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ORIGINAL RESEARCH

# Tubular IL-1β Induces Salt Sensitivity in Diabetes by Activating Renal Macrophages

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**BACKGROUND:** Chronic renal inflammation has been widely recognized as a major promoter of several forms of high blood pressure including salt-sensitive hypertension. In diabetes, IL (interleukin)-6 induces salt sensitivity through a dysregulation of the epithelial sodium channel. However, the origin of this inflammatory process and the molecular events that culminates with an abnormal regulation of epithelial sodium channel and salt sensitivity in diabetes are largely unknown.

**METHODS:** Both in vitro and in vivo approaches were used to investigate the molecular and cellular contributors to the renal inflammation associated with diabetic kidney disease and how these inflammatory components interact to develop salt sensitivity in db/db mice.

**RESULTS:** Thirty-four-week-old db/db mice display significantly higher levels of IL-1β in renal tubules compared with nondiabetic db/+ mice. Specific suppression of IL-1β in renal tubules prevented salt sensitivity in db/db mice. A primary culture of renal tubular epithelial cells from wild-type mice releases significant levels of IL-1β when exposed to a high glucose environment. Coculture of tubular epithelial cells and bone marrow-derived macrophages revealed that tubular epithelial cell-derived IL-1β promotes the polarization of macrophages towards a proinflammatory phenotype resulting in IL-6 secretion. To evaluate whether macrophages are the cellular target of IL-1β in vivo, diabetic db/db mice were transplanted with the bone marrow of IL-1R1 (IL-1 receptor type 1) knockout mice. db/db mice harboring an IL-1 receptor type 1 knockout bone marrow remained salt resistant, display lower renal inflammation and lower expression and activity of epithelial sodium channel compared with db/db transplanted with a wild-type bone marrow.

**CONCLUSIONS:** Renal tubular epithelial cell-derived IL-1β polarizes renal macrophages towards a proinflammatory phenotype that promotes salt sensitivity through the accumulation of renal IL-6. When tubular IL-1β synthesis is suppressed or in db/db mice in which immune cells lack the IL-1R1, macrophage polarization is blunted resulting in no salt-sensitive hypertension.

**GRAPHIC ABSTRACT:** A graphic abstract is available for this article.

**Key Words:** epithelial sodium channels ■ diabetic kidney disease ■ hypertension ■ inflammation ■ macrophages

Editorial, see p 74

Hypertension is twice as prevalent in patients with diabetes compared with nondiabetic populations and is a major risk factor for the progression of diabetic kidney disease (DKD).[1] A high-salt diet has been considered an important contributor to hypertension during diabetes. Specifically, type 2 diabetes has been associated with increased renal sodium reabsorption and volume expansion that predisposes to salt-sensitive hypertension.[2] The renin-angiotensin system, mainly through Ang II (angiotensin II) and aldosterone secretion, promotes a sodium retentive state by activating several sodium transporters along the nephron.[3–5] However, research has shown that whole-kidney Ang II levels can increase, decrease, or remain unchanged during DKD,[6–12] and no evidence of systemic renin-angiotensin system activation has been found in diabetes.[13,14] This suggests that other mechanisms might impair the sodium homeostasis during diabetes.

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## Novelty and Significance

### What Is Known?

- Chronic renal inflammation associated with diabetes has been widely recognized as a major promoter of salt-sensitive hypertension.
- The origin of this inflammatory process and the molecular events that culminate with an abnormal regulation of sodium and the development of salt sensitivity are still unknown.

### What New Information Does This Article Contribute?

- Renal tubular epithelial cells, and not immune cells, are a major source of IL (interleukin)-1β in diabetic kidney disease and specific tubular IL-1β suppression in vivo prevents salt sensitivity in db/db diabetic mice.
- Tubular epithelial cell-derived IL-1β promotes the polarization of macrophages towards a proinflammatory phenotype resulting in IL-6 secretion, a major promoter of sodium retention and salt sensitivity during diabetes.
- Diabetic db/db mice in which immune cells cannot respond to IL-1β (IL-1 receptor type 1 knockout) display reduced renal inflammation and no salt sensitivity.

Renal inflammation is a contributor to the development of diabetic kidney disease and hypertension in both humans and animal models. However, the origin of this inflammatory process remains unknown. In this study, we identify IL-1β as an initial promoter of the renal inflammatory response associated with diabetes. Indeed, we observe that renal tubular epithelial cells, and not immune cells, are the major source of IL-1β in response to a high glucose environment. We also establish a molecular connection between nonimmune and immune cells within the kidney during diabetes. Indeed, tubular cell-derived IL-1β polarizes macrophages towards a proinflammatory phenotype that further contributes to expand the inflammation associated with diabetic kidney disease. Two approaches were used to assess the in vivo significance of these observations: (1) we specifically suppressed IL-1β expression in tubular cells of diabetic (db/db) mice, and (2) we used bone marrow transplantation to create a db/db mouse in which immune cells lack the IL-1R1. These mice displayed less abundance of proinflammatory macrophages in the kidney and, when exposed to a high-salt diet, did not develop salt sensitivity. In total, we propose a novel molecular mechanism behind the development of renal inflammation and salt sensitivity during diabetes.

## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **Ang II** | angiotensin II |
| **DKD** | diabetic kidney disease |
| **ELISA** | enzyme-linked immunoassay |
| **ENaC** | epithelial sodium channel |
| **IL-1R1** | IL-1 receptor type 1 |
| **IL-1R1KO** | IL-1R1 knockout |
| **IL-1β** | interleukin 1β |
| **IL-6** | interleukin 6 |
| **NLRP3** | NLR family pyrin domain containing 3 |
| **SGK-1** | serum- and glucocorticoid-induced kinase 1 |

Research has shown that a chronic low-grade inflammatory response in the kidney contributes to the pathogenesis of DKD.[15] These renal inflammatory processes play a critical role in the abnormal sodium handling and development of hypertension in both human and animal models of diabetes.[16] The accumulation of inflammatory mediators, such as cytokines and reactive oxygen species, causes damage and promotes renal dysfunction that ultimately, lead to hypertension.[17] Indeed, proinflammatory cytokines, such as IL (interleukin)-1β, IL-6, or IL-17A, either directly or indirectly, activate several sodium transporters in the kidney and play a critical role in sodium retention and hypertension.[18–20] In line with these findings, recent studies from our laboratory have demonstrated that IL-6 is a critical promoter of salt sensitivity during diabetes.[21] Specifically, reducing the IL-6 levels prevented the development of salt-sensitive hypertension in db/db mice, a model of type 2 diabetes.[21] Nonetheless, despite abundant evidence supporting a causative role of inflammation in the progression of kidney injury and hypertension associated with diabetes, the origin of this inflammatory process remains elusive.

In this study, we observed that tubular epithelial cells are a major source of IL-1β in diabetic db/db mice. The specific suppression of IL-1β in renal tubules prevented salt sensitivity in db/db mice. A primary culture of renal tubular epithelial cells releases IL-1β when exposed to a high glucose environment. Coculture of tubular epithelial cells and bone marrow-derived macrophages revealed that tubular epithelial cell-derived IL-1β promotes the polarization of macrophages towards a proinflammatory phenotype resulting in IL-6 secretion. To evaluate whether macrophages are the cellular target of IL-1β in vivo, we created a mouse in which immune cells cannot respond to IL-1β. For this, diabetic db/db mice were transplanted with the bone marrow of IL-1R1 (IL-1 receptor type 1)

knockout (IL-1R1KO) mice. This receptor mediates most of the inflammatory effects of IL-1β.[22] We observed that db/db mice harboring an IL-1R1KO bone marrow remained salt resistant, display lower renal inflammation and lower expression and activity of the epithelial sodium channel (ENaC) compared with db/db mice transplanted with a wild-type bone marrow. Thus, the activation of renal macrophages by tubular epithelial cell-derived IL-1β emerges as an initial event in the inflammatory process that dysregulates renal sodium transporters and predisposes to salt sensitivity during diabetes.

# METHODS

## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

The detailed methods section is available in the Supplemental Material.

## Mice and Study Design

All animal procedures were approved by the Cedars-Sinai Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on db/db male mice (B6.BKS(D)-Lepr$^{db}$/J, RRID: IMSR_JAX:000697), a mouse model of obesity and type 2 diabetes, and their respective db/+ (heterozygous) controls purchased from Jackson Laboratories (Bar Harbor, ME). Given that female db/db mice do not develop salt-sensitive hypertension,[21] only male mice were used in this study. Power calculations for the number of mice used in each experimental group were based on similar results from previous studies.[21] Mice were treated humanely, and all efforts were made to minimize the animals suffering and the number of mice used in the study. All results are reported in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Mice were randomly assigned to each experimental group by flipping a coin. Animals were maintained under controlled light and temperature conditions and had free access to water.

