Barutta F; Grimaldi S; Franco I; Bellini S; Gambino R; Pinach S; Corbelli A; Bruno G; Rastaldi MP; Aveta T; Hirsch E; Di Marzo V; Gruden G. Deficiency of cannabinoid receptor of type 2 worsens renal functional and structural abnormalities in streptozotocin-induced diabetic mice. KIDNEY INTERNATIONAL. 86 (5) pp: 979-990.
DOI: 10.1038/ki.2014.165

Deficiency of cannabinoid receptor of type 2 worsens renal functional and structural abnormalities in streptozotocin-induced diabetic mice

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A functionally active endocannabinoid system is present within the kidney. The cannabinoid receptor type 2 (CB2) is expressed by both inflammatory cells and podocytes, and its activation has beneficial effects in experimental diabetic nephropathy. To further explore the role of CB2 in diabetic nephropathy, we studied renal functional and structural abnormalities in streptozotocin-induced diabetic CB2 knockout mice. In diabetic mice, deletion of the CB2 receptor albuminuria, the downregulation of podocin and nephrin, mesangial expansion, overexpression of extracellular matrix components, monocyte infiltration, and reduced renal function were all exacerbated. To investigate the relative contributions of podocytes and monocytes to the phenotype of diabetic knockout mice, bone marrow transplantation experiments were performed. The lack of CB2 on bone marrow–derived cells was shown to be important in driving the enhanced glomerular monocyte accrual found in diabetic knockout mice. Absence of CB2 on resident glomerular cells had a major role in worsening diabetic nephropathy, both functional and structural abnormalities, likely by enhanced MCP-1 and CB1 signaling. Studies in cultured podocytes demonstrated that CB2 expression is not altered by a high glucose milieu but is downregulated by mechanical stretch, mimicking glomerular capillary hypertension. Thus, CB2 deletion worsens diabetic nephropathy, independent of bone marrow–derived cells.

Diabetic nephropathy (DN) is characterized by increased glomerular permeability to proteins and excessive extracellular matrix (ECM) deposition in the mesangium.[1] Both hyperglycemia and hypertension are key determinants in the development of DN.[2] In addition, increasing evidence suggests that a low-grade inflammatory response also has a role in disease etiology. In particular, the potent chemokine monocyte chemoattractant protein 1 (MCP-1) is an important mediator of both functional and structural abnormalities of the diabetic kidney.[3] The slit diaphragm, a junction connecting foot processes (FP) of neighboring podocytes, represents the major restriction site to protein filtration.[4] In human DN, there is a downregulation of the slit diaphragm proteins, nephrin, and podocin, and diabetes-related insults, such as advanced glycated products, MCP-1, and mechanical stretch, which mimics glomerular capillary hypertension, downregulate nephrin in cultured podocytes.[5, 6 and 7]

Endocannabinoids (EC), anandamide (AEA) and 2-arachidonoylglycerol (2-AG), bind to two G-protein-coupled seven transmembrane cannabinoid receptors named CB1 and CB2. In experimental diabetes, the CB1 is overexpressed by podocytes, and CB1 blockade ameliorates albuminuria and nephrin loss.[8] The CB2 is mainly expressed by cells of hematopoietic origin including monocytes–macrophages,[9] and CB2 activation is protective in animal models of chronic inflammatory diseases, such as atherosclerosis[10] and liver fibrosis,[11] whereas CB2 deletion exacerbates tissue damage by enhancing inflammatory, oxidative, and fibrotic processes.[12, 13 and 14] We have previously demonstrated that CB2 receptors are also expressed both *in vitro* and *in vivo* by podocytes and undergo downregulation in human DN. Furthermore, in experimental diabetes, AM1241, a specific CB2 agonist, reduces albuminuria by preventing loss of podocyte proteins important in preserving glomerular permselectivity. [15]

To further explore the role of the CB2 receptor in DN, we have assessed both functional and structural abnormalities of DN in CB2 knockout (KO) mice made diabetic with streptozotocin.

