Macrophage Accumulation and Lipid Loading in Diabetic Kidney Disease

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Abstract

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Diabetes is becoming increasingly problematic in the United States with 34.2 million total people diagnosed with either Type 1 or Type 2 in 2018.[1] Diabetic individuals who have elevated cholesterol, triglycerides, and blood glucose levels have an increased risk of complications. One complication that individuals are at risk for is diabetic kidney disease (DKD). Previous studies have shown that elevated triglyceride-rich lipoproteins (TRLs) are elevated in those diagnosed with diabetes and are associated with DKD progression.[3,4,5] Further, lipids are thought to contribute to kidney function decline through altering different cells in the kidney glomerulus namely endothelial cells, podocytes, sand mesangial cells.[6] Even with the vast knowledge and previous research on how lipids interact with different cells in the progression to DKD, the exact role of dyslipidemia and hyperglycemia on progression to DKD in diabetic patients is still not well understood.

Therefore, in this study, we used mouse models that were diabetic and dyslipidemic to mimic human DKD progression, to investigate the direct effects of TRL accumulation in DKD progression. Immunohistochemistry (IHC) was performed on sacrificed diabetic and dyslipidemic mouse kidneys to assess whether lipids were accumulating and where. After our initial findings indicated around half of the lipid droplets did not overlap with macrophages, we explored other cell types that may be impacted by diabetic conditions. We extracted endothelial cells, which are known to take up lipids and are sensitive to lipotoxic stimuli, from mouse kidneys and stimulated them with diabetic and dyslipidemic conditions. Additionally, kidney cells from diabetic and wildtype mice were extracted to explore whether changes to endothelial cells also occur *in vivo.* In this study, we observed that macrophages were accumulating and are lipid-loaded in diabetic and dyslipidemic mice. Further, kidney endothelial cells responded primarily to elevated glucose levels resulting in increased expression of *Ccl2*, a key mediator of monocyte recruitment. These findings are a starting point in understanding how dyslipidemia and diabetic conditions affect different cells in the glomerulus and drive DKD progression and can help development treatments and prevention of DKD progression.

Introduction

Diabetes is increasingly problematic in the United States with 1.5 million new cases in 2018 totaling 34.2 million people diagnosed with either Type 1 or Type 2 diabetes (T1D and T2D).[1] Individuals with diabetes are at higher risk of complications if they also have higher cholesterol, triglyceride (TG), and blood glucose levels.[1] Of those diagnosed with diabetes, 37% were diagnosed with diabetic kidney disease (DKD), a major complication of diabetes.[1] Even with such a high disease prevalence and high incidence of DKD, the exact underlying mechanism on the role of elevated lipids and glucose in progression to DKD is not well understood. Therefore, further investigation into the role of lipids and glucose in driving DKD is necessary to get more insight into potential treatments.

Diabetes is associated with elevated levels of triglyceride-rich lipoproteins (TRLs; including very-low density lipoproteins (VLDL) and chylomicrons) and reduced levels of high-density lipoprotein (HDL). This combination is often referred to as diabetic dyslipidemia. Elevated TRLs, rather than low-density lipoproteins (LDL), are associated with renal function decline in diabetes.[2] Apolipoprotein C3 (APOC3) is a key regulator of TG metabolism and APOC3 is elevated in people with impaired renal function .[3,4,5]

Neutral lipid accumulation, such as TGs and cholesterol, had been reported within the kidney glomerulus in diabetes. These lipid droplets are found in most cell types present in the glomerulus, namely endothelial cells, mesangial cells and podocytes.[6] Previous research has shown that lipid accumulation contributes to declining kidney function through the alteration of different cells in the kidney glomerulus. The Kanter lab has recently demonstrated that induction of dyslipidemia by overexpressing the inducible degrader of the LDL receptor (IDOL) results in augmented DKD in a mouse model.[7] The accelerated DKD was associated with increased macrophage accumulation and induction of inflammatory markers including cytokines, chemokines, and adhesion molecules. Further, Ducasa et al. identified impaired cholesterol efflux in podocytes as a contributor to podocyte lipid loading and DKD. Reduced levels of the cholesterol effluxer ABCA1 resulted in increased accumulation of lipids in podocytes, which in turn was associated with altered podocyte function and injury that ultimately led to podocyte loss and augmented albuminuria.[8,9]

A previous study that used the APOC3 ASO showed dramatic reduction in the elevated TRLs associated with diabetes and completely blocked diabetes-accelerated atherosclerosis, another common comorbidity of diabetes.[10] In this study, we wanted to explore the direct effects of TRL accumulation in the kidney in DKD progression and whether the APOC3 silencing using antisense oligonucleotides (ASO) could suppress the progression.

