

Briefly, periodic acid–Schiff staining revealed an increase in mesangial matrix, hyalinosis, and interstitial cellular

Figure 1 | (continued) lymphocytes (CD3, CD4, and γδ), and neutrophils (myeloperoxidase [MPO]). (**a**) Representative images of light microscopy and immunohistochemistry (bars = 20 μm) of at least 3 mice per group. Renal gene expression levels of the proinflammatory marker *monocyte chemoattractant protein-1* (*mcp-1*) (**b**) and of the biomarkers of renal damage *neutrophil gelatinase-associated lipocalin* (*ngal*) (**c**) and *kidney injury molecule-1* (*kim-1*) (**d**) were assessed at different time points. Mice were killed at indicated times (every 2 weeks) and total RNA was extracted from renal tissue. Gene expression levels were evaluated by real-time polymerase chain reaction, and mRNA levels in each sample were normalized by cyclophilin 1. At each time point, data were normalized by the mean value of their corresponding controls. Figures show individual gene expression values of each mouse and the mean $\pm$ SEM of each group. $N = 6$–8 mice per group. *$P < 0.05$ versus control. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
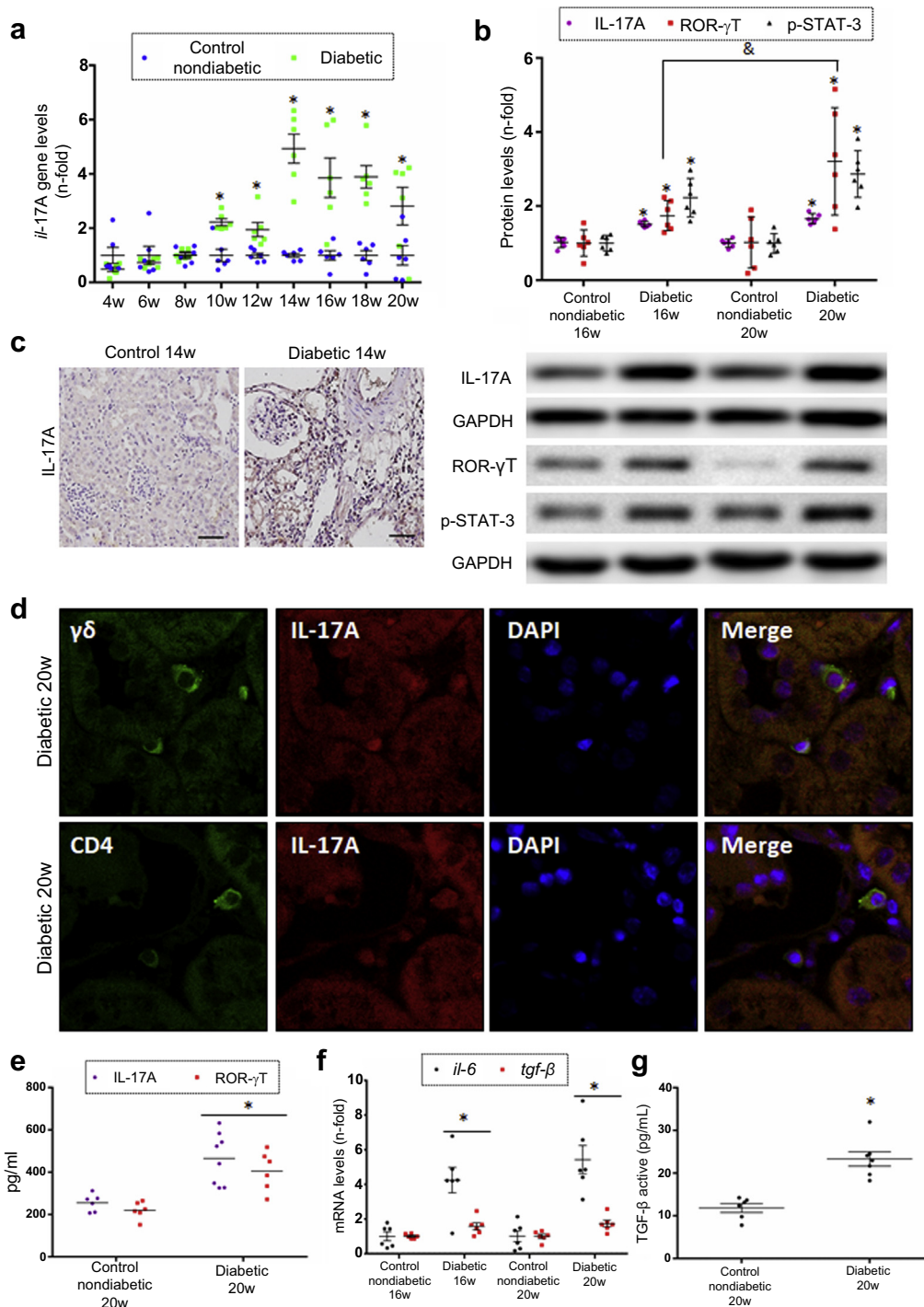
Figure 2 | Increased interleukin (IL)-17A production and activation of Th17-related factors in the kidneys of diabetic mice. (a) In renal samples of BTBR ob/ob mice (diabetic mice) and their corresponding controls, *Il-17A* gene expression levels were studied from 4 to 20 weeks of age. (b) Protein levels of IL-17A and the Th17-related factors, retinoic acid–related orphan receptor (ROR)-γT and phosphorylated signal transducer and activator of transcription 3 (p-STAT-3), were evaluated at indicated times by Western blotting (a representative blot is shown in the upper panel, and densitometric analysis of normalized data is shown below). (c) IL-17A was detected in kidney sections by immunohistochemistry. A representative image of control and diabetic mice (14 weeks) is shown (bars = 20 μm). (d) IL-17A–expressing cells were evaluated by double immunofluorescence in diabetic mice (20 weeks). IL-17A was detected with a secondary Alexa 633 (red), and the different cell types were determined using a specific anti–T-cell receptor γδ antibody (γδ lymphocytes) or anti-CD4 antibody (for CD4+ cells) labeled with a secondary Alexa 488 (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). IL-17A, p-STAT-3 (e), and transforming growth factor (TGF)–β active (g) protein levels were measured by enzyme-linked immunosorbent assay in diabetic and control mice at 20 weeks of age. (f) *Il-6* and *tgf-β* mRNA levels were analyzed at the indicated times. Gene expression levels were evaluated by real-time polymerase chain reaction, and mRNA levels in each sample were (continued)

infiltration in IgG-treated diabetic mice compared with control mice. In response to IL-17A neutralization, the previously described lesions also were noted, but to a lesser extent (Figure 4b). However, at the time point analyzed, interstitial fibrosis was not detected by Masson's trichrome staining in any of the evaluated groups (data not shown).