RESULTS

Metabolic and physiological parameters in wild-type (WT) and CB2-KO mice

As shown in Table 1, after 16 weeks of diabetes both blood glucose and glycated hemoglobin levels were significantly higher in diabetic (DM) than in nondiabetic (ND) mice. Furthermore, compared with controls, DM mice showed a significant decrease in body weight and a significant increase in kidney weight–to–body weight (KW/BW) ratio. Lack of CB2 receptors did not affect the degree of glycemic control in DM mice and did not alter body weight, KW/BW ratio, and systolic blood pressure in either ND or DM animals.

Table 1. Metabolic and physiological parameters in wild-type and $CB2^{-/-}$ knockout mice

	ND-WT	ND-KO	DM-WT	DM-KO
Body weight (g)	28.37±0.57	28.40±0.77	24.20±0.82a	23.86±0.51a
BG (mg/dl)	101±8.56	104±14.61	309±11.76a	338±15.68a
Glycated Hb (%)	5.01±0.10	5.02±0.09	10.94±0.22a	11.15±0.55a
SBP (mmHg)	109±7.27	115±7.08	117±6.46	115±5.69
KW/BW ratio	5.96±0.23	5.32±0.14	7.53±0.24a	7.57±0.22a
SCr (μmol/l)	6.33±0.31	6.27±0.35	6.45±0.31	8.46±1.02b
NAG (U/g)	86.27±2.42	81.29±6	142.74±10.05a	130.98±5.33a
AER (μg/18h)	87.4 (70.5–106.2)	87.3 (74.9–104.9)	216.2 (192–274.4)a	345.8 (329.3–352.8)a,b

Abbreviations: AER, albumin excretion rate; BG, blood glucose; DM, diabetic mice; KO, $CB2^{-/-}$ knockout; KW/BW, kidney weight/body weight; NAG, *N*-acetylglucosamine activity/creatinine ratio; ND, nondiabetic mice; SBP, systolic blood pressure; SCr, serum creatinine; WT, wild type. Data are shown as means±s.e.m. or geometric means (25th–75th percentile). a: $P<0.001$ DM-WT and DM-KO vs. ND-WT and ND-KO. b: $P<0.05$ DM-KO vs. others.

Albumin excretion rate (AER), urinary *N*-acetylglucosamine (NAG), and serum creatinine

There was a significant increase in urinary AER in DM as compared with ND animals. The absence of CB2 did not alter albuminuria in the controls but significantly enhanced AER in DM mice. NAG activity/creatinine ratio, a marker of tubular injury, was also significantly augmented in DM mice but did not differ in WT and KO DM animals. Diabetes did not enhance serum creatinine values in WT mice, and a significant reduction in renal function was exclusively observed in DM-KO mice (Table 1)

Podocyte abnormalities

As shown in Figure 1, there was a significant diminution in the glomerular staining for both nephrin and podocin in DM as compared with ND mice, and this effect was significantly worsened in DM-KO mice. Immunoblotting analyses confirmed a greater downregulation of both nephrin and podocin in DM-KO mice. The number of TUNEL (terminal deoxinucleotidyl transferase-mediated dUTP-fluorescein nick end labeling)- and cleaved caspase 3–positive cells per glomerular cross-sectional area displayed in a podocyte distribution was very modest and did not differ between groups (TUNEL: ND-WT: 1.9.0±0.6; DM-WT: 2.1±0.5; ND-KO: 2.0±0.2; DM-KO: 2.1±0.5. Cleaved caspase-3: ND-WT: 1.2±0.2; DM-WT: 1.7±0.3; ND-KO^{-}: 1.3±0.7; DM-KO: 1.5±0.5, positive cells/100 glomeruli, *P*=NS). Electron microscopy analysis showed areas of podocyte–FP fusion in DM mice, but the extent of FP effacement was similar in DM-WT and DM-KO mice (Figure 2a).