We observed that macrophages were accumulating and are lipid-loaded in diabetic and dyslipidemic mice. Further, kidney endothelial cells responded primarily to elevated glucose levels resulting in increased expression of *Ccl2*, a key mediator of monocyte recruitment. These findings are a starting point in understanding how dyslipidemia and diabetic conditions affect different cells in the glomerulus and drive DKD progression.

Methods

Description of mice used for the study

Male and female BTBR (black and tan, brachyury) wildtype (WT) or BTBR mice homozygous for the leptin-deficiency mutation (*Lepob*; OB) mice were used in this study. Leptin deficiency results in elevated blood glucose levels from ~5 weeks of age. Human-like dyslipidemia was induced in the mice using an ASO against the LDL receptor (LDLR). Half of all the mice were injected with APOC3 ASO to determine what effect APOC3 has on diabetic kidney disease. At the initiation of dyslipidemia with the LDLR ASO injections, the animals were fed a semi-purified, high fat, cholesterol-containing diet (40 % of calories from fat, 1.25% added cholesterol; TD 00244) for 14 weeks. At baseline, no significant differences were observed between the treatment groups. Plasma cholesterol and TGs were elevated at 2 weeks with LDLR ASO treatment and maintained elevated throughout the study in mice treated with LDLR ASO. APOC3 ASO treatment dramatically reduced circulating APOC3 levels but did not significantly reduce plasma cholesterol levels. In line with the known role for APOC3 in TG-metabolism, APOC3 ASO treatment resulted in a dramatic reduction in plasma TG levels, which resulted in mainly a reduction in VLDL levels. Note: the animal study was approved by IACUC and completed prior to the start of the master's project.

Lectin labeled endothelial cell extraction

3-week-old diabetic and lean mice were sedated with isoflurane and injected with biotinylated lectin (Lycopersicon esculentum) 15 min prior to being sacrificed with CO_2. The kidney cortex was isolated and digested using 2mg/ml Collagenase Type 1 for 2 x 15 min. Biotin-positive cells (endothelial cells) were isolated using a Biotin positive selection kit according to the manufacturer's instructions. Instead of plating the cells, 350µl of lysis buffer was added into the tube to lyse the samples. The lysis buffer solution was moved into a 1.5ml tube until RNA extraction. All solutions from this isolation included Pol 2 inhibitor diluted 1:10,000. The specificity of the lectin labeling was verified by staining for biotinylated lectin using streptavidin-Alexa-488 on frozen sections from the sacrificed mice.

Immunohistochemistry (IHC) – quantification of lipid staining in macrophages

Kidneys from the above mice were embedded in paraffin and sectioned. Sections were deparaffinized, rehydrated and antigen retrieved (10mM sodium citrate + 0.05% tween, pH 6.0 with HCl). The sections were incubated with primary antibodies to perilipin-2, Mac-2, or a combination overnight at 4°C (see table for concentrations). Mac-2 was visualized using an anti-rat Alexa 488 and perilipin-2 was visualized using an anti-rabbit Alexa-568. The slides were mounted with DAPI containing mounting media to identify nuclei. Negative controls included a rat IgG and a rabbit IgG and single stained sections to verify spectral specificity in each channel (Table 1).

Table 1

	Brand	**Concentration**
Mac-2	CL8942AP, Cedarlane	5 µg/ml
Perilipin-2	NB110-40877, Novus Biologicals	5 µg/ml
Rat IgG	CLCR2A00, Cedarlane	5 µg/ml
Rabbit IgG	Invitrogen	5 µg/ml
Alexa – 488	Thermo Fisher	8 µg/ml
Alexa – 568	Thermo Fisher	8 µg/ml

Microscopy

All images were acquired on a Leica fluorescent microscope with all negative controls taken on the same day. Images were taken at 20x magnification with variable exposures from 150 sec to 500 sec. Images were taken in red, green, and blue channels for perilipin-2, Mac-2, and DAPI respectively.

Stimulation of Primary Renal Endothelial Cells

Mice were sacrificed at around 3 weeks of age and the kidney endothelial cells were extracted. Extracted kidneys were washed briefly, digested in digestion media (2mg/mL Collagenase Type 1, 0.25% BSA, RPMI 1640 medium), filtered with a 40μm cell strainer, and plated onto a collagen-coated 10cm petri plate with base media (Dulbecco's Modified Eagle Medium, 10% horse serum, heparin, L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, penicillin) for at least two hours in 37°C. Growth media (base media, ECGS 100μg/ml, VEGF 10ng/ml) was added to the plate until the cells were about 70-90% confluent. The kidney cells were purified for endothelial cells following the eBioscience CD102/ICAM (eBioscience #13-1021-85) antibody kit protocol. The cells were plated on a collagen-coated 10cm tissue culture plate for at least 1 hour and then growth media was added to allow for 70-90% confluence. Once confluent, cells were passaged onto a 12, 16, or 96-well collagen-coated plate with at least 20,000 cells/well before being stimulated. Cells would not be stimulated until 70-90% confluent in the 12, 16, or 96-well plate. One day prior to stimulation, the cells were starved in low serum conditions (low-glucose DMEM, 2% horse serum, heparin, L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, penicillin) overnight. The treatments were added in triplicate. The treatments included low-glucose media, low-glucose media with VLDL, low-glucose media with TNFα, high-glucose, high-glucose media with VLDL, and high-glucose media with TNFα (Table 2). The cells were incubated at 37°C for 6 hours. After the 6 hours, the wells were washed 2x with PBS and 350μl of lysis buffer LBP was added in from the Macherey-Nagel NucleoSpin RNA plus kit.