To further assess glomerular damage, an electron microscopy analysis was carried out. Diabetic kidneys (IgG-treated diabetic group) showed thickening of the glomerular basement membrane, irregular laminations, and localized protrusions in some segments, along with greater effacement of foot processes, thus resembling lesions found in diabetic patients; these changes were not observed in control mice (Figure 4c, d, f, g, i, and j). In response to anti–IL-17A treatment, diabetic mice presented less glomerular basement membrane thickening (Figure 4e and i). Furthermore, the mesangial matrix expansion detected in IgG-treated diabetic mice (Figure 4g and j) was significantly decreased in mice treated with the anti–IL-17A antibody (Figure 4h and j).

IL-17A neutralization restores podocyte number in DN mice

Podocyte damage is a key characteristic of DN. To assess the podocyte number in the glomeruli of diabetic mice, we examined Wilms tumor protein-1 (WT-1) staining, using this as a podocyte marker protein. Of note, in IgG-treated diabetic mice, the podocyte number was strongly decreased compared with control mice (Figure 5a–c), clearly indicating podocyte damage in this model as described in human patients.

In BTBR ob/ob mice, IL-17A neutralization treatment led to a recovery of the number of podocytes when compared with IgG-treated diabetic mice (Figure 5a and 5d). Additionally, IL-17A blockade restored the downregulation of gene expression of several podocyte markers in IgG-treated diabetic mice, including *nephrin (nphs-1), podocin (nphs-2), wt-1,* and *synaptopodin (synpo)* (Figure 5e).

IL-17A blockade inhibits the diabetic-induced renal inflammatory response

In diabetic mice, IL-17A neutralization treatment caused a significant diminution in the number of inflammatory infiltrating cells in the kidney, mainly monocytes/macrophages (F4/80$^+$ cells) and T-lymphocytes (CD3$^+$ and CD4$^+$ cells) when compared with IgG-treated diabetic mice (Figure 6a and b).

Diabetic kidneys presented overexpression of several proinflammatory genes, including chemokines and cytokines, involved in the recruitment of inflammatory cells.[34] In BTBR ob/ob mice, treatment with the neutralizing anti-IL17A antibody markedly diminished proinflammatory gene upregulation compared with its corresponding control (Figure 6c).

Toll-like receptor–4 (TLR-4) promotes tubule-interstitial inflammation in DN and is essential for IL-17A generation.[35,36] Blockade of IL-17A significantly diminished *tlr-4* mRNA upregulation in diabetic kidneys (Figure 6c).

Many signaling pathways are involved in renal inflammation; of these, the nuclear factor-κB signaling pathway (NF-κB) is the most widely studied.[37] Diabetic BTBR ob/ob kidneys presented activation of NF-κB, as described in other experimental models of diabetes and in human DN.[38] Blockade of IL-17A prevented renal NF-κB activation, as shown by downregulation of phosphorylated IκBα and p65 levels, reaching values similar to healthy control mice of the same age (Figure 6d).

These data clearly demonstrate the involvement of IL-17A in renal inflammation in DN, likely due to the modulation of the NF-κB pathway and downstream proinflammatory mediators.

DISCUSSION

Despite the many experimental and clinical studies performed in the field of DN, no currently available treatments can prevent the development and progression of the disease. Recognized therapeutic strategies used in patients with DN, such as strict control of glucose levels and blood pressure, as well as blockade of the renin-angiotensin system, retard the progression of renal damage but offer incomplete protection. BTBR ob/ob mice develop lesions that mimic key features of advanced human DN.[18,39]

Here we have found that a therapeutic regimen consisting of a neutralizing antibody against IL-17A, administered after renal lesions have developed, reversed the structural abnormalities of DN, including mesangial matrix accumulation, glomerular basement membrane thickening, and inflammatory infiltrate. These beneficial effects were temporally associated with discrete better glycemic control. One important finding of our study is that IL-17A blockade elicited a marked decrease in albuminuria (higher than 50% compared with diabetic mice, without treatment) and an improvement in renal lesions. These results suggest that IL-17A blockade could be a potential therapeutic option for DN and a worthwhile approach to testing in a well-designed clinical trial.

IL-17A has been associated with many inflammatory diseases, including psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, lupus, renal allograft rejection, and obesity.[9,40,41] Within the field of kidney diseases, many studies have demonstrated the involvement of Th17 and IL-17A in immune and nonimmune renal diseases.[42–45] In BTBR ob/ob mice, we found a local activation of the Th17 immune response, including activation of