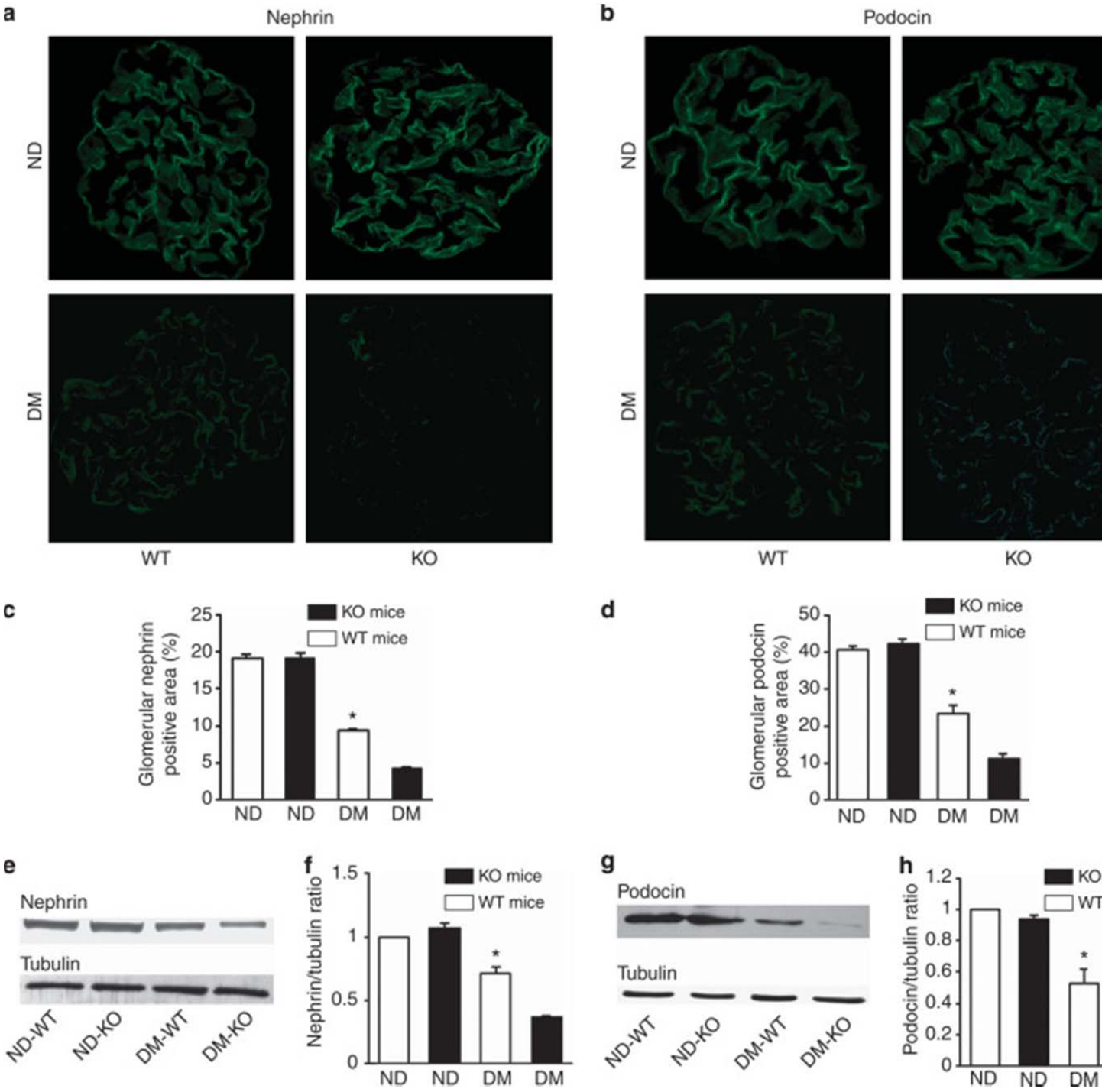

Figure 1.