# RESULTS

## Diabetic Mice Display Increased Levels of IL-1β in Tubular Epithelial Cells

The renal levels of IL-1β were assessed by enzyme-linked immunoassay (ELISA) in cortex and medulla isolated from 34-week-old db/+ and db/db mice. Cortical IL-1β was significantly higher in db/db compared with db/+ mice (Figure 1A). No significant differences were observed in medulla (Figure 1B). Further assessment of IL-1β levels was performed by flow cytometry analysis of a cell suspension prepared from kidney samples of db/+ and db/db mice.[23] The expression of IL-1β in E-cadherin$^+$ (tubular epithelial cell marker) cells was significantly higher in db/db compared with db/+ mice (Figure 1C). However, IL-1β levels in renal macrophages (CD45$^+$F4/80$^+$) were similar between db/+ and db/db mice (Figure 1D). Immunofluorescence analysis also revealed higher levels of renal IL-1β in db/db compared with db/+ mice in cortex (Figure 1E). In line with flow cytometry data, IL-1β staining colocalized with E-cadherin indicating that tubular epithelial cells produce IL-1β during diabetes. No significant differences in the expression of IL-1β were observed in glomeruli and renal medulla (Figure 1E and Figure S5A). To test the specificity of IL-1β staining, a cortical sample from a db/db mouse was incubated with the primary anti-IL-1β antibody preabsorbed with pure recombinant IL-1β (Figure S5B). The specific staining of the anti-mouse IgG antibody used to detect IL-1β was confirmed by removing the primary antibody (Figure S5C). The specific staining of the anti-goat IgG antibody used to detect E-cadherin was confirmed by removing the primary antibody (Figure S5D). We also analyzed the levels of IL-1β by ELISA in cortical tubules, glomeruli, total medulla, lymphocytes (CD45$^+$CD3$^+$), and myeloid cells (CD45$^+$CD11b$^+$) isolated from the kidney of db/+ and db/db mice. We observed higher levels of IL-1β in cortical tubules of db/db compared with db/+. Low levels of IL-1β were also observed in myeloid cells, although no differences were observed between cells isolated from diabetic and non-diabetic mice. No detectable or very low levels of IL-1β were measured in glomeruli, medulla, and lymphocytes (Figure S6). In total, these data suggest that tubular epithelial cells are a major source of IL-1β during diabetes.

## Tubular Epithelial Cells Release IL-1β in Response to High Glucose

To get further insight into the mechanism of IL-1β production by renal cells, a primary tubular epithelial cell culture was established from wild-type mice and exposed to either low (5 mmol/L) or high (15 mmol/L) D-glucose for 72 hours. For osmotic control, additional groups of tubular cells were exposed to 5 mmol/L D-glucose plus 10 mmol/L mannitol. Pro-caspase-1, caspase-1, and NLRP3 (NLR family pyrin domain containing 3), key components of the NLPR3 inflammasome that mediates the synthesis of IL-1β, were analyzed by Western blot. Cells exposed to high D-glucose displayed significantly higher levels of caspase-1 and lower levels of pro-caspase-1 compared with cells cultured under low D-glucose conditions (Figure 2A through 2C). This suggests an increased cleavage and activation of caspase-1 in response to D-glucose. Higher levels of caspase-1 were associated with increased NLRP3 expression in high D-glucose-treated cells (Figure 2A and 2D). Finally, IL-1β levels in the culture media, assessed by ELISA, were significantly higher in cells exposed to high D-glucose compared with those exposed to low D-glucose concentration (Figure 2E). No changes in caspase-1, NLRP3, or IL-1β

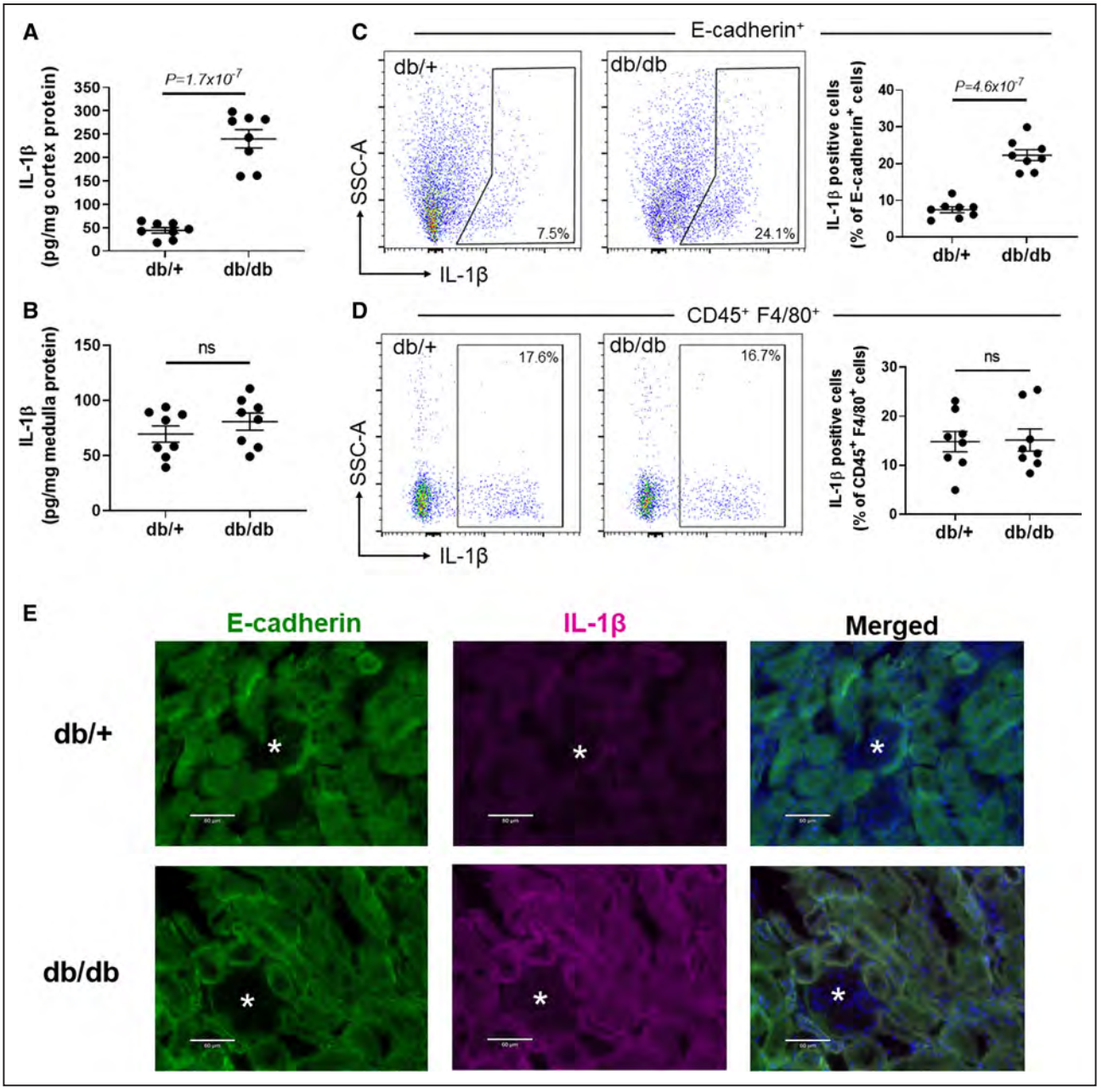


**Figure 1. Increased levels of IL (interleukin)-1β in kidney of db/db mice.**
IL-1β was evaluated in homogenates from kidney (**A**) cortex and (**B**) medulla of 34-wk-old db/+ and db/db mice by enzyme-linked immunoassay (ELISA). Results are expressed as mean pg of IL-1β per mg of tissue protein±SD. Analysis by unpaired Student *t* test, n=8 per group. **C** and **D**, Kidney cell suspensions from db/+ and db/db mice were prepared. Live cells were identified using a LIVE/DEAD Fixable Yellow staining. For surface marker staining, cells were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific antibody binding. To evaluate whether macrophages or renal epithelial cells produce IL-1β, cells were stained with antibodies anti-CD45-Pacific blue, anti-F4/80-FITC (fluorescein isothiocyanate), and anti-E-cadherin-PE (phycoerythrin). After fixation and permeabilization cells were stained with an anti-IL-1β followed by a secondary anti-rabbit IgG1-APC (allophycocyanin) antibody. Results are expressed as mean percentage of IL-1β$^+$ cells per E-cadherin$^+$ or CD45$^+$F4/80$^+$ cells±SD. Analysis by unpaired Student *t* test, n=8 per group. **E**, Immunofluorescence staining of IL-1β in kidney samples of db/+ and db/db mice. Scale bar=60μm. The white asterisk indicates a glomerulus. SSC-A indicates side scatter area.

were observed in cells exposed to D-mannitol negating an osmotic effect (Figure 2A through 2E). The synthesis of IL-1β by tubular epithelial cells was confirmed by flow cytometry. We observed that tubular epithelial cells (E-cadherin$^+$) produce higher levels of IL-1β in response to high D-glucose compared with cells exposed to low D-glucose or mannitol (Figure 2F).