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Figure 2 | (continued) normalized by cyclophilin 1. Protein levels were evaluated in total renal extracts and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values in each sample. At each time point, data were normalized by the mean value of their corresponding controls. Figures show individual gene or protein expression values of each mouse and the mean ± SEM of each group. $N = 6$–8 mice per group. *$P < 0.05$ versus control. $^\&P < 0.05$ versus the 16th week. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
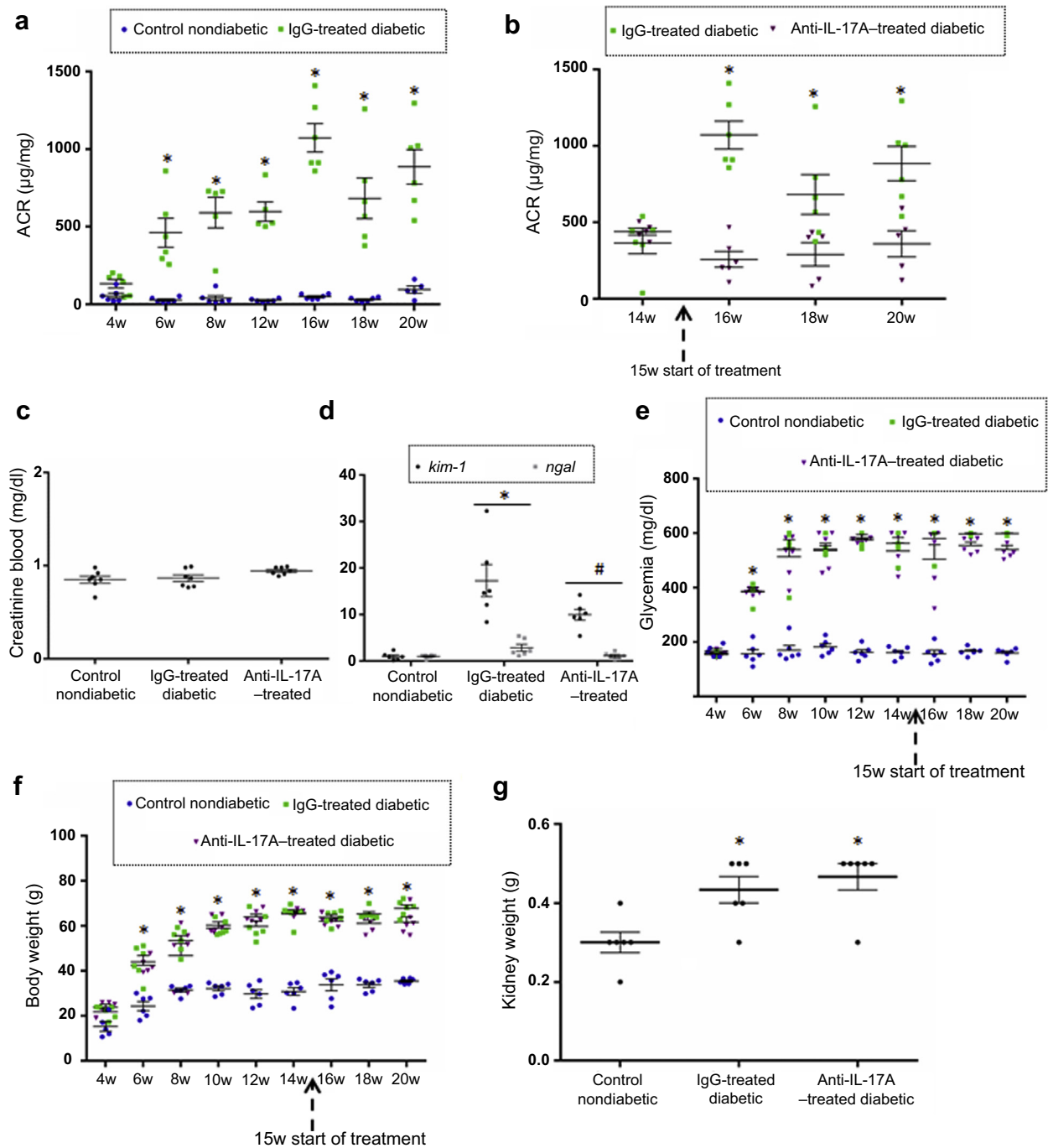
Figure 3 | Effect of interleukin (IL)-17A–neutralization treatment in biochemical parameters in experimental diabetes. BTBR ob/ob mice (diabetic mice) were studied from the 4th to 20th weeks of age, and biochemical time-course determinations were done every 2 weeks. At 15 weeks, mice were randomly distributed into 2 groups: mice were treated with an IL-17A–neutralizing antibody (called anti-IL-17A–treated diabetic) and its corresponding control (IgG-treated diabetic mice) and studied until week 20 (blood and kidney determinations were performed at the time the mice were killed). Arrow indicates treatment initiation time point (week 15). As a control group, nondiabetic mice (BTBR wild-type [WT] mice) also were studied. Evolution of albuminuria, determined as the albuminuria/creatinine ratio (ACR), is shown for each mouse. (**a**) The comparison between control nondiabetic versus diabetic (IgG-treated) mice is shown. (**b**) The effect of IL-17A neutralization treatment in ACR in diabetic mice (comparison of IgG-treated vs. anti-IL-17A–treated). (**c**) Evaluation of creatinine levels in blood. (**d**) Renal expression levels of *kidney injury molecule-1* (*kim-1*) and *neutrophil gelatinase-associated lipocalin* (*ngal*) were determined by quantitative real-time polymerase chain reaction, and normalized versus control mice, as previously described. Evaluation over time of (**e**) glycemia and (**f**) body weight. (**g**) Kidney weight at the final time point. Figures show individual data of each animal and the mean ± SEM of the different groups in each time point, $N = 6–8$ mice per group. *$P < 0.05$ versus control. #$P < 0.05$ versus IgG-treated diabetic.
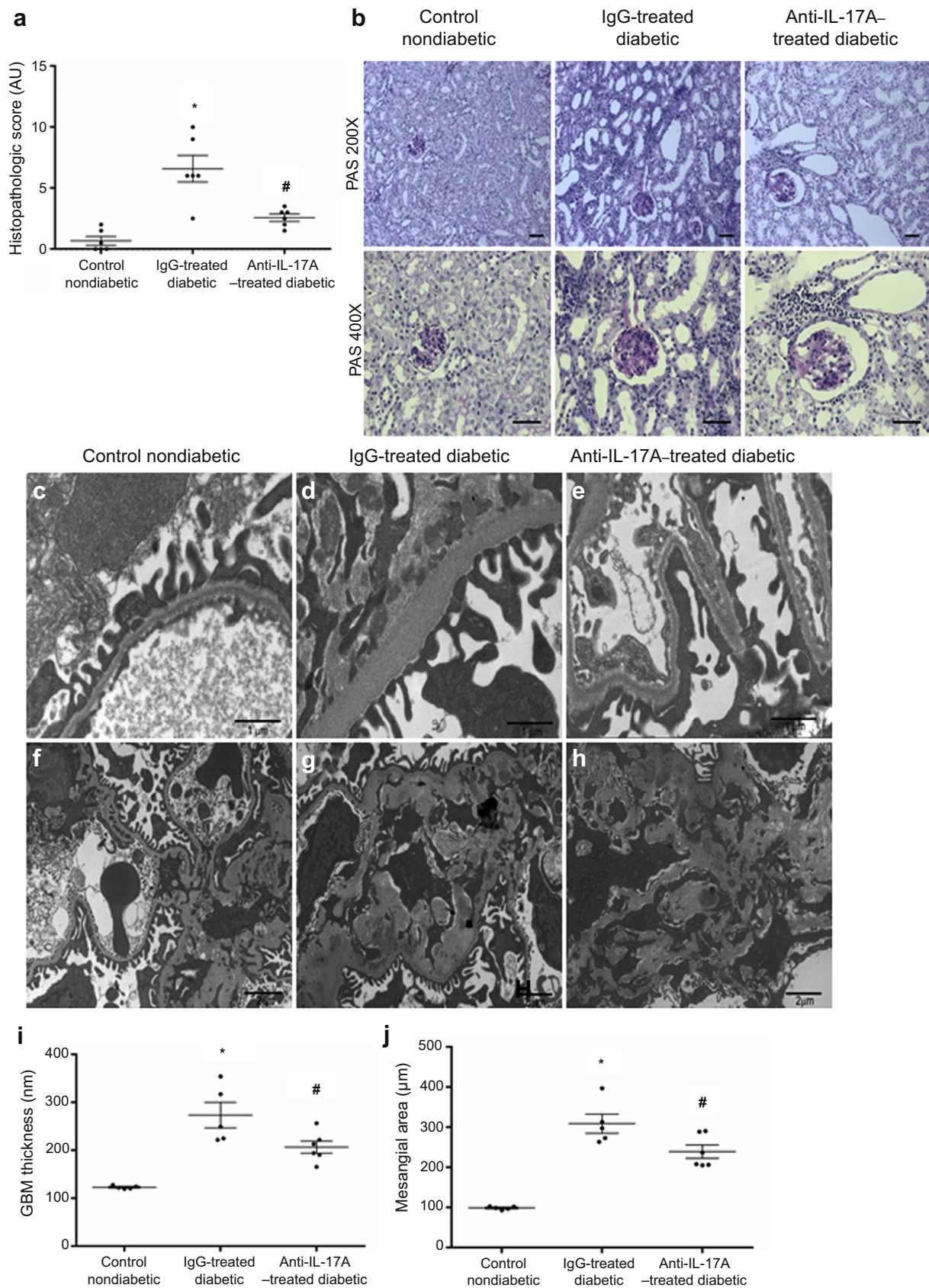
Figure 4 | Effect of interleukin (IL)-17A neutralization treatment on renal lesions in a BTBR ob/ob experimental model. (a) Quantification of histopathologic score at week 20. (**b**) Representative images of light microscopy of kidney sections of (continued)