Effect of the CB2 deficiency on diabetes-induced nephrin and podocin downregulation. Both nephrin and podocin expressions were assessed in ND-WT (*n*=12), ND-KO (*n*=6), DM-WT (*n*=9), and DM-KO (*n*=7) mice by immunofluorescence and immunoblotting. Representative immunofluorescence images of (**a**) nephrin and (**b**) podocin are shown (× 400), and (**c**, **d**) quantification of glomerular staining is reported in the graphs (**P*<0.001 DM-WT vs. ND-WT, ND-KO, and DM-KO). (**e**, **g**) Representative immunoblots of nephrin and podocin protein expression in total protein extracts from the renal cortex are shown. Tubulin was used as internal control. Results of densitometry analyses are reported in panel (**f**) for nephrin (**P*<0.01 DM-WT vs. ND-WT, ND-KO, and DM-KO) and in panel (**h**) for podocin (**P*<0.001 DM-WT vs. ND-WT and ND-KO; $^{\#}P$<0.05 DM-KO vs. DM-WT). DM, diabetic; KO, $CB2^{-/-}$ knockout; ND, nondiabetic; WT, wild type.

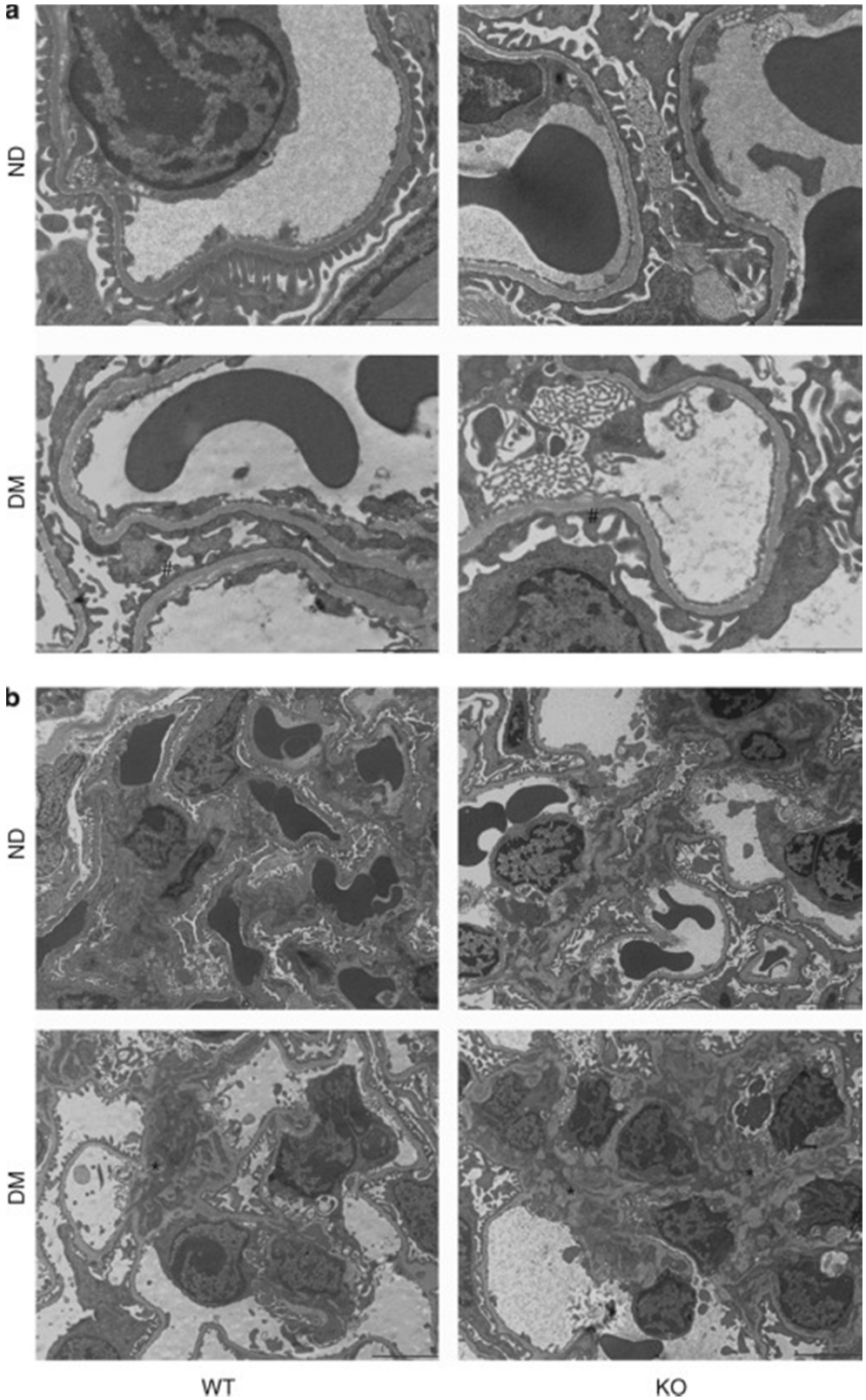

Figure 2.