## Tubular Epithelial Cell-Derived IL-1β Can Induce a Proinflammatory Phenotype in Macrophages

To evaluate whether IL-1β derived from tubular epithelial cells can activate macrophages, we established a direct cell-cell coculture of wild-type tubular cells and wild-type bone marrow-derived macrophages. Again,

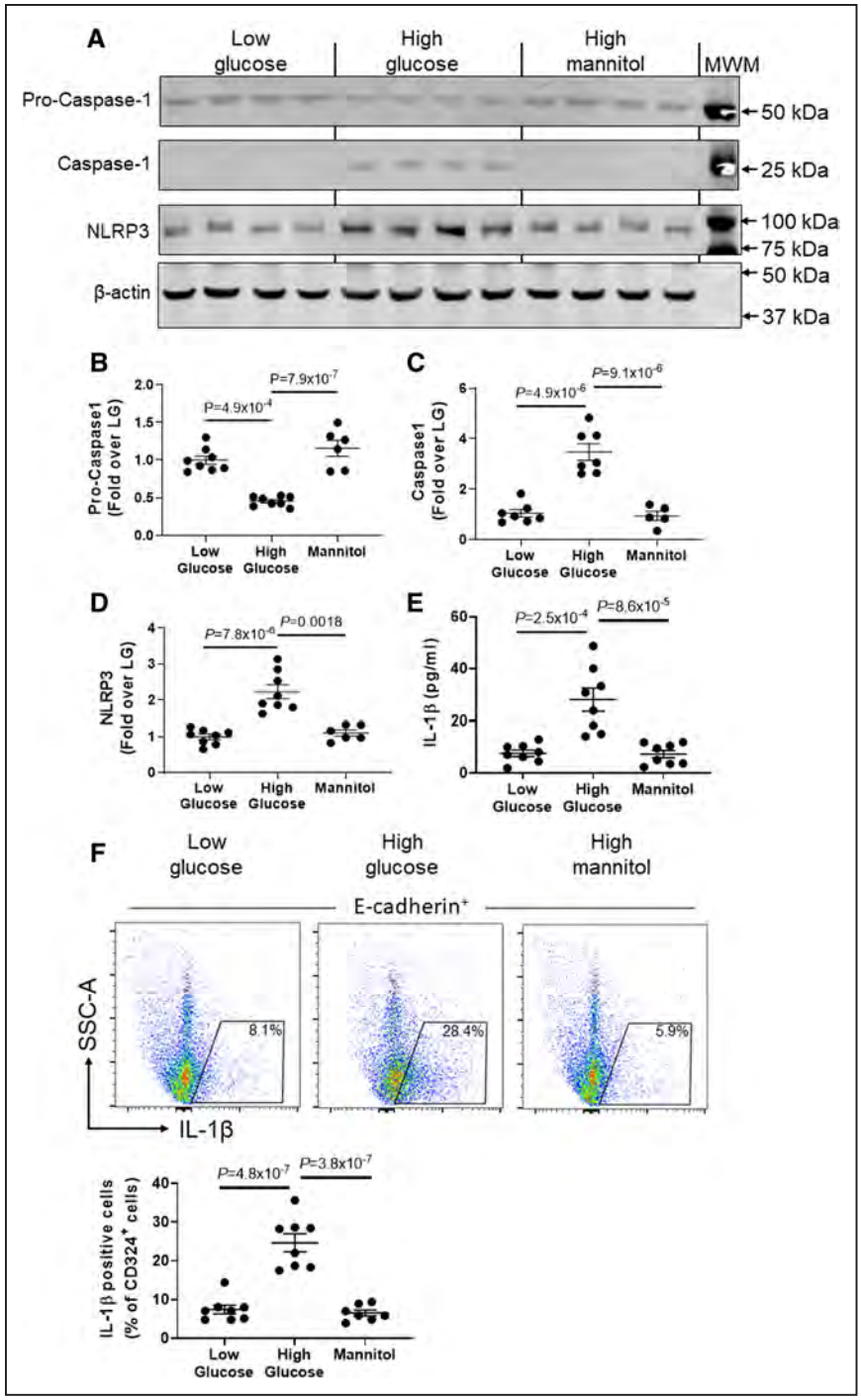


**Figure 2. Tubular epithelial cells exposed to high glucose release IL (interleukin)-1β.**
Primary tubular epithelial cells were cultured from collagenase-digested cortical fragments of kidneys isolated from 4-wk-old wild-type male mice. After 7 d, cells were organized as a confluent monolayer. During the last 72 h of cell culture, cells were stimulated with culture media containing 15 mmol/L glucose. Control cells were maintained in 5 mmol/L glucose. Cells stimulated with 5 mmol/L glucose and 10 mmol/L mannitol were used as osmotic controls. At the end of the experiment, **A**–**D**, (pro)-caspase-1 and NLRP3 (NLR family pyrin domain containing 3) were assessed by Western blot in cell homogenates. Data are expressed as mean±SD. Analysis by 1-way ANOVA followed by Tukey multiple comparisons test. Low glucose: n=8, high glucose: n=8, and mannitol: n=6. Representative blots are displayed with the closest molecular weight markers indicated in the rightmost band. Uncropped blots are displayed in the Supplemental Material. **E**, IL-1β levels were measured in the culture supernatant by enzyme-linked immunoassay (ELISA). Data are expressed as mean±SD. Analysis by 1-way ANOVA followed by Tukey multiple comparisons test. n=8 per group. **F**, IL-1β was also evaluated by flow cytometry using E-cadherin as a marker of tubular epithelial cells as described in Figure 1. Data are expressed as mean±SD. Analysis by 1-way ANOVA followed by Tukey multiple comparisons test. Low glucose: n=8, high glucose: n=8, and mannitol: n=7. SSC-A indicates side scatter area.

cells were exposed to either low or high D-glucose for 72 hours. Control cells were treated with mannitol. Cells were resuspended and stained with antibodies against the surface markers CD45, F4/80, CD80 (proinflammatory), and CD206 (anti-inflammatory) macrophage markers.[24] Macrophages cocultured with tubular cells under a high D-glucose condition display a significantly higher abundance of $CD80^+$ macrophages ($CD45^+$, $F4/80^+$, $CD80^+$, and $CD206^-$), less $CD206^+$ macrophages ($CD45^+$, $F4/80^+$, $CD80^-$, and $CD206^+$), and higher $CD80^+/CD206^+$ ratio compared with wild-type macrophages cocultured with renal tubular cells under a low D-glucose condition (Figure 3A). No effect was observed when D-glucose was substituted by mannitol (Figure 3A). The $CD80^+$ macrophage polarization induced by high D-glucose was blunted in the presence of an anti-IL-1β neutralizing antibody (Figure 3B). Similarly, no $CD80^+$ polarization was observed when the experiment was performed using macrophages lacking the IL-1R1 derived from IL-1R1KO mice (Figure 3C). We observed that high D-glucose induced a mild increase of CD80 expression in wild-type macrophages even in