Th17 differentiation factors and production of IL-17A in diabetic kidneys, associated with the presence of inflammatory cell infiltration and upregulation of proinflammatory mediators, such as MCP-1. Several immune cells can produce IL-17A, including lymphocytes, neutrophils, and mast cells.[9,46–48] In the diabetic kidney we have detected the presence of Th17 (IL-17A^{+}/CD4^{+}) cells, the main source of IL-17A producing cells, and γδ lymphocytes, which also express IL-17A. Previous studies in other models of experimental diabetes also have described activation of the Th17 immune response, as in streptozotocin-induced diabetes,[49,50] confirming our findings.

Many experimental studies have focused on the role of immune cells in experimental type 1 diabetes. The earliest experimental studies in nonobese diabetic mice found increased IL-17A serum levels and an elevated number of pancreatic IL-17A–producing Th17 cells and interferon-γ–producing Th1 cells.[13] In agreement with our results, reduction of IL-17A attenuated DN progression induced by streptozotocin and autoimmune diabetes in nonobese diabetic mice.[49–52] Moreover, blockade of Th17 differentiation by using a selective RORα/γ inverse agonist in nonobese diabetic mice ameliorated diabetes incidence and insulitis and downregulated proinflammatory cytokine overexpression.[53]

The beneficial effect of IL-17A blockade also has been described in other experimental models of immune- and nonimmune-mediated renal damage, suggesting that blocking the Th17/IL-17A axis could be useful in the treatment of inflammatory renal diseases.[30,54–56] Importantly, Th17/IL-17A inhibition ameliorated diabetes-associated damage observed in other target tissues, including the retina.[57,58] However, other experimental studies in diabetes have produced controversial results. Therapy with low doses of recombinant IL-17A prevented and reversed DN,[17] whereas IL-17A knockout mice delayed the onset of immune-mediated type 1 diabetes.[52] Similarly, therapies using cytokines in experimental models of renal injury also have found controversial results, as observed in response to angiotensin-II administration in anti-Thy glomerulonephritis.[59,60] Even though IL-17A has emerged as a crucial regulator of immune response and diseases, its regulation is still poorly understood. Interestingly, in atherosclerosis, a vascular inflammatory disease, IL-17A plays a dual role, carrying out pro- and anti-atherogenic roles,[61] thus supporting such opposite roles of IL-17A in some pathologic conditions.

The loss of podocytes is a key event in human DN[3] that also can be found in BTBR ob/ob diabetic mice and other experimental models. Our study also revealed a significant decrease in podocyte number associated with a down-regulation of podocin (*nphs2*) gene expression, the latter consistent with the podocytopenia. An important finding of our study is that IL-17A blockade restored both the podocyte number and the *nphs2* gene expression levels and induced *de novo* expression of WT-1 (a specific marker of podocytes). This protective effect may be key to the preservation of renal function observed in response to IL-17A–neutralization treatment, suggesting that IL-17A blockade can be used to protect podocytes as part of a therapeutic strategy.