Transmission electron microscopy analysis. Representative electron microscopy images showing glomeruli from both nondiabetic (ND) and diabetic (DM), wild-type (WT) and $CB2^{-/-}$ knockout (KO) mice. (**a**) The extent of foot processes fusion (#) was similar in WT and KO diabetic mice. (**b**) Glomeruli from DM-KO mice showed a more prominent mesangial expansion (*) than DM-WT mice.

Glomerular hypertrophy, mesangial expansion, and expression of ECM components

Glomerular volume was significantly increased in DM as compared with ND animals, and this effect was not altered by CB2 deletion (ND-WT: 140±9.05; DM-WT: 345±12.03; ND-KO: 134±4.05; DM-KO: 357±5.23 μm^3, $P<0.05$ DM-WT and DM-KO vs. ND). Histological assessment by PAS (periodic acid–Schiff) staining revealed a mild mesangial expansion and scattered tubulointerstitial damage in DM mice. The degree of glomerular injury was greater in DM-KO mice. Indeed, a more prominent mesangial expansion was observed by both light and electron microscopy (Figure 2b and Figure 3a and b). As shown in Figure 3e and h, the rise in both fibronectin and type IV (α4) collagen mRNA levels induced by diabetes was significantly greater in KO mice and an increase in type I collagen was exclusively observed in DM-KO animals (Figure 3d). Although changes in total renal cortex expression may also reflect tubulointerstitial abnormalities, immunohistochemical analysis and picrosirius red staining confirmed that glomerular expression of both collagen and fibronectin was greater in DM-KO compared with DM-WT mice (Figure 3c, f, and g). Finally, there was a significant increase in both transforming growth factor β1 and connective tissue growth factor mRNA expression in DM as compared with ND mice, and the expression of these prosclerotic cytokines was significantly greater in DM-KO than in DM-WT mice (Figure 3i and j).