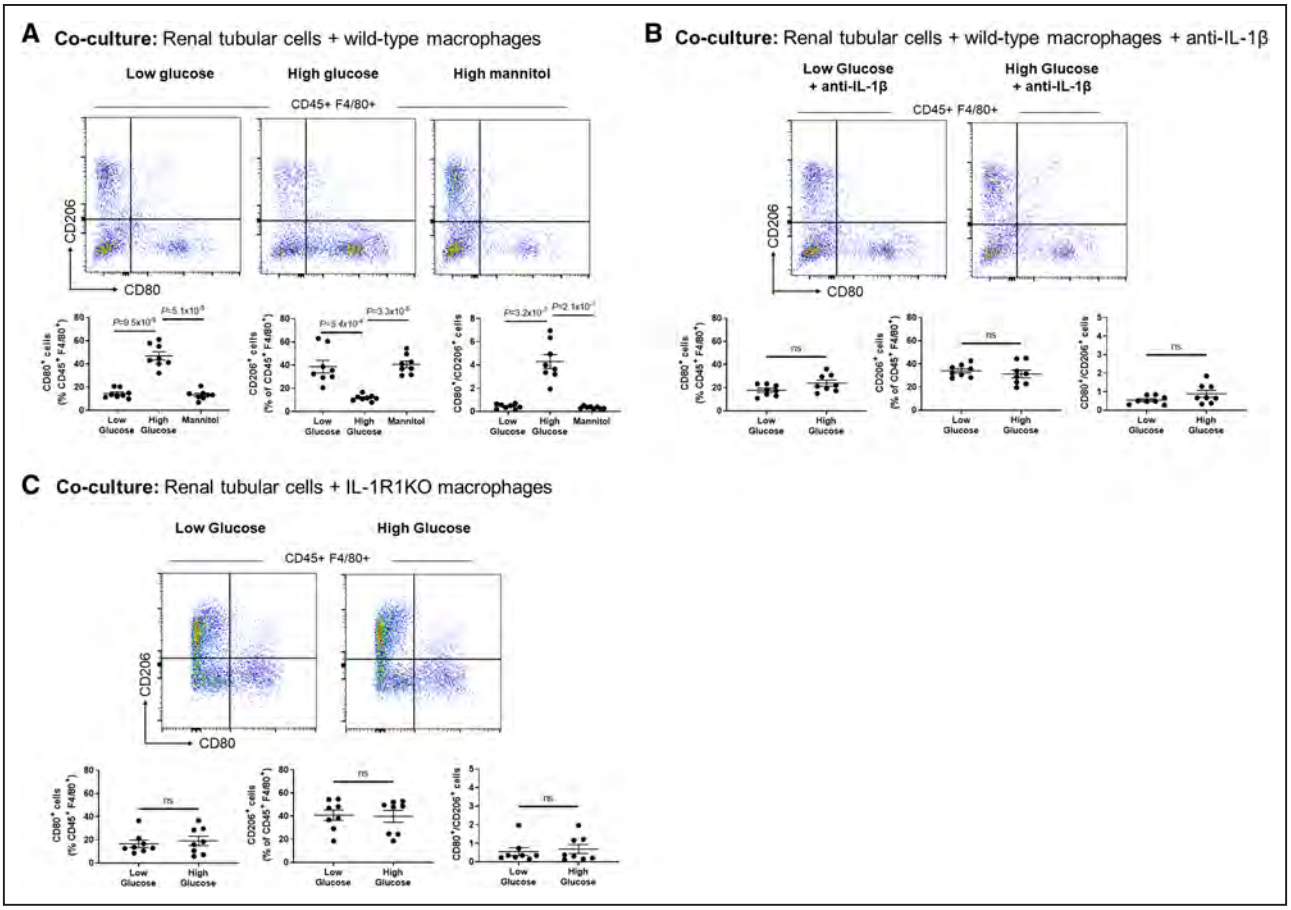


**Figure 3. Tubular epithelial cells polarize macrophages towards a proinflammatory phenotype.**
**A**, Tubular epithelial cells from wild-type mice were cocultured by direct contact with wild-type bone marrow-derived macrophages for 72 h under low (5 mmol/L) or high (15 mmol/L) glucose conditions. To test the role of IL (interleukin)-1β, an additional group of cells were incubated in the presence of **B** an anti-IL-1β neutralizing antibody or (**C**) with bone marrow-derived macrophages isolated from IL-1 receptor type 1 knockout (IL-1R1KO) mice. For osmotic control, cells were incubated with 5 mmol/L glucose plus 10 mmol/L mannitol. At the end of the experiment, cells were resuspended with 0.25% trypsin. Live cells were identified using a LIVE/DEAD Fixable Yellow staining. For surface marker staining, cells were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific antibody binding. Cells were stained with antibodies anti-CD45-Pacific blue, anti-F4/80-FITC (fluorescein isothiocyanate), anti-CD80-PE/Cy7 (phycoerythrin/cyanine7; proinflammatory marker), and anti-CD206-APC (allophycocyanin; anti-inflammatory marker). Results are expressed as the mean percentage of $CD80^+$ and $CD206^+$ cells per $CD45^+F4/80^+$ cells±SD, and the mean $CD80^+$-to-$CD206^+$ ratio±SD. Analysis by 1-way ANOVA followed by Tukey multiple comparisons test (n=8 for all groups).

the absence of tubular epithelial cells (Figure S7A and S7D). However, the magnitude of this response was significantly lower to that observed when macrophages are grown in the presence of tubular epithelial cells. Also, this response was not affected by the presence of an anti-IL-1β neutralizing antibody or in macrophages obtained from IL-1R1KO (Figure S7B through S7D). No change of CD206 expression was observed in wild-type macrophages exposed to high D-glucose in the absence of tubular epithelial cells (Figure S7A through S7E). An additional group of bone marrow-derived macrophages exposed to high glucose media in the presence of mouse recombinant IL-1β for 72 hours showed a proinflammatory polarization similar to that observed in macrophages cocultured with tubular cells (Figure S8). This confirms a role of IL-1β in macrophage polarization towards a proinflammatory phenotype.

## Reducing Renal Tubular Epithelial Cell-Derived IL-1β Prevents the Development of Salt Sensitivity in db/db Mice

We have demonstrated that 30-week-old male db/db mice exposed to a high-salt diet display a progressive increase in blood pressure.[21] To evaluate the role of tubular-derived IL-1β, we used an in vivo administration of IL-1β siRNA delivered in nanoparticles targeting kidney tubules. Previous data showed that polymeric mesoscale nanoparticles selectively and stably localized in the proximal tubule epithelium.[25,26] IL-1β siRNA nanoparticles were administered IV in 28-week-old male db/db (db/$db^{IL1β\text{-}siRNA}$) and db/+ (db/$+^{IL1β\text{-}siRNA}$) mice. db/db and db/+ mice treated with nontargeting siRNA (db/$db^{ctrl\text{-}siRNA}$ and db/$+^{ctrl\text{-}siRNA}$) were used as controls. Two weeks later, all mice were exposed to a 4-week high-salt diet

as described earlier.[21] The nanoparticles continued to be administered every 2 weeks until the end of the experiment. After 4 weeks of high-salt diet, body weight, plasma glucose, and plasma insulin were higher in db/db groups compared with db/+ groups and were not modified but the IL-1β siRNA treatment (Table S1). We confirmed the suppression of IL-1β in renal tubular cells by flow cytometry analysis as described above. Tubular epithelial (E-cadherin$^+$) cells of db/db$^{IL1β\text{-}siRNA}$ mice display lower IL-1β levels compared with db/db$^{ctrl\text{-}siRNA}$ mice (13±6% versus 28±7% of IL-1β$^+$E-cadherin$^+$ cells, Figure 4A). Indeed, IL-1β expression in IL-1β-siRNA-treated db/db mice was indistinguishable from levels observed in db/+ mice (Figure 4A). The specific suppression of IL-1β in renal tubular cells was further confirmed by immunofluorescent analysis of renal cortical samples (Figure S9). The levels of IL-1β in macrophages (CD45$^+$F4/80$^+$) were similar among all experimental groups indicating a specific renal tubular IL-1β knockdown (Figure 4B). The suppression of IL-1β in tubular cells correlated with a significant reduction of IL-1β in total cortex homogenate (Figure 4C). No suppression of IL-1β was observed in extrarenal anatomic sites such as plasma, heart, aorta, liver, or spleen (Figure S10). Notably, at the end of the experiment, mean arterial pressure of db/db$^{IL1β\text{-}siRNA}$ mice was significantly lower compared with db/db$^{ctrl\text{-}siRNA}$ mice (109±5 versus 123±8 mm Hg, Figure 4D). No significant blood pressure changes were observed in the db/+ groups (Figure S11A). All experimental groups reached sodium (Figure 4E) and potassium (Figure S11B) balance at the end of the experiment but at the expense of hypertension in db/db control mice.

## The Absence of Salt Sensitivity in db/db$^{IL1β\text{-}siRNA}$ Is Associated With Lower ENaC Activity and Expression

To evaluate ENaC activity in vivo, 4 days before the end of the 4-week high-salt diet, we performed an amiloride test.[12,27] The natriuretic response to amiloride was similar in both of db/+$^{IL1β\text{-}siRNA}$ and db/+$^{ctrl\text{-}siRNA}$ indicating similar ENaC activity (Figure 4F). In db/db$^{ctrl\text{-}siRNA}$, the natriuretic response was significantly higher suggesting increased ENaC activity (Figure 4F). This agrees with previous data from our laboratory showing that salt sensitivity in db/db mice is associated with higher ENaC activity compared with nondiabetic db/+ controls exposed to the same diet.[21] The absence of salt sensitivity in db/db$^{IL1β\text{-}siRNA}$ was associated with a suppression of ENaC activity (Figure 4F). The amiloride-induced potassium retention was significantly reduced in db/db$^{IL1β\text{-}siRNA}$ compared with db/db$^{ctrl\text{-}siRNA}$ mice adding further evidence supporting the decreased ENaC activity in the IL-1β-siRNA-treated group (Figure 4F). We also evaluated the renal expression of full-length α-, β-, and γ-ENaC subunits and the cleaved (active form) of α and γ subunits in renal cortical homogenates by Western blot.[12,21,28–30] Full-length α- and β-ENaC subunits and the cleaved fragments of α- and γ-ENaC subunits were significantly lower in db/db$^{IL1β\text{-}siRNA}$ compared with db/db$^{ctrl\text{-}siRNA}$ (Figure 4G). Also, the levels of renal SGK-1 (serum- and glucocorticoid-induced kinase 1), a critical regulator of ENaC abundance in the plasma membrane,[31] were significantly lower in db/db$^{IL1β\text{-}siRNA}$ compared with db/db$^{ctrl\text{-}siRNA}$ mice (Figure 4G). In total, these data show that the salt sensitivity of db/db$^{ctrl\text{-}siRNA}$ is associated with higher activity and expression of ENaC and its main regulator SGK-1. Knocking down IL-1β from tubular epithelial cells results in lower ENaC expression and no salt sensitivity.

## The db/db$^{IL1β\text{-}siRNA}$ Mice Display Lower Renal Inflammation

We have previously shown that renal inflammation plays a critical role in the development of salt sensitivity in db/db mice.[21] Indeed, db/db display an accumulation of proinflammatory CD80$^+$ macrophages and a reduction of anti-inflammatory CD206$^+$ macrophages in the kidney compared with db/+ mice.[21] Our in vitro studies showed that tubular-derived IL-1β can polarize macrophages into an inflammatory phenotype. Thus, we aimed to determine whether the absence of salt sensitivity in db/db$^{IL1β\text{-}siRNA}$ was associated with less proinflammatory macrophages in the kidney. The analysis of kidney samples by flow cytometry revealed that db/db$^{IL1β\text{-}siRNA}$ have a reduced number of CD80$^+$ and a higher number of CD206$^+$ macrophages compared with db/db$^{ctrl\text{-}siRNA}$ mice (CD80$^+$: 20±14% versus 50±12% and CD206$^+$: 34±12% versus 12±5% of total CD45$^+$F4/80$^+$ cells, Figure 5A through 5D). Further analysis showed that the increased abundance of CD80$^+$ macrophages observed in db/db$^{ctrl\text{-}siRNA}$ correlates with higher levels of intracellular IL-6 expression in renal macrophages (Figure 5E). In db/db$^{IL1β\text{-}siRNA}$ mice, macrophage intracellular IL-6 expression was significantly reduced compared with db/db$^{ctrl\text{-}siRNA}$ mice (20±10% versus 40±14% of total CD45$^+$F4/80$^+$ cells, Figure 5E and 5F). The lower abundance of IL-6$^+$ macrophages in db/db$^{IL1β\text{-}siRNA}$ mice was also associated with reduced levels of IL-6 in total renal cortex homogenates (Figure 5G).