Our current hypothesis is that in diabetic conditions the elevated renal IL-17A production produced by infiltrating immune cells can activate resident renal cells and directly cause kidney damage, mainly inducing the production of additional proinflammatory mediators, which could contribute to the recruitment of inflammatory cells into the diabetic kidney, thereby amplifying the inflammatory response. It has been described that IL-17A can bind to its receptors expressed in tubular, mesangial, and endothelial cells, as well as fibroblasts, inducing the release of cytokines and chemokines such as IL-6, MCP-1, and regulated on activation, T cell expressed, and secreted, among others.[62] Therefore, the beneficial effects of IL-17A–neutralization treatment observed in our study may be due to the blockade of IL-17A proinflammatory actions in the diabetes-induced kidney damage. Many preclinical studies have observed that the blockade of chemokines and cytokines, including MCP-1 and TLR4, ameliorates experimental diabetic renal damage.[63,64] In addition, some of these compounds have been tested in clinical trials, supporting the idea that antiinflammatory therapies could be a feasible option for the treatment of diabetic nephropathy.[65]

Among the mechanisms activated by IL-17A in renal cells, the transcription factor NF-κB has special relevance in human and experimental DN.[9,40,66–69] Numerous drugs used in clinical practice that have protective effects in DN also reduced the NF-κB activation. Moreover, direct inhibition of NF-κB activation by BAY 11-7082 reduced renal injury, inflammation, and hyperglycemia in experimental diabetes.[70]

We found that IL-17A–neutralization treatment blocked NF-κB activation and the subsequent proinflammatory gene upregulation linked to the inhibition of inflammatory cell infiltration in the kidney, suggesting one potential mechanism involved in the beneficial effect of IL-17A blockade in DN. Another important signaling system involved in diabetic renal

Figure 4 | (continued) control mice, IgG-treated diabetic mice, and anti-IL-17A–treated diabetic mice stained with periodic acid–Schiff (PAS). Original magnification: ×200 and ×400. Bars = 20 μm. IL-17A blockade reduces glomerular basement membrane (GBM) thickening and mesangial matrix accumulation. Electron micrographs of glomeruli of representative animals for (**c,f**) control mice, (**d,g**) IgG-treated diabetic mice, and (**e,h**) anti-IL-17A–treated diabetic mice show changes in GBM and mesangial matrix. (**c–e**) Bar = 1 μm, original magnification ×16,500. (**f–h**) Bar = 2 μm, original magnification ×16,500. The quantitative measure of (**i**) GBM thickness and (**j**) mesangial area. Figures show individual histologic score data of each animal and the mean ± SEM of the different groups, N = 6–8 mice per group. *P < 0.05 versus control #P < 0.05 versus IgG-treated diabetic mice. AU, arbitrary units. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
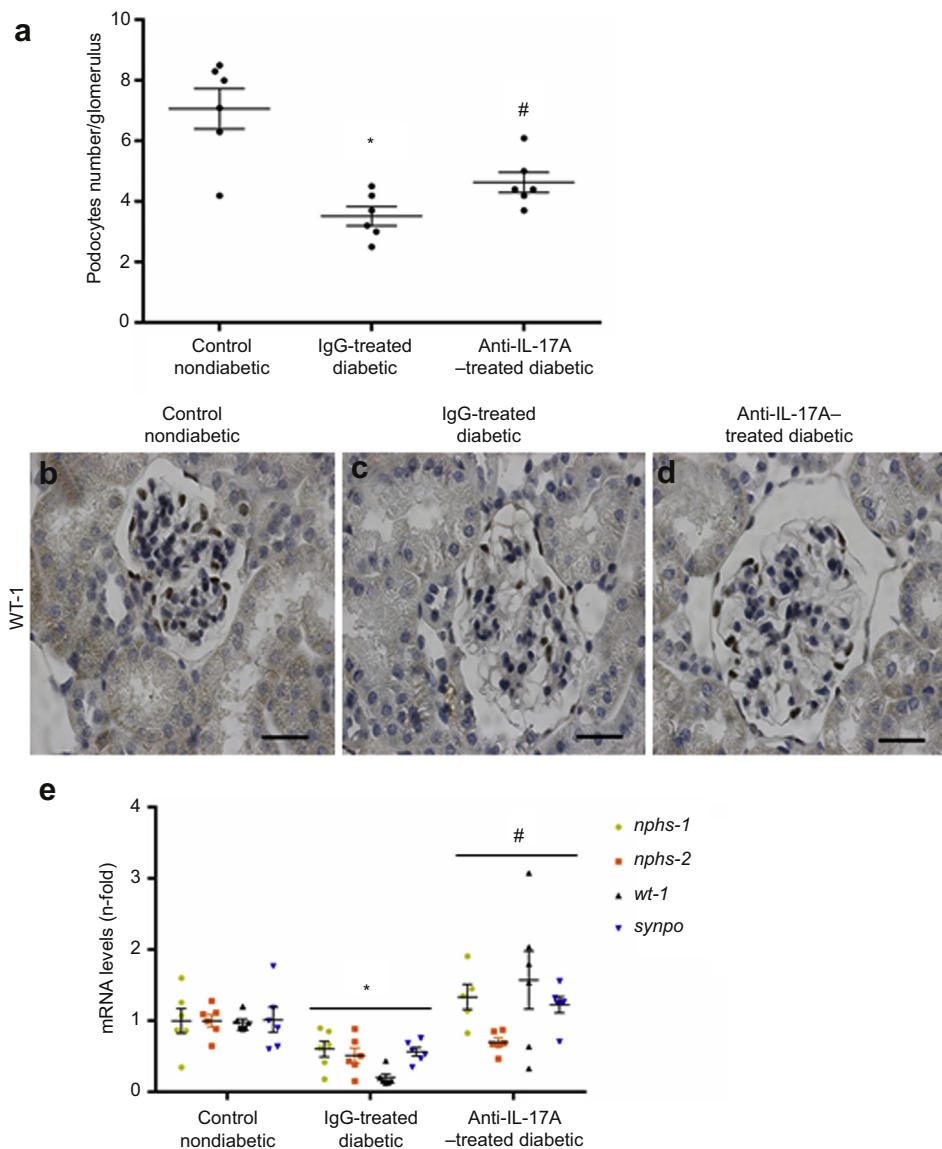
Figure 5 | Podocyte number and gene expression of podocyte markers in BTBR ob/ob experimental model. (a) The average number of podocytes observed of a total of 25 glomeruli per animal, and the mean $\pm$ SEM of control mice, IgG-treated diabetic mice, and anti-interleukin (IL)-17A–treated diabetic groups. Representative images of immunohistochemistry against podocyte marker Wilms tumor protein 1 (WT-1; brown stained nuclei) for (b) control, (c) IgG-treated diabetic, and (d) anti-IL-17A–treated diabetic mice. Original magnification $\times 400$. Bars = 20 μm. (e) Gene expression levels of *nphs1, nphs2, wt-1,* and *synpo* were evaluated by reverse transcriptase–polymerase chain reaction. mRNA levels in each sample were normalized by cyclophilin 1. At each time point, data were normalized by the mean value of their corresponding controls. Figures show individual gene expression values of each mouse and the mean $\pm$ SEM of each group. $N = 6–8$ mice per group. $*P < 0.05$ versus control. $^{\#}P < 0.05$ versus IgG-treated diabetic. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

injury is the STAT pathway. The selective compound targeting STAT3 (nifuroxazide) inhibited hyperglycemia-induced cell responses and ameliorated renal damage in experimental DN.[71] We also observed that blockade of IL-17A diminished STAT3 activation in the diabetic kidney. All these findings support the notion that the beneficial effects of IL-17A neutralization could be due to its antiinflammatory actions, blocking these important signaling pathways involved in the genesis of diabetic renal damage.