- Low glucose media for stimulation: 1mg/ml glucose, Dulbecco's Modified Eagle Medium, 2% horse serum, heparin, L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, penicillin.
- High glucose media for stimulation: 5mg/ml glucose, Dulbecco's Modified Eagle Medium, 10% horse serum, heparin, L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, penicillin.

Table 2

	Glucose concentration	**VLDL concentration**	**Oxidized LDL**	**Palmitate**	**TNFα**
Low Glucose	1mg/ml	20 - 50µg/ml	20ng/ml	200µM	20ng/ml
High Glucose	5mg/ml	20 - 50µg/ml	20ng/ml	200µM	20ng/ml

RNA extraction

RNA from stimulated kidney endothelial cells or lectin labeled endothelial cells were extracted using Macherey-Nagel NucleoSpin RNA plus kit. The RNA was stored in a freezer until use.

qPCR

A reverse transcriptase reaction was run with the extracted RNA to make cDNA. Then, the cDNA was used to run qPCR reactions for *18s, Ccl2, Sele, Icam1* and *Vcam1* using SYBR green as the marker (Table 3).

Table 3

Gene	**Forward Primer Sequence**	**Reverse Primer Sequence**
18s	CATTAAATCAGTTATGGTTCCTTTGG	CCCGTCGGCATGTATTAGCT
Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Sele	ATGAAGCCAGTGCATACTGTC	CGGTGAATGTTTCAGATTGGAGT
Icam1	GGCATTGTTCTCTAATGTCTCC	GCTCCAGGTATATCCGAGCTTC
Vcam1	TGCACAGTCCCTAATGTGTATCC	GACTTTATGCCCATTTCCTCCA

Data and Statistical analysis

IHC Statistical Analysis

A minimum of 10 glomeruli per mouse was analyzed for perilipin-2, Mac-2, and overlay staining using Image J software. The staining values were averaged for each mouse with at least five mice per experiment group. A one-way or two-way ANOVA with posthoc analysis was run using GraphPad PRISM software and the data is presented as mean ± SEM.

In Vitro Statistical Analysis

Each endothelial cell extraction, stimulation, RNA extraction, and qPCR for the genes *Ccl2, Sele, Icam1*, and *Vcam1* were conducted at least 4 times and run in triplicates. A representative triplicate is shown in each figure. A one-way or two-way ANOVA with posthoc analysis was run using GraphPad PRISM software and data is presented as mean ± SEM.

Lectin Statistical Analysis

The blood glucose, TG, and cholesterol levels of the male OB and lean mice were compared with an unpaired T-test. Each dot in the graph represents one animal. The results from this test are presented as mean ± SEM. The qPCR fold-over comparison between the lean and OB mice for the genes *Ccl2*, *Sele, Icam1*, and *Vcam1* were analyzed with a Mann-Whitney T-test. The tests were run using GraphPad PRISM software and the results are presented as mean ± SEM.

Results

Diabetes results in neutral lipid accumulation, which is reduced with APOC3 ASO

To test if dyslipidemia plays a role in diabetic kidney disease, non-diabetic WT and diabetic OB mice were treated with an ASO to the LDLR. This creates a human-like dyslipidemia with elevated LDL and VLDL cholesterol levels. To specifically ask if APOC3, an apolipoprotein that regulates TG metabolism, plays a role in mediating some of the deleterious effects of dyslipidemia, half of all the mice were treated with APOC3 ASO. Diabetic OB mice were hyperglycemic, hypercholesterolemic, and hypertriglyceridemic compared to the WT mice (cholesterol: 320 ± 14 mg/dl in WT mice and 723 ± 66 mg/dl in OB mice; TGs: 93 ± 12 mg/dl in WT versus 463 ± 32 mg/dl in OB mice). APOC3 ASO treatment did not affect plasma cholesterol but dramatically reduced plasma TG (165 ± 19

mg/dL in OB mice treated with LDLR and APOC3 ASO). Consistent with the BTBR OB model being a model of DKD and that dyslipidemia worsens that, OB mice had an increased albumin creatinine ratio (Figure 1A).[11] Interestingly, this was partially reversed in the OB mice treated with the APOC3 ASO, suggesting that APOC3-driven dyslipidemia plays a role in DKD. Glomeruli from people with diabetic kidney disease are known to accumulate neutral lipids, and to test if neutral lipid accumulation was altered by APOC3 ASO treatment, frozen sections were stained with Oil-Red O.[6] Similar to what was seen in humans with DKD, OB mice had significantly more glomeruli with neutral lipids accumulating compared to WT mice, and this was reduced by APOC3 ASO treatment (Figure 1 B–C).