## Diabetic db/db Mice Transplanted With a Bone Marrow From IL-1R1KO Mice Do Not Display Salt Sensitivity

To determine whether immune cells are a cellular target of tubular-derived IL-1β in salt sensitivity, 8-week-old db/db mice were transplanted with bone marrow from IL-1R1KO (db/db$^{IL1R1KO}$). This created a db/db mouse in which immune cells including macrophages cannot respond to IL-1β. Male db/db mice transplanted with the bone marrow of wild-type mice (db/db$^{WT}$), db/+

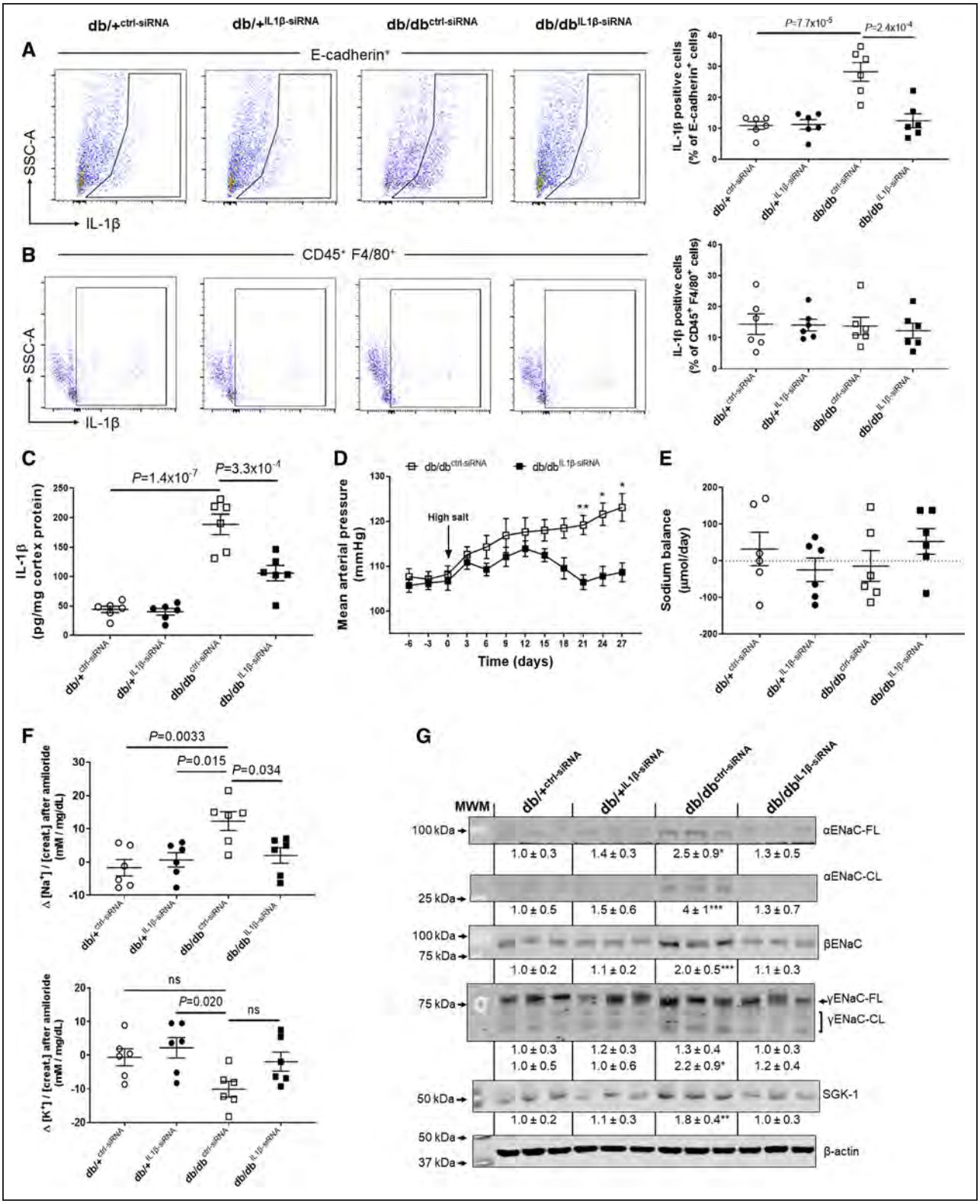


**Figure 4. Specific IL (interleukin)-1β suppression in renal tubular cells prevent salt sensitivity in db/db mice.**
Kidney cell suspensions from db/+$^{ctrl\text{-}siRNA}$, db/+$^{IL1\beta\text{-}siRNA}$, db/db$^{ctrl\text{-}siRNA}$, and db/db$^{IL1\beta\text{-}siRNA}$ mice were prepared. Live cells were identified using a LIVE/DEAD Fixable Yellow staining. For surface marker staining, cells were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific antibody binding. To evaluate the synthesis of IL-1β by (**A**) renal epithelial cells or (**B**) macrophages, cells were stained with antibodies anti-CD45-Pacific blue, anti-F4/80-FITC (fluorescein isothiocyanate), and anti-E-cadherin-PE (phycoerythrin). After fixation and permeabilization cells were stained with an anti-IL-1β followed by a secondary anti-rabbit IgG1-APC (allophycocyanin) antibody. Results are expressed as mean percentage of IL-1β$^+$ cells per E-cadherin$^+$ or CD45$^+$F4/80$^+$ cells±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6 per group. (*Continued*)

transplanted with IL-1R1KO bone marrow (db/+$^{IL1R1KO}$), and db/+ transplanted with wild-type bone marrow (db/+$^{WT}$) were used as control. At 30 weeks of age, mice were exposed to an additional 4-week high-salt diet (NaCl, 4% w/w) as described before.[21] At the end of the experiment (week 34), body weight, plasma glucose, and plasma insulin were higher in db/db compared with db/+ groups and were not modified by the bone marrow received (Table S2). Mean arterial pressure was significantly higher in db/db$^{WT}$ compared with db/db$^{IL1R1KO}$ (122±9 versus 110±7 mm Hg, $P<0.05$, Figure 6A). The blood pressure of db/+$^{IL1R1KO}$ and db/+$^{WT}$ was not increased by the high-salt diet (Figure S12A). All experimental groups reached sodium (Figure 6B) and potassium (Figure S12B) balance at the end of the experiment. To confirm a successful bone marrow transplantation, the expression of IL-1R1 was evaluated in total CD45$^+$ cells and macrophages by flow cytometry analysis of kidney cell suspensions. db/db$^{WT}$ mice show that ≈20% of total immune (CD45$^+$) cells and 50% of macrophages (CD45$^+$F4/80$^+$) express detectable levels of IL-1R1 whereas in db/db$^{IL1R1KO}$, IL-1R1 was only observed in roughly 1% of analyzed cells (Figure S13).

## db/db$^{IL1R1KO}$ Display Lower ENaC Activity and Expression

ENaC activity assessed by the amiloride test was similar in both of db/+$^{IL1R1KO}$ and db/+$^{WT}$ mice (Figure 6C). The amiloride-induced sodium excretion and potassium retention were significantly higher in db/db$^{WT}$ compared with db/db$^{IL1R1KO}$ suggesting lower ENaC activity in the latter (Figure 6C). Full-length α- and β-ENaC subunits, the cleaved fragments of α- and γ-ENaC subunits, and SGK-1 were significantly higher in db/db$^{WT}$ compared with all other groups (Figure 6D). As observed for db/db$^{IL1β\text{-}siRNA}$ mice, the absence of salt sensitivity in db/db$^{IL1R1KO}$ was associated with lower expression of ENaC subunits and SGK-1. In total, these data show that removing the IL-1R1 from immune cells results in lower ENaC expression.

## Renal Inflammation Is Blunted in db/db$^{IL1R1KO}$ Mice

Initially, we assessed the levels of IL-1β and IL-6 in renal cortical homogenates by ELISA. IL-1β levels were higher in db/db compared with db/+ mice irrespective of the bone marrow received and no significant differences were observed between db/db$^{WT}$ and db/db$^{IL1R1KO}$ mice (Figure 7A). Similarly, flow cytometry analysis revealed that epithelial (E-cadherin$^+$) cells from both db/db$^{WT}$ and db/db$^{IL1R1KO}$ have higher abundance of IL-1β compared with db/+ mice (Figure S14). Cortical IL-6 levels were also increased in db/db$^{WT}$ compared with db/+ groups. However, the absence of the IL-1R1 in immune cells of db/db$^{IL1R1KO}$ blunted the accumulation of cortical IL-6 (Figure 7B). Flow cytometry analysis of renal macrophages from db/db$^{WT}$ displayed a proinflammatory polarization evidenced by higher CD80 expression (Figure 7C) and higher intracellular IL-6 levels (Figure 7D) compared with db/+ groups. In db/db$^{IL1R1KO}$ mice, these parameters were significantly reduced. Indeed, renal CD80$^+$ macrophage abundance and intracellular IL-6 were indistinguishable between db/db$^{IL1R1KO}$ and db/+ groups (Figure 7C through 7E). Total renal macrophages were slightly increased in db/db$^{WT}$ compared with db/+$^{WT}$ mice. In db/db$^{IL1R1KO}$ mice, the level of macrophages was not significantly reduced compared with db/db$^{WT}$ mice (Figure 7F).