Although future studies are needed, our experimental data, which show that IL-17A blockade ameliorated damage

associated with DN, support the concept of IL-17A neutralizing antibody as a promising tool for chronic inflammatory diseases, including chronic kidney diseases.

METHODS
Design of the experimental model
The principles of laboratory animal care were followed, and the mice were killed after administration of anesthesia in accordance with the protocols approved by the Ethics Committee for Animal Experiments of the Universidad Austral de Chile (permit No. 222-2015) and according to National Institutes of Health guidelines. The establishment of BTBR ob/ob animals, diabetic and obese mice
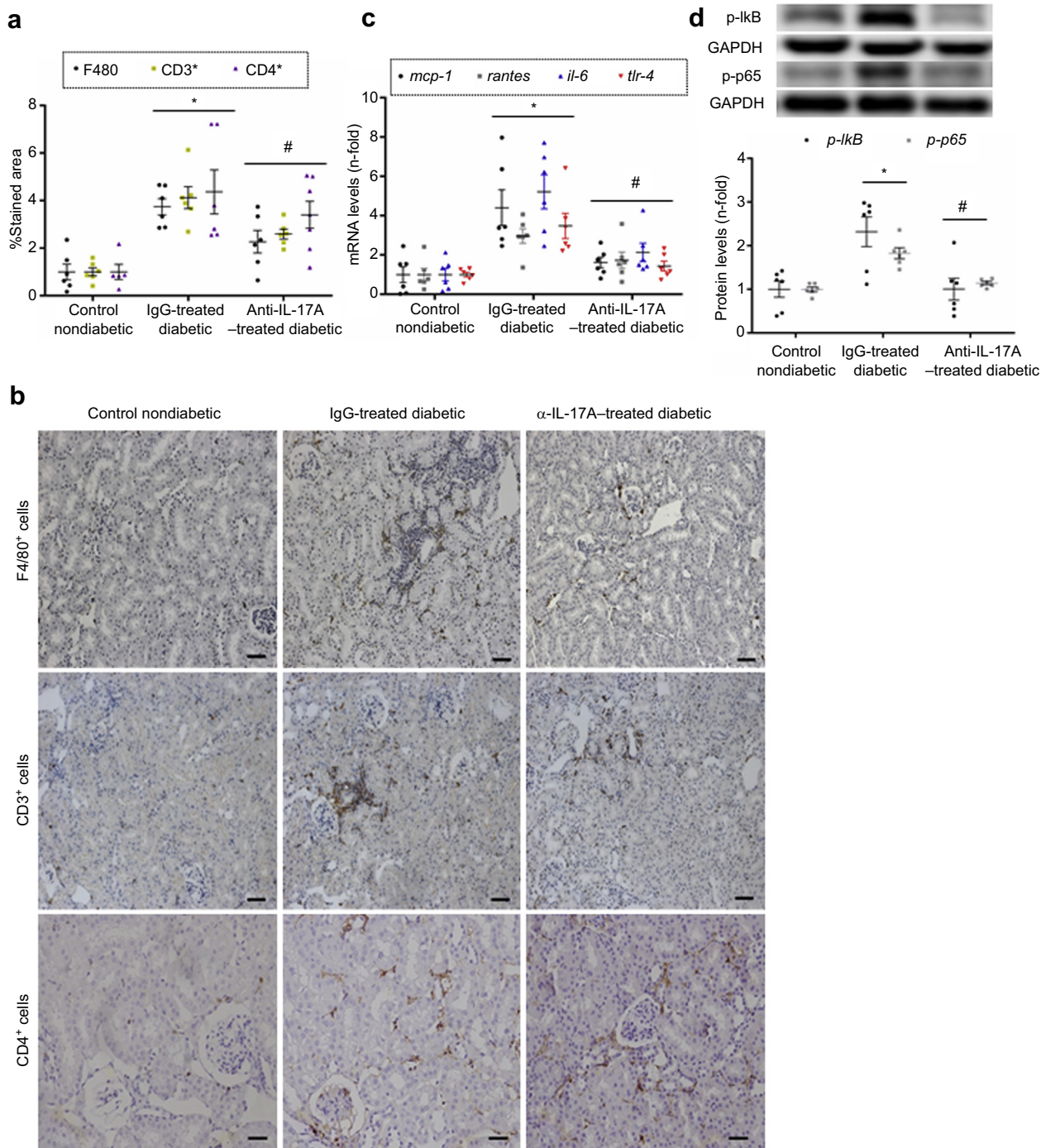
Figure 6 | Interleukin (IL)-17A neutralization inhibited renal-inflammatory responses in the mouse model of diabetic nephropathy.
(**a**) Quantification of F4/80^{+}, CD3^{+}, and CD4^{+} cells as described in the Methods section, as n-fold change of positive staining versus total area, normalized by values of control mice and data of each animal and mean ± SEM. (**b**) Representative images of immunohistochemistry for control, IgG-treated, and anti-IL-17A–treated animals. Original magnification ×200. Bars = 20 μm. (**c**) Renal chemokines mRNA expression (*monocyte chemoattractant protein-1* [*mcp-1*]; *regulated on activation, T-cell expressed, and secreted* [*rantes*]; *Il-6*; and *Toll-like receptor* [*tlr*]*-4*) in each sample, normalized by cyclophilin 1. At each time point, data were normalized by the mean value of their corresponding controls. Figures show individual gene expression values of each animal and the mean ± SEM of each group. *N* = 6–8 mice per group. (**d**) Activation of renal nuclear factor (NF)–κB was evaluated, determining levels of p-IkB and p65 NF-kB subunit by Western blotting (representative immunoblots are shown in the upper panel and the summary of normalized quantification appears below). Figures show individual values of each mouse and are expressed as the mean ± SEM of 6–8 mice per group. *$P < 0.05$ versus control. #$P < 0.05$ versus IgG-treated diabetic mice. GADPH, glyceraldehyde-3-phosphate dehydrogenase. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Table 1 | Western blot antibodies

Antibody	Dilution	Reference
IL-17A	1/500	ab79056
p-STAT3	1/500	#9131 Cell Signaling
ROR-γT	1/500	14-6981 eBioscience
Phosphorylated p65 (Ser536)	1/500	#3033 Cell Signaling
Phosphorylated IκB-α (Ser32)	1/500	sc-8404 Santa Cruz
GAPDH (loading control)	1/5000	MAB374 Chemicon Int

GADPH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; p-STAT3, phosphorylated signal transducer and activator of transcription 3; ROR-γT, retinoic acid–related orphan receptor–γT.