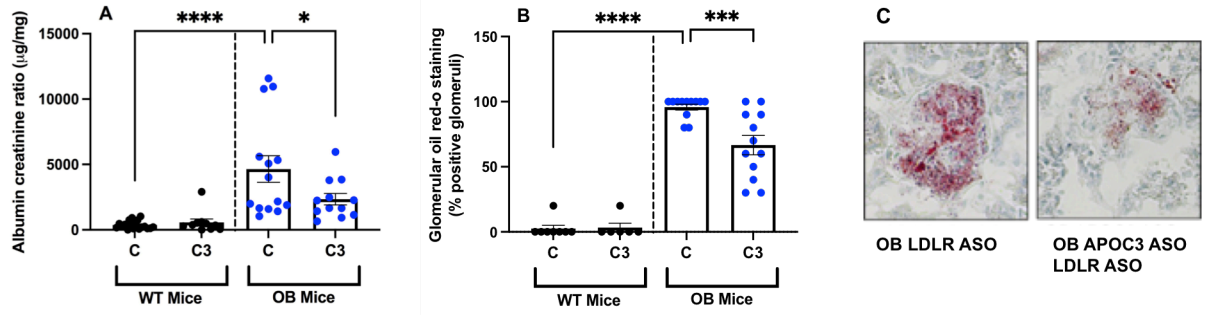

Figure 1. **Reduced APOC3 suppresses DKD.** A. Albumin creatinine ratio measured at week 13 of WT and OB mice. B. Proportion of positive glomerular Oil-Red-O staining. C. Example of glomerular Oil-Red-O staining. N=10-14, Data expressed as mean ± SEM, 1-way ANOVA followed by Tukey's post test, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001.

Lipid and macrophages are accumulating in DKD

Kidney sections from BTBR WT and OB mice, known to be diabetic and dyslipidemic, were stained with perilipin-2, a lipid-droplet marker, to assess if there was intracellular lipid deposition in the glomeruli of the kidney (Figure 2). Only sections stained with the perilipin-2 antibody showed positive staining, whereas the negative isotype control showed no staining (Figure 2). The OB mice had significantly increased perilipin-2 staining compared to the WT mice and the APOC3 ASO treated OB mice had visibly reduced perilipin-2 staining. Further, the diabetic and dyslipidemic mice had significantly increased lipid droplet accumulation in the glomeruli compared to all the other groups (Figure 3 B). A

significant decrease in the proportion of the glomeruli that were lipid loaded in the mice that received the APOC3 ASO suggests the APOC3 ASO might reverse some lipid deposition in the kidney glomeruli.

To investigate whether the lipid accumulation overlaps with inflammatory cells, the kidney sections from diabetic and dyslipidemic mice were stained with Mac-2, a macrophage marker (Figure 2). Again, only sections stained with the macrophage marker showed positive staining, whereas the negative control showed no staining (Figure 2). Macrophages in the kidneys were visibly concentrated in the glomeruli. In contrast, there are less macrophages inside the glomeruli of the WT mice. Further, the APOC3 ASO treated mice have decreased macrophage accumulation in the glomeruli again suggesting the APOC3 ASO might reduce macrophage accumulation in the kidney glomeruli. Analysis of the extent of glomerular perilipin-2 staining within macrophages in OB and WT mice showed a significantly increased proportion of macrophages that overlapped with lipid droplets in the OB mice compared to the WT and APOC3 ASO treated OB mice (Figure 3 C). The reduction in macrophage and perilipin-2 overlap in the glomeruli of the OB APOC3 ASO treated mice suggests that APOC3 can reverse some lipid-loaded macrophage accumulation in the kidney glomeruli. The increased lipid deposition and increased overlap of lipids and macrophages in the glomeruli suggest that APOC3 plays a role in lipid deposition and macrophage accumulation in the glomeruli in diabetic and dyslipidemic mice.