In total, these data together with the experiments performed on db/db$^{IL1β\text{-}siRNA}$ mice suggest that tubular-derived IL-1β induces salt sensitivity by promoting a proinflammatory polarization of renal macrophages that results in increased IL-6 accumulation and higher ENaC activity in the kidney.

# DISCUSSION

Although renal inflammation is now recognized as a critical mediator of high blood pressure and the development of DKD in both humans and animal models,[15,16] the origin of this inflammatory process remains largely unknown. In this study, we propose a novel molecular mechanism

**Figure 4 Continued. C**, IL-1β was evaluated in homogenates from kidney cortex by enzyme-linked immunoassay (ELISA). Results are expressed as mean pg of IL-1β per mg of tissue protein±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6 per group. **D**, Mean arterial pressure was measured by telemetry in db/db$^{ctrl\text{-}siRNA}$ and db/db$^{IL1β\text{-}siRNA}$ mice exposed to a high-salt diet (4% NaCl w/w) for 4 wk and expressed as mean±SD. Analysis by multiple Mann-Whitney tests (*$Q$=0.035, **$Q$=0.026). **E**, At the end of the experiment, mice were individually housed in metabolic cages for a 24-h urine collection. The intake and urinary excretion of sodium determined by flame photometry were used to calculate sodium balance and expressed as μmol of Na$^+$ excreted per day±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. **F**, The amiloride test to assess epithelial sodium channel (ENaC) activity in vivo was performed during the last week of high-salt diet as described before.[21] The difference between sodium and potassium excretion before and after amiloride injection (5 μg/g body wt in 100 μL 0.9% NaCl) was calculated for each mouse and considered a surrogate of ENaC activity. Data are expressed as mean differential (Δ) Na$^+$ or K$^+$ urinary concentration per mg/dL creatinine±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. **G**, The expression of α, β, and γ subunits of ENaC and SGK-1 (serum- and glucocorticoid-induced kinase 1) were assessed by Western blot in homogenates of renal cortices. β-actin (RRID: AB_476697; Sigma-Aldrich; Catalog no. A2228) was measured to verify uniform protein loading. Representative blots are displayed with the closest molecular weight markers indicated in the leftmost band. Data are expressed as mean±SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs other groups (uncropped blots and exact $P$ values are displayed in the Supplemental Material). Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. CL indicates cleaved; FL, full-length; MWM, molecular weight marker; and SSC-A, side scatter area.

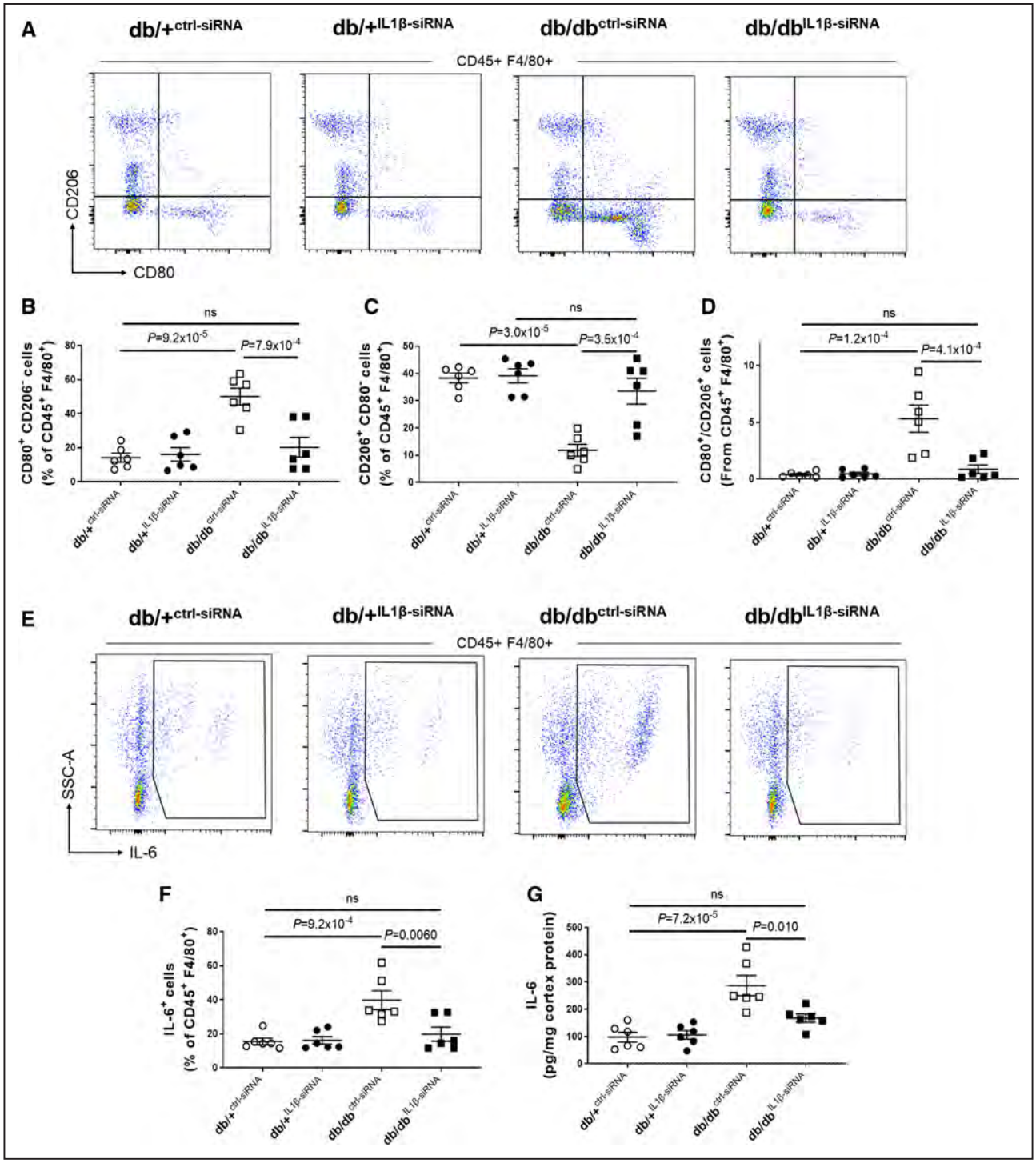


**Figure 5. db/db$^{IL1β\text{-}siRNA}$ mice display lower renal inflammation.**

**A**, Renal macrophages were analyzed by flow cytometry in kidney cell suspensions. Live cells were identified using a LIVE/DEAD Fixable Yellow staining. For surface marker staining, cells were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific antibody binding. Cells were stained with antibodies anti-CD45-Pacific blue, anti-F4/80-FITC (fluorescein isothiocyanate), anti-CD80-PE/Cy7 (phycoerythrin/cyanine7; proinflammatory marker), and anti-CD206-APC (allophycocyanin; anti-inflammatory marker). Results are expressed as the mean percentage of (**B**) CD80$^+$ and (**C**) CD206$^+$ cells per CD45$^+$F4/80$^+$ cells, and the (**D**) mean CD80$^+$-to-CD206$^+$ ratio±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. **E**, For intracellular IL (interleukin)-6 staining, additional aliquots of isolated kidney cells were incubated with 5 μg/mL of the protein transport inhibitor brefeldin A for 3 h. Live cells were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific antibody binding and then stained with antibodies anti-CD45-Pacific blue, anti-F4/80-FITC. After fixation and permeabilization, cells were stained with an anti-IL-6-PE. **F**, Results were expressed as mean IL-6$^+$ macrophages per total CD45$^+$F4/80$^+$ cells±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. **G**, The total levels of IL-6 were measured in renal cortex homogenate by enzyme-linked immunoassay (ELISA) and expressed and pg of IL-6 per mg of cortex protein±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. SSC-A indicates side scatter area.