(referred to here as "diabetic mice") and their corresponding controls (BTBR WT litter mate mice) has been previously described.[72] These mice rapidly develop morphologic renal lesions characteristic of both early and advanced human DN.[18]

Characterization of DN throughout the study

Male BTBR ob/ob diabetic mice and corresponding control subjects were killed serially every 2 weeks, starting at week 4 and up until 20 weeks of age ($N = 6$ for each group).

Body weight was checked once a week. At the time the mice were killed, serum was collected and mice were anesthetized with 2% 2,2,2-tribromethanol (Sigma-Aldrich, Darmstadt, Germany) dissolved in 2-methyl-buthanol (Sigma-Aldrich). The kidneys were removed, decapsulated, and cut along the sagittal plane. A portion of the left kidney was fixed in 4% formaldehyde or 2% glutaraldehyde and the right kidney was immediately frozen in liquid nitrogen and processed for RNA and protein extraction.

Once a week, blood glucose levels were checked with Accu-Chek Performa (Roche Diagnostics GmbH, Mannheim, Germany) by caudal vein puncture. Serum creatinine levels were measured by Jaffe reaction (Creatinine LiquiColor, Human Biochemica und Diagnostica GmbH, Wiesbaden, Germany). Spot urine samples were collected once a week from all mice and analyzed for albumin by enzyme-linked immunosorbent assay (ALPCO, Salem, NH) and for creatinine by Jaffe reaction (Creatinine LiquiColor, Human Biochemica und Diagnostica GmbH, Germany) to obtain the urine albumin/creatinine ratio.

IL-17A neutralization model

Neutralizing antibody against murine IL-17A or its corresponding treatment control, the mouse IgG1-K isotype (eBioscience, Vienna,

Austria), were administered to BTBR ob/ob mice via subcutaneous osmotic minipumps (Alza Corp., Mountain View, CA) at a dose of 50 mg/kg/d, as in previous studies[30,72] ($N = 6$–8 animals per group). This experimental approach was chosen to ensure correct antibody dose delivery, because previously described weekly i.p. injection[30,72] could be difficult in this obese model because of the elevated abdominal fat of these mice after 6 weeks. IL-17A neutralization was started in the 15th week of life of the BTBR ob/ob mice, when mice already have developed the characteristic lesions of DN (therapeutic approach), and treatment was continued for 5 weeks. For neutralization experiments, BTBR ob/ob (diabetic mice) were studied as of 4 weeks of age, and at 15 weeks the mice were randomly distributed among 2 groups: one treated with an anti–IL-17A neutralizing antibody ("anti-IL-17A–treated") and the other with IgG1-K isotype ("IgG-treated") and studied at week 20 ($N = 6$–8 mice per group). In some experiments, these mice were compared with untreated BTBR ob/ob mice or control mice of the same age.

Protein studies

Total proteins from frozen renal tissues were isolated in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL). Protein levels were quantified using a Pierce BCA protein assay kit (Thermo Scientific). Renal protein levels were evaluated by Western blot analysis. Proteins (100 μg/lane) were separated on 10% to 12% polyacrylamide–sodium dodecylsulfate gels under reducing conditions. Protein quality and efficacy of transfer were evaluated by Ponceau red staining (data not shown). Primary antibodies (Table 1) were detected with a horseradish peroxidase–conjugated secondary antibody, developed by Luminata Forte (MilliporeSigma, Billerica, MA), and scanned using the G:BOX Chemi XRQ (Syngene, Frederick, MD). Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control.

IL-17A (eBioscience), pSTAT3 (Invitrogen, Rockford, IL), and TGF-β active (BioLegend, San Diego, CA) renal protein levels were evaluated by enzyme-linked immunosorbent assay.

Histologic analysis and immunohistochemistry

The specimens fixed in 4% formaldehyde were embedded in paraffin and cut into 4-μm tissue sections for further histologic (periodic acid–Schiff/Masson) and immunohistochemistry studies. The tissue fixed in 2% glutaraldehyde (Merck KGaA, Darmstadt, Germany) was postfixed with 1% osmium tetroxide (Ted Pella Inc., Hedding, CA) included in resin EMbed 812 (Electron Microscopy Sciences,

Table 2 | Primers used for quantitative polymerase chain reaction

Gene	Forward	Reverse
il-17A	5'-TCTCCACCGCAATGAAGACC-3'	5'-GACCAGGATCTCTTGCTGGA-3'
ngal	5'-GCCCTGAGTGTCATGTGTCT-3'	5'-GAACTGATCGCTCCGGAAGT-3'
kim-1	5'-TGTCGAGTGGAGATTCCTGGATGGT-3'	5'-GGTCTTCCTGTAGCTGTGGGCC-3'
mcp-1	5'-AGCTCTCTCTTCCTCCACCA-3'	5'-GGCGTTAACTGCATCTGGCT-3'
rantes	5'-AGAGGACTCTGAGACAGCACA-3'	5'-CGAGCCATATGGTGAGGCAG-3'
il-6	5'-CCCCAATTTCCAATGCTCTCC-3'	5'-CGCACTAGGTTTGCCGAGTA-3'
tlr-4	5'-CTGGTTGCAGAAAATGCCAGG-3'	5'-TCATCAGGGACTTTGCTGAGTT-3'
nphs1	5'-AGGGTCGGAGGATCGAA-3'	5'-GGGAAGCTGGGGACTGAAGT-3'
nphs2	5'-CCAAAGTGCGGGTGATTGC-3'	5'-TGA TGC TCC CTT GTG CTC TG-3'
wt-1	5'-CAGCGAAAGTTTTCCCGGTC-3'	5'-TGTTGTGATGGCGGACCAAT-3'
synpo	5'-TCTCCTCGAGCCAAGCA-3'	5'GAGAAGGGGACAAGACAGGC-3'
cyc	5'-GGCAATGCTGGACCAAACACAA-3'	5'-GTAAAATGCCCGCAAGTCAAAG-3'