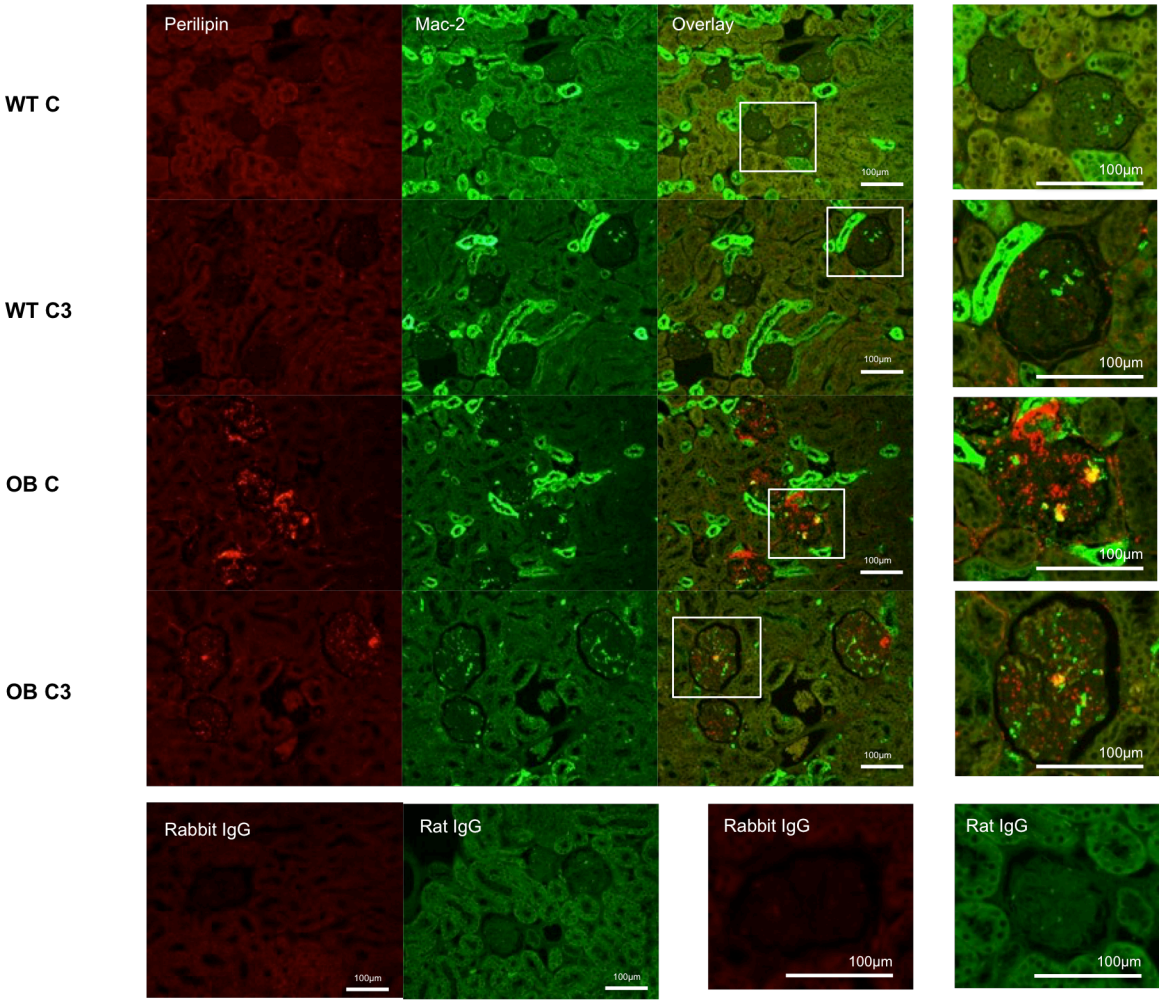

Figure 2. **APOC3 ASO reverses macrophage lipid loading in DKD.** The kidney sections from LDLR mice were stained with perilipin-2 and Mac-2 to assess lipid and macrophage accumulation in the glomeruli. The yellow staining indicates an overlap between perilipin-2 and Mac-2. Magnified glomeruli images of each group are on the left. Rabbit and Rat IgG used as controls are on the bottom. N= 11 – 13.