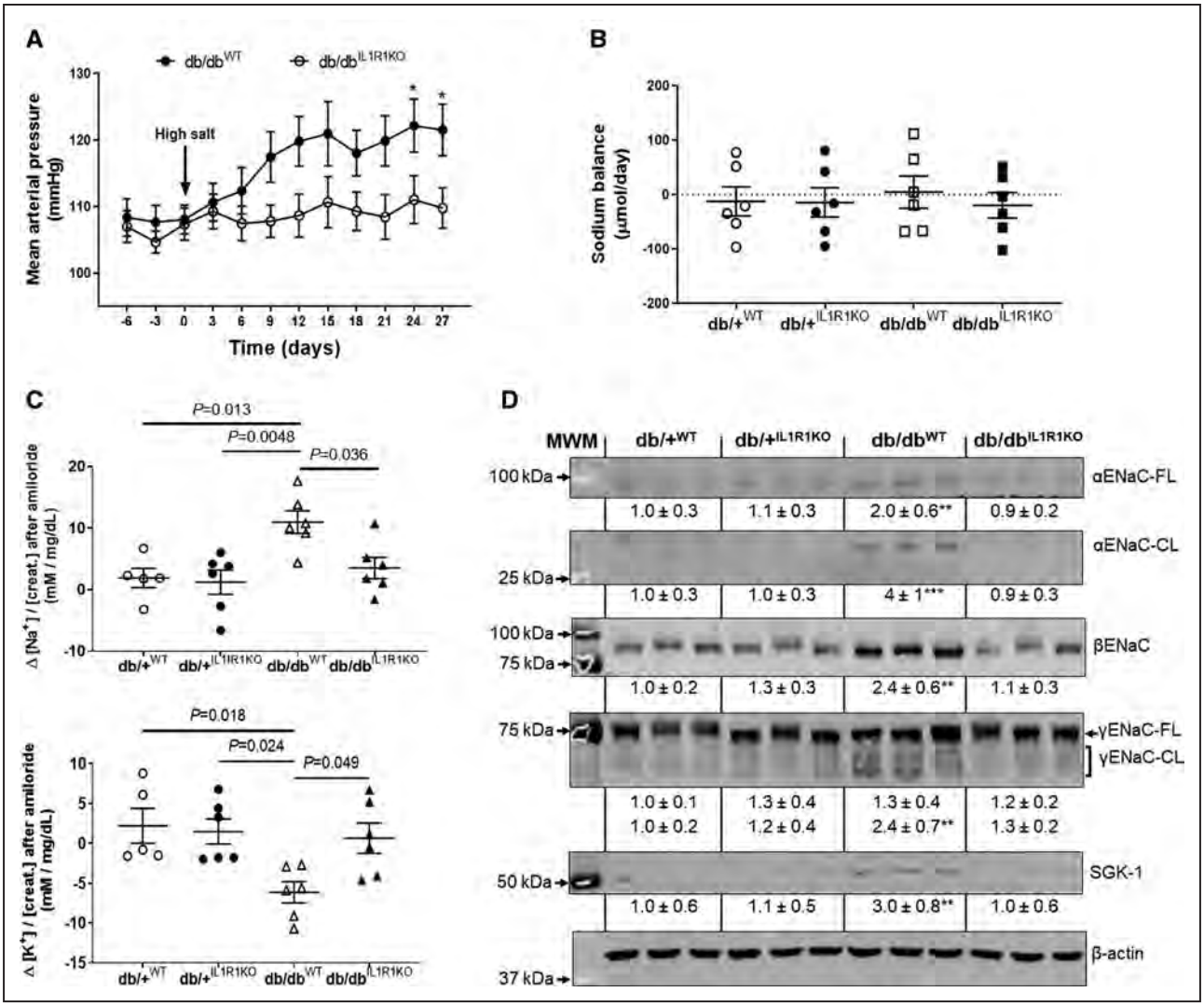


**Figure 6. db/db$^{IL\text{-}1RKO}$ mice display no salt sensitivity and lower epithelial sodium channel (ENaC) activity.**
**A**, Mean arterial pressure was measured by telemetry in db/db$^{IL\text{-}1RKO}$ and db/db$^{WT}$ mice exposed to a high-salt diet (4% NaCl w/w) for 4 wk and expressed as mean±SD. Analysis by multiple Mann-Whitney tests (*$Q$=0.039). **B**, At the end of the experiment, mice were individually housed in metabolic cages for a 24-h urine collection. The intake and urinary excretion of sodium determined by flame photometry were used to calculate sodium balance and expressed and µmol of $Na^+$ excreted per day±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. **C**, The amiloride test to assess ENaC activity in vivo was performed during the last week of high-salt diet as described before.[21] The difference between sodium and potassium excretion before and after amiloride injection (5 µg/g body wt in 100 µL 0.9% NaCl) was calculated for each mouse and considered a surrogate of ENaC activity. Data are expressed as mean differential (Δ) $Na^+$ or $K^+$ urinary concentration per mg/dL creatinine±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. One value from the db/+$^{WT}$ group was noted as a significant outlier and discarded. **D**, The expression of α, β, and γ subunits of ENaC and SGK-1 (serum- and glucocorticoid-induced kinase 1) were assessed by Western blot in homogenates of renal cortices. β-actin was measured to verify uniform protein loading. Representative blots are displayed with the closest molecular weight markers indicated in the leftmost band. Data are expressed as mean±SD. **$P$<0.01 vs other groups (uncropped blots and exact $P$ values are displayed in the Supplemental Material). Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. CL indicates cleaved; FL, full-length; and MWM, molecular weight marker.

behind the development of renal inflammation and salt sensitivity during diabetes. Specifically, we identified IL-1β as an initial promoter of the renal inflammatory response associated with diabetes. We also observed that renal tubular epithelial cells, and not immune cells, are the major source of IL-1β. This was confirmed in both a primary culture of tubular epithelial cells exposed to a high glucose environment and in kidney samples from diabetic mice. Finally, we established a molecular connection between nonimmune and immune cells within the kidney during diabetes. Indeed, tubular cell-derived IL-1β polarizes macrophages towards an inflammatory phenotype that further contributes to expand the inflammatory response associated with DKD.

Macrophages have been classically associated with IL-1β production and release.[32] However, several studies have demonstrated that different renal cell types can also synthesize this inflammatory cytokine under pathological

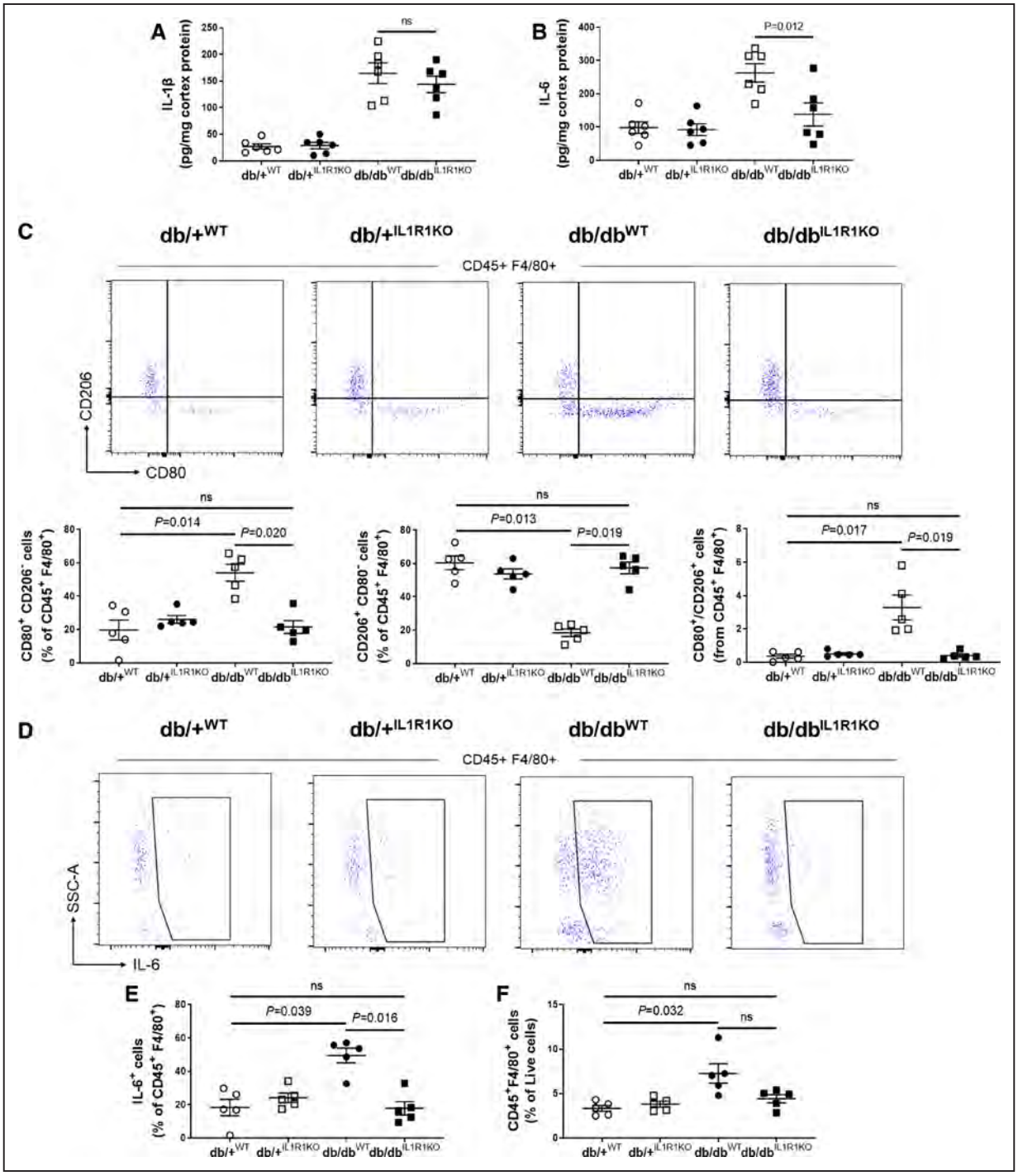


**Figure 7. db/db$^{IL\text{-}1RKO}$ mice display lower renal inflammation.**

**A**, IL (Interleukin)-1β and (**B**) IL-6 were measured in renal cortex of db/+$^{WT}$, db/+$^{IL\text{-}1RKO}$, db/db$^{WT}$, and db/db$^{IL\text{-}1RKO}$ mice after a 4-wk high-salt diet by enzyme-linked immunoassay (ELISA). Results were expressed as pg of cytokine per mg of kidney cortex protein±SD. Analysis by 2-way ANOVA followed by Tukey multiple comparisons test, n=6. **C**, Renal macrophages were analyzed by flow cytometry in kidney cell suspensions. Live cells were identified using a LIVE/DEAD Fixable Yellow staining. For surface marker staining, cells were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific antibody binding. Cells were stained with antibodies anti-CD45-Pacific blue, anti-F4/80-FITC (fluorescein isothiocyanate), anti-CD80-PE/Cy7 (phycoerythrin/cyanine7; proinflammatory marker), and anti-CD206-APC (allophycocyanin; anti-inflammatory marker). Results are expressed as the mean percentage of CD80$^{+}$ and CD206$^{+}$ cells per CD45$^{+}$F4/80$^{+}$ cells, and the mean CD80$^{+}$-to-CD206$^{+}$ ratio±SD. Analysis by Kruskal-Wallis test followed by a Dunn multiple comparisons test, n=5. **D** and **E**, For intracellular IL-6 staining, additional aliquots of isolated kidney cells were incubated with 5 μg/mL of the protein transport inhibitor brefeldin A for 3 h. Live cells were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific antibody binding and then stained with antibodies anti-CD45-Pacific blue, anti-F4/80-FITC. After fixation and permeabilization, cells were stained with an anti-IL-6-PE. Results were expressed as mean IL-6$^{+}$ macrophages per total macrophages±SD. Analysis by Kruskal-Wallis test followed by a Dunn multiple comparisons test, n=5. **F**, Total macrophages were measured and expressed per total live cells±SD. Analysis by Kruskal-Wallis test followed by a Dunn multiple comparisons test, n=5. SSC-A indicates side scatter area.