cyc, cyclophilin; il, interleukin; kim-1, kidney injury molecule-1; mcp-1, monocyte chemoattractant protein-1; ngal, neutrophil gelatinase-associated lipocalin; nphs1, nephrin; nphs2, podocin; rantes, regulated on activation, T cell expressed, and secreted; synpo, synaptopodin; tlr, Toll-like receptor; wt-1, Wilms tumor protein-1.

Hatfield, PA), cut, stained, and observed under a Philips Tecnai 12 BioTWIN electron microscope (Philips Research, Eindhoven, The Netherlands) at 80 kV.

Glomerular and tubulo-interstitial lesions were graded according to their histopathologic score (from 0 to 4) as previously described.[33] Immunohistochemistry for detection of WT-1 as a podocyte marker was performed with use of a heat-induced antigen retrieval system (Tris-base 10 mM, ethylenediamine tetraacetic acid 1 mM, 0.05% Tween 20, pH 9.0) for 10 minutes in a microwave oven. Sections were incubated overnight with Monoclonal Mouse Anti WT1 protein Clone 6F-H2, M3561 (dilution: 1:100; Dako, Carpinteria, CA) followed by incubation with the Mouse on Mouse (M.O.M.) Immunodetection Kit (PK 2200, Vector Laboratories, Burlingame, CA) and ImmPACT DAB Peroxidase Substrate (Vector Laboratories).

IL-17A and interstitial infiltrating cells were detected by means of IL-17A, F4/80 (monocytes/macrophages) and CD3 (T lymphocytes), CD4 (T-helper lymphocytes), γδ lymphocytes and myeloperoxidase (neutrophils) antibodies. F4/80 was detected with use of the MA1-91124 antibody (dilution: 1/100, Thermo Scientific) followed by ImmPRESS Reagent Kit (MP 7444, Vector Laboratories). IL-17A (ab79056 antibody, dilution: 1/100, Abcam, Cambridge, UK) and CD3 (A 0452 antibody, dilution: 1:200, Dako) were detected using Trilogy epitope retrieval (Cell Marque Corp., Rocklin, CA) and followed by horseradish peroxidase streptavidin (dilution 1:500, SA-5004, Vector Laboratories), revealed with DAB SK4105 (Vector Laboratories), and counterstained with hematoxylin. Gamma-delta lymphocytes (dilution: 1:250; BioLegend), myeloperoxidase (A0398 antibody, dilution: 1:1000, Dako), and CD4 (IS649 antibody, ready to use) staining were performed using the Dako Autostainer (Dako), as described previously.[31] First, endogenous peroxidase was blocked and then sections were incubated for 30 minutes at room temperature or overnight at 4° C with primary antibodies. Slides were then treated with the EnVision DuoFLEX Doublestain System (Agilent Technologies, Santa Clara, CA) using 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with Carazzi's hematoxylin and evaluated by optical microscopy.

Image analysis and quantification of the immunohistochemistry signals were performed using the KS300 imaging system, version 3.0 (Zeiss, Oberkochen, Germany). For each sample, the mean staining area was obtained by analysis of 20 randomly chosen fields (original magnification ×200) using Image-Pro Plus software (Media Cybernetics, Rockville, MD). Data are expressed as positive-stained area compared with total area analyzed.

Podocyte involvement was calculated by enumerating podocyte nuclei stained for WT-1–positive glomerular cells by counting stained cells by immunohistochemistry in 25 glomeruli.

For double immunofluorescence staining, primary antibodies were followed by their corresponding anti-IgG Alexa488-conjugated or Alexa633-conjugated secondary antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Samples were mounted in ProLong Gold antifade reagent (Invitrogen) and examined using a Leica DM IRB confocal microscope (Leica, Wetzlar, Germany).

Gene expression studies

Total RNA from renal tissue was isolated with TRIzol reagent (Invitrogen) according to the method provided by the manufacturer and treated with DNase I to remove potential contamination with genomic DNA. cDNA was synthesized by the ImProm-II Reverse Transcription System (Promega Corp., Madison, WI) using 2 μg of total RNA primed with random hexamer primers. Quantitative gene expression analysis was performed on a Rotor-Gene Q (Qiagen,

Hilden, Germany) using primers designed by Integrated DNA Technologies (Coralville, IA) and the reagent KAPA SYBR FAST qPCR Kit Master Mix (2X) (Kapa Biosystems, Wilmington, MA) to determine the expression of genes of interest. Primers used for quantitative polymerase chain reaction are shown in Table 2. Polymerase chain reaction product specificity was verified by melting curve analysis, and all real-time polymerase chain reactions were performed in triplicate. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression levels.

Statistical analysis

Results throughout the text are expressed as the n-fold increase over control subjects (mean ± SEM). Differences between groups were assessed with the Kruskal-Wallis test. Statistical significance was assumed when a null hypothesis could be rejected at $P < 0.05$. Statistical analysis was performed using SPSS statistical software, version 16.0 (IBM Corp., Armonk, NY).

AUTHOR CONTRIBUTIONS
CL contributed to the conception, design, and performance of the experiments; acquisition, analysis, and interpretation of all data; drafting of the manuscript; and in securing financial support for the study. YSM contributed to the performance of the experiments and the acquisition and analysis of data. MO contributed to the analysis and interpretation of data.

JDC contributed to the analysis and interpretation of data. AD contributed to the performance of the experiments. JE contributed to interpretation of the data, the critical review of the manuscript, and in securing financial support for the study. SM contributed to interpretation of the data, the critical review of the manuscript, and in securing financial support for the study. MR-O contributed to the design of the experiments, analysis and interpretation of the data, drafting of the manuscript, and in securing financial support for the study. All authors reviewed the manuscript and approved the final version to be published.

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