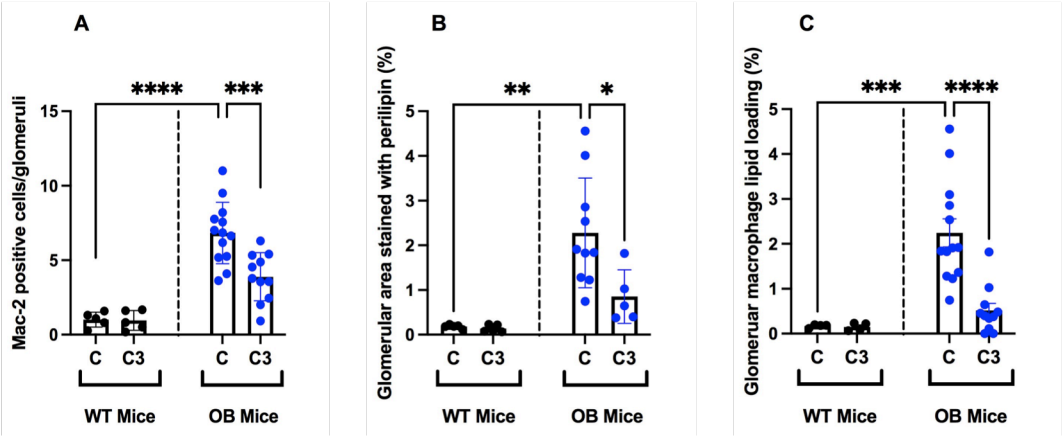

Figure 3. **Reduced APOC3 decreases macrophage and lipid accumulation in kidney glomeruli.** Kidney sections from LDLR WT and OB mice were stained with perilipin-2 and Mac-2. A. Average number of Mac-2 positive cells per glomeruli. B. Glomerular area stained with perilipin-2. C. Glomerular area with lipid loaded macrophages. N=11 – 16, Data expressed as mean ± SEM, 2-way ANOVA followed by Sidak's multiple comparison test, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001.

Other cells apart from macrophages are also lipid-laden in DKD

Oil Red-O staining showed a dramatic increase in glomerular lipid deposition in the OB mice compared to the WT mice (Figure 1 C). However, in this experiment, only 52.8% of the perilipin-2 staining co-stained with macrophages, suggesting that almost ½ of all intracellular lipid droplets are not localized to macrophages, clearly indicating that lipid droplets are accumulating in other cell types within the glomerulus, such as endothelial cells, mesangial cells and/or podocytes.

Hyperglycemia stimulates expression in *Ccl2*, which may elevate *Ccl2* in kidney endothelial cells and may drive macrophage accumulations

Previous studies have shown that endothelial cells and podocytes can take up lipids, which may elicit kidney deterioration.[9,12] But hyperglycemia is also hypothesized to play a role in DKD.[13] To investigate whether diabetic and dyslipidemic environmental stimuli impacted endothelial cells in the kidney, primary endothelial cells from WT mice were extracted and stimulated *in vitro*. Cells were stimulated with low (1mg/ml) and high (5mg/ml) glucose and TNFα to mimic hyperglycemic conditions. Elevated levels of TNFα are associated with DKD progression, so TNFα was included to induce inflammatory marker expression to test if hyperglycemia could augment the effect of TNFα.[14] *Ccl2, Icam1, Vcam1*, and *Sele* mRNA levels were measured because they are key in regulating the recruitment of leukocytes into tissues and previous studies showed that elevated *Ccl2, Icam1, Vcam1,* and *Sele* were related to increased endothelial cell dysfunction.[12] With the lipid accumulation seen in macrophages in the diabetic and dyslipidemic mice, the increased inflammatory markers may drive macrophage recruitment in the endothelial cells and lead to DKD progression. In this experiment, cells stimulated with high glucose and TNFα showed an increase in *Ccl2* and *Icam1* mRNA compared to the low glucose and TNFα treated cells (Figure 4 A and C). Interestingly, *Vcam1* and *Sele* were not elevated under high glucose and TNF alpha conditions (Figure 4 B and D). Additionally, when the cells were treated with VLDL or palmitate, there were no significant increases in any of the inflammatory markers. This may suggest that for endothelial cells, VLDL and palmitate do not directly affect and were insufficient to be a primary driver of up-regulating the inflammatory markers. Additionally, TNFα elevations may play a role in the increased *Ccl2*

and *Icam1*, but since TNFα levels were not measured, this may be something to investigate in future research.