conditions..[33–35] Studies performed in rats have shown that cortical tubular epithelial cells are a major source of IL-1β, and its expression is markedly upregulated in kidney disease.[36] Our current data show that high glucose can increase the expression of NLRP3 inflammasome components and induce IL-1β synthesis in renal tubular epithelial cells. Further confirmation of these findings was obtained in vivo. In db/db mice, ELISA analysis showed an increased expression of IL-1β in renal cortex compared with db/+ mice. Also, renal tubular epithelial cells (E-cadherin positive) isolated from diabetic kidneys display higher levels of IL-1β compared with tubular cells isolated from nondiabetic control mice. Interestingly, although high glucose has been previously associated with an upregulation of IL-1β synthesis in monocytes,[37] the expression of IL-1β by renal macrophages was not different between diabetic and nondiabetic mice. These data suggest that most of renal IL-1β accumulation in db/db mice has a tubular and not an immune origin. Notably, specific silencing of IL-1β in tubular epithelial cells prevented renal inflammation and salt sensitivity in db/db mice. This was associated with lower expression and cleavage of ENaC subunits, and less activity of this sodium channel assessed by the amiloride test compared to diabetic mice treated with control siRNA. The lower expression of ENaC subunits was accompanied by reduced levels of its main regulator SGK-1. This suggests that tubule-derived IL-1β might act as an initial trigger of the inflammatory response that impairs the normal kidney function in diabetes. Although the exact locus of renal tubular-derived IL-1β remains to be elucidated, our immunofluorescence analysis and the assessment of IL-1β by ELISA in isolated cortical tubules suggest that IL-1β might be synthesized by several cortical nephron segments, including proximal tubules, distal tubules, and collecting ducts while no significant expression of IL-1β was observed in glomeruli or renal medulla.

IL-1β has been shown to promote the differentiation of monocytes into proinflammatory macrophages classically identified as CD80$^+$ macrophages.[22,38] In line with these findings, we observed that IL-1β secreted by high glucose-treated renal tubular epithelial cells can activate and induce a proinflammatory phenotype of naive bone marrow-derived macrophages. To investigate the significance of these observations in vivo, we used a bone marrow transplantation approach, to create a db/db mouse in which immune cells do not express the IL-1R1 (db/db$^{IL1R1KO}$). This experimental approach did not modify metabolic parameters such as body weight, blood glucose, or plasma insulin. However, after 4 weeks of a high-salt diet, db/db$^{IL1R1KO}$ mice did not develop salt sensitivity. The analysis of renal cortical IL-1β and IL-6 by ELISA revealed that both db/db$^{WT}$ and db/db$^{IL1R1KO}$ have similar levels of IL-1β. These data suggest that IL-1β is unlikely to be directly involved in controlling ENaC. However, db/db mice harboring an IL-1R1KO bone marrow

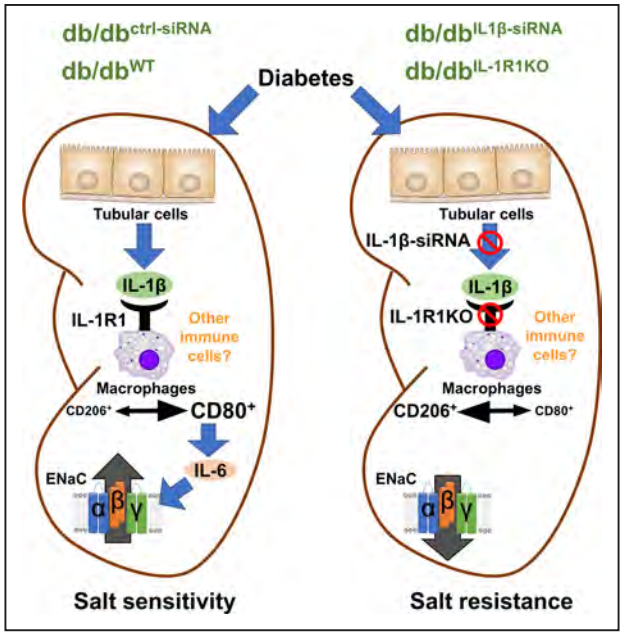


**Figure 8. Schematic representation of renal tubular epithelial cells as a major source of IL (interleukin)-1β during diabetes.** Tubular-derived IL-1β plays a key role in driving the polarization of renal macrophages towards a proinflammatory phenotype that promotes salt sensitivity through the synthesis and release of IL-6. When tubular IL-1β synthesis is suppressed (db/db$^{IL1β-siRNA}$) or in db/db mice in which immune cells lack the IL-1R1 (db/db$^{IL-1R1KO}$), the macrophage proinflammatory polarization is blunted resulting in less accumulation of renal IL-6, lower activity of epithelial sodium channel (ENaC), and no salt-sensitive hypertension.

have significantly lower levels of cortical IL-6 compared with db/db$^{WT}$ mice. These data indicate that renal IL-6 is produced in response to IL-1β. Further evidence supporting this hypothesis came from db/db$^{IL1β-siRNA}$ in which suppressing IL-1β expression resulted in lower renal IL-6 accumulation. IL-6 is a potent activator of SGK-1,[39] and previous studies from our laboratory have shown that this cytokine is a key promoter of salt-sensitive hypertension in db/db mice by dysregulating ENaC.[21] Taken together, these data suggest that the absence of salt sensitivity and the lower expression of ENaC in both db/db$^{IL1β-siRNA}$ and db/db$^{IL1R1KO}$ mice is a consequence of lower renal IL-6 accumulation. IL-6 is among the most abundant cytokines secreted by CD80$^+$ macrophages during chronic kidney disease.[40] Our current studies also show that the lower abundance of renal IL-6 in db/db$^{IL1β-siRNA}$ and db/db$^{IL1R1KO}$ mice was associated with less CD80$^+$ and higher CD206$^+$ macrophages compared with control groups. Indeed, macrophages from db/db$^{IL1β-siRNA}$ and db/db$^{IL1R1KO}$ mice express less IL-6 compared with macrophages from control groups.

There are some limitations associated with our bone marrow transplantation experiment. Since this is a global approach, it replaces immune cells from virtually all tissues. However, the observation the salt sensitivity is prevented by specifically silencing IL-1β in renal tubules suggests that

the IL-1β-mediated activation of immune cells in the kidney is critical to generate salt-sensitive hypertension. It is also worth mentioning that during bone marrow transplantation, other immune cells besides macrophages are transferred to the donor. Thus, although not explored in this article, it is possible that the absence of IL-1R1 in other immune cells might also be responsible for the protective phenotype of db/db$^{IL1R1KO}$ mice. This is especially true for T cells, which express IL-1R and have been linked to the development of renal inflammation and hypertension.[17,41]

In conclusion, we identified renal tubular epithelial cells as a major source of IL-1β during diabetes. This cytokine triggers an initial inflammatory response by driving the polarization of renal macrophages towards a proinflammatory phenotype characterized by high IL-6 secretion. IL-6 ultimately promotes salt sensitivity by upregulating ENaC as described before.[21] In db/db mice in which tubular IL-1β is suppressed or immune cells lack the IL-1R1, macrophages are not polarized towards an inflammatory phenotype resulting in less accumulation of IL-6, lower ENaC activity, and no salt-sensitive hypertension (Figure 8).

## ARTICLE INFORMATION

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### Author Contributions

L.C. Veiras and J.F. Giani conceived and designed research; L.C. Veiras, E.A. Bernstein, D. Cao, D. Okwan-Duodu, Z. Khan, A. Roach, R. Skelton, R.M. Williams, and J.F. Giani performed experiments; L.C. Veiras, D.R. Gibb, R.M. Williams, K.E. Bernstein, and J.F. Giani analyzed data; L.C. Veiras and J.F. Giani prepared figures and drafted the article; L.C. Veiras, K.E. Bernstein, and J.F. Giani edited and revised article; all authors approved the final version of article.

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### Disclosures

R.M. Williams is a scientific advisor with equity interest in Goldilocks Therapeutics, Inc. The other authors report no conflicts.

### Supplemental Material

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