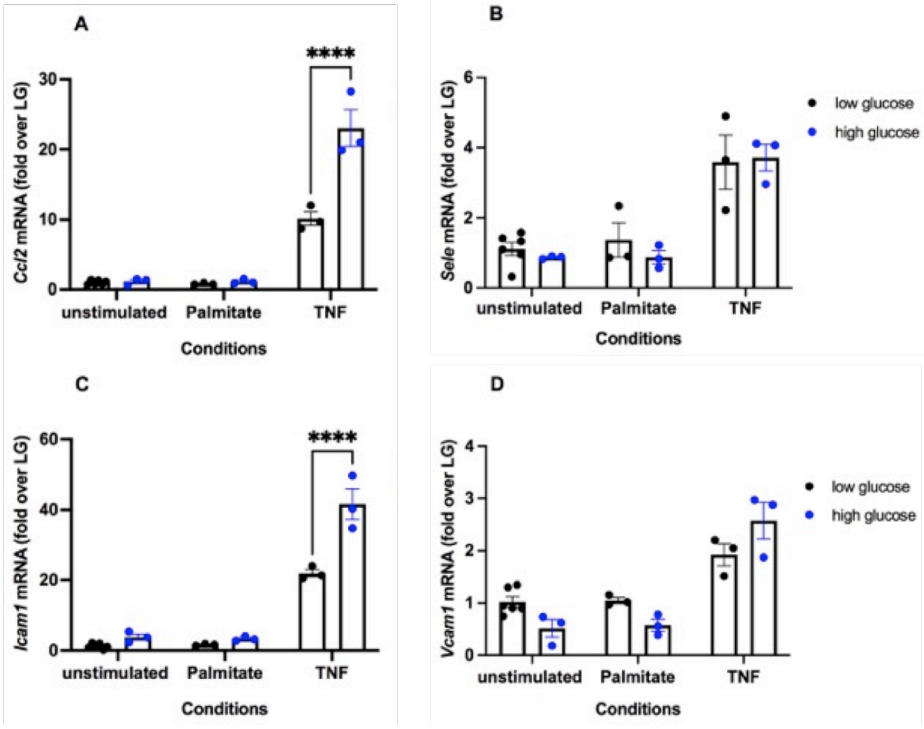

Figure 4. ***Ccl2* and *Icam1* are elevated under hyperglycemic conditions *in-vitro.*** Endothelial cells were stimulated with hyperglycemic conditions and mRNA levels of key inflammatory markers were assessed. A. *Ccl2* mRNA levels folded over low glucose B. *Sele* mRNA levels fold over low glucose C. *Icam1* mRNA levels fold over low glucose D. *Vcam1* mRNA levels fold over low glucose. N=4, Run in triplicate, Representative data shown, Data expressed as mean ± SEM, 2-way ANOVA followed by Sidak's multiple comparison test, ****p<0.0001.

To explore if these changes to endothelial cells occur *in vivo*, endothelial cells were labeled *in vivo* using a lectin that specially binds endothelial cells. Diabetic, but not dyslipidemic (not injected with LDLR ASO) OB mice and WT mice were injected with lectin and kidney endothelial cells were extracted and assessed for inflammatory markers. The OB mice had significantly elevated blood glucose levels. Although TG and cholesterol levels were statistically elevated in OB mice compared to WT mice, the levels were much lower than in the mice treated with the LDLR ASO and none of it is in VLDL and little in LDL suggesting that the elevated inflammatory markers were mainly driven by hyperglycemia.

Mice injected with lectin successfully labeled endothelial cells in the kidney, which can be observed in the positive streptavidin labeled endothelial cell staining compared to the control (Figure 5). The extracted endothelial cells were then assessed for inflammatory

markers that regulate monocyte recruitment, such as *Ccl2, Sele, Icam1*, and *Vcam1* mRNA. *Ccl2*, a chemokine that aids in monocyte recruitment, was significantly elevated in OB mice compared to the lean mice (Figure 6 A). Although not significant, *Sele*, an adhesion molecule that is necessary for monocytes to adhere to endothelial cells, might also be elevated in OB mice compared to lean mice (Figure 6 B).

The OB mice had elevated *Ccl2,* which may play a role in monocyte recruitment to the kidney endothelial cell. The only inflammatory marker that was elevated under *in vitro* and *in vivo* conditions was *Ccl2*, which may suggest that *Ccl2* is the only marker elevated by hyperglycemia. Therefore, elevated *Ccl2* levels may suggest a pathway for the recruitment of macrophages in the glomeruli under hyperglycemic and dyslipidemic conditions.

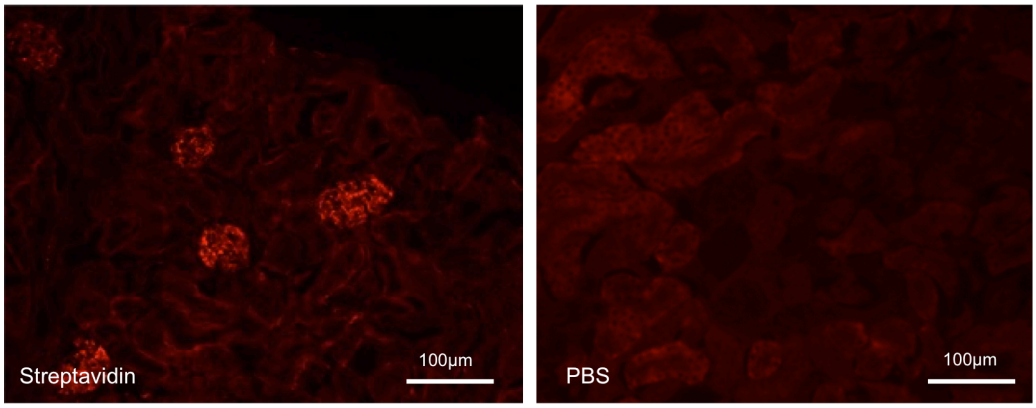

Figure 5. **Lectin successfully labels endothelial cells.** Mice were injected with lectin to isolate kidney endothelial cells. Endothelial isolation was confirmed with biotinylated lectin using streptavidin-Alexa-488 on frozen sections from the sacrificed mice.

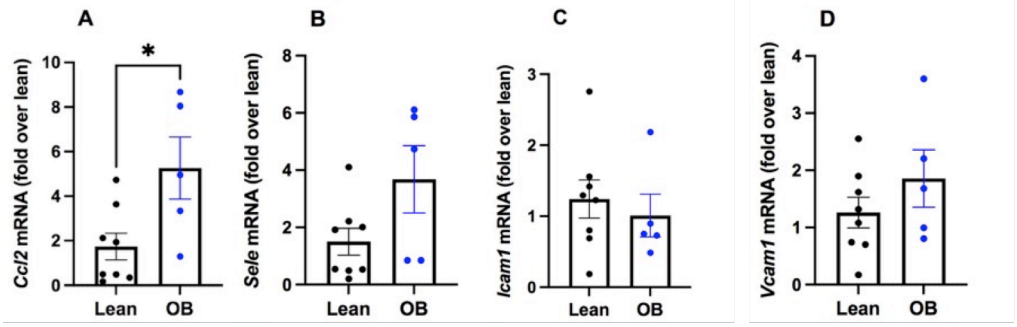

Figure 6. ***Ccl2* is elevated under hyperglycemic conditions *in-vivo***. Endothelial cells are isolated from lectin-injected mice. Key inflammatory marker's mRNA levels were assessed using qPCR. A. *Ccl2* mRNA levels fold over lean mice levels B. *Sele* mRNA levels fold over lean mice levels C. *Icam1* mRNA levels fold over lean mice levels D. *Vcam1* mRNA levels fold over lean mice levels. N=4 -8, Data expressed as mean ± SEM, Mann-Whitney T-test, *p<0.05.

Discussion

Kidney disease and lipids

In this current study, we observed that lipids accumulate in glomerular macrophages in diabetic and dyslipidemic mice. Further, the mice treated with APOC3 ASO had a significantly decreased proportion of glomerular lipid loading and glomerular macrophage lipid loading. In this study, the lipid accumulation was reduced in the APOC3 ASO treated kidneys, which suggest that the accumulated lipid particles are thought to be VLDL particles. Further, the decreased macrophage accumulation and their lipid loading in APOC3 ASO treated mice suggests that APOC3 plays a role in macrophage accumulation and lipid loading that may progress DKD. Additionally, these findings build on the results from Bornfeldt et al.'s study where glomerular macrophage accumulation resulted in worsened albuminuria and therefore the progression to DKD may be due to the lipid accumulation in glomerular macrophages. Lastly, the findings from this study are in line with results from human studies that have shown that lipids accumulate in the kidney glomeruli in participants with T2D, which may suggest that patients with diabetes also have neutral lipid accumulation in the kidneys.[6] The findings from this study further strengthen the relationship between APOC3 and renal lipid accumulation in DKD.

Hyperglycemia and increased lipids as a direct inducer of kidney disease

Our study also showed that hyperglycemic conditions elevated *Ccl2* levels in kidney endothelial cells, which may drive the recruitment of macrophages and monocytes. Our data was consistent with data from T2D humans, which were observed to have endothelial dysfunction as a marker for progression to DKD.[12] Further, the IRMA 2 study showed that increased baseline biomarkers that indicate endothelial dysfunction, such as elevated *Icam1, Vcam1*, and *Sele*, were associated with the development of macro-and microalbuminuria in T2D patients.[12] Therefore, elevated *Ccl2* levels may suggest a pathway for the recruitment of macrophages in the glomeruli under hyperglycemic conditions.

Study limitations and future directions

Previous studies indicate that there is potential lipid accumulation in other cells in the kidneys that may contribute to kidney function decline and DKD progression.[6] In this study, only macrophages were assessed for lipid loading in the glomeruli. For further investigation, the kidney sections from this experiment should be assessed for other cell types such as podocytes and endothelial cells to gauge whether there is lipid loading in these cells as well. Further, kidney sections were stained from already sacrificed mice, so we only know that lipids accumulate in the kidney glomeruli under diabetic and dyslipidemic conditions. Therefore, we do not know whether the accumulation of lipids is reflective of the increased TRLs in diabetic and dyslipidemic conditions or whether lipids accumulate as DKD progression worsens. Further investigation on whether blocking the lipid accumulation could prevent DKD progression is needed to understand whether the lipid accumulation is part of the pathological process of the disease. Lastly, a similar experiment should be conducted using samples from human kidneys to understand this mechanism in humans, since we conducted our research on mice kidneys.

Even with the vast knowledge and previous research on how lipids interact with different cells in the progression to DKD, the exact role of dyslipidemia associated with diabetes and hyperglycemia on progression to DKD in people with diabetes is still not well understood. By getting preliminary data on the association between increased lipid loading and macrophages in the glomeruli under diabetic and dyslipidemic conditions and observing the elevated levels of the inflammatory marker *Ccl2,* we can start to grasp a mechanism by which elevated lipids and hyperglycemic conditions lead to DKD progression. Understanding the mechanism and process can help develop treatments for the prevention of DKD progression.

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