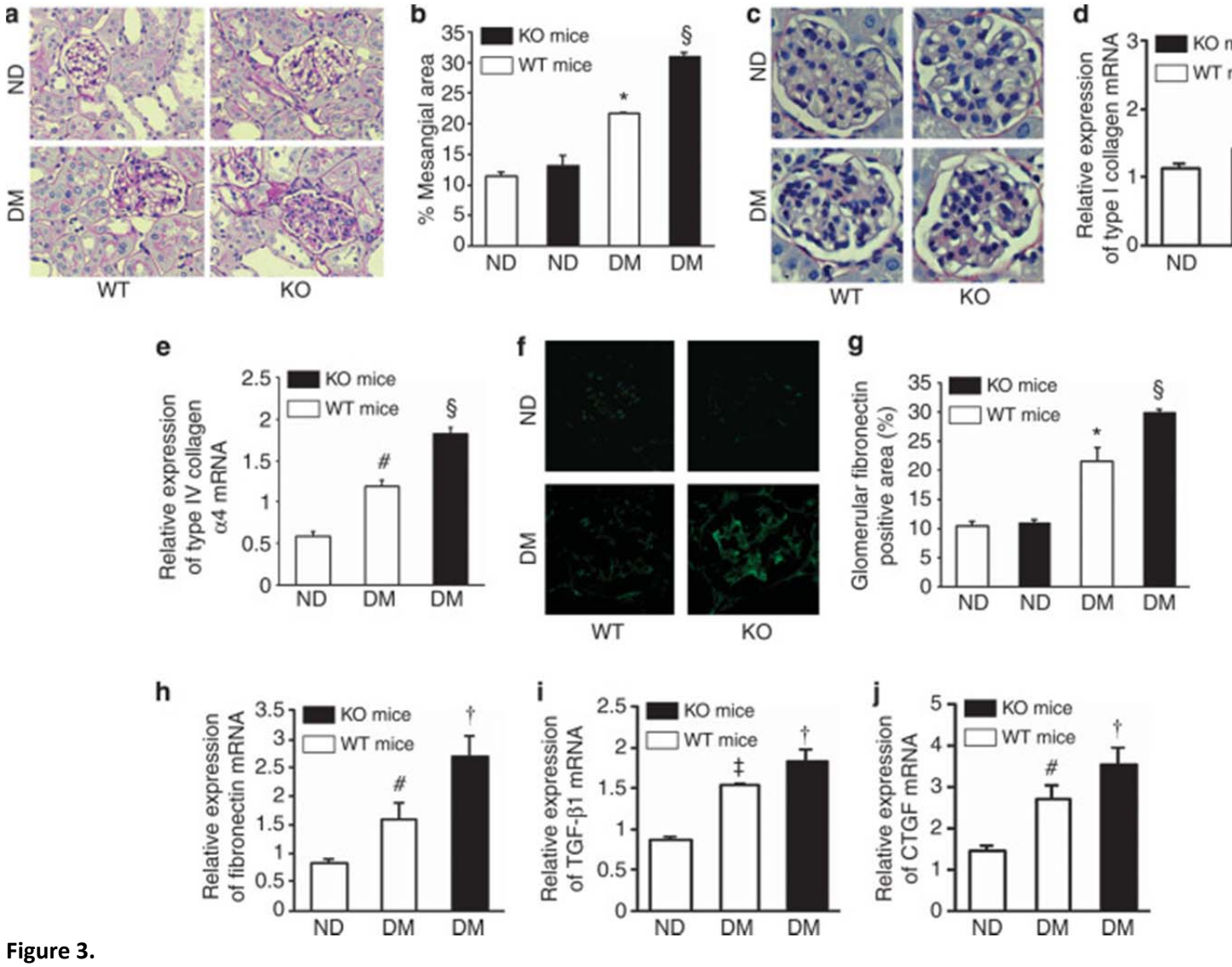

Figure 3.

CB2 absence worsens diabetes-induced glomerular structural abnormalities and enhances expression of fibrosis markers. Renal cortex samples from ND-WT (*n*=12), ND-KO (*n*=6), DM-WT (*n*=9), and DM-KO (*n*=7) mice were studied 16 weeks after diabetes induction. (**a**) Representative PAS staining images (× 400) and (**b**) quantitation of percentage of mesangial areas are shown. (**c**) Collagen staining and both (**d**) type I and (**e**) type IV collagen mRNA expression were studied by picrosirius red staining and real-time PCR, respectively. (**f**, **g**) Glomerular fibronectin expression was assessed by immunofluorescence and (**h**) renal cortex fibronectin gene expression by real-time PCR. (**i**) Transforming growth factor (TGF)-β1 and (**j**) connective tissue growth factor

(CTGF) mRNA levels were measured by real-time PCR in total renal cortex. ($^{\#}P<0.05$, $^{\ddagger}P<0.01$, and $^{*}P<0.001$ DM-WT vs. ND-WT; $^{\dagger}P<0.05$, and $^{\S}P<0.01$ DM-KO vs. DM-WT.) DM, diabetic; KO, $CB2^{-/-}$ knockout; ND, nondiabetic; WT, wild type.

Glomerular monocyte infiltration and MCP-1/C-C motif chemokine receptor 2 (CCR2) system

The number of glomerular cells positive for the monocyte marker F4/80 was significantly augmented in DM-WT as compared with ND mice. The absence of CB2 further increased diabetes-induced monocyte accrual without affecting monocyte infiltration in ND mice (Figure 4a and b). Expression of GR1, a marker of the M1 subpopulation of proinflammatory monocytes,[16] was greater in DM mice, and this effect was exacerbated by CB2 deficiency (Figure 4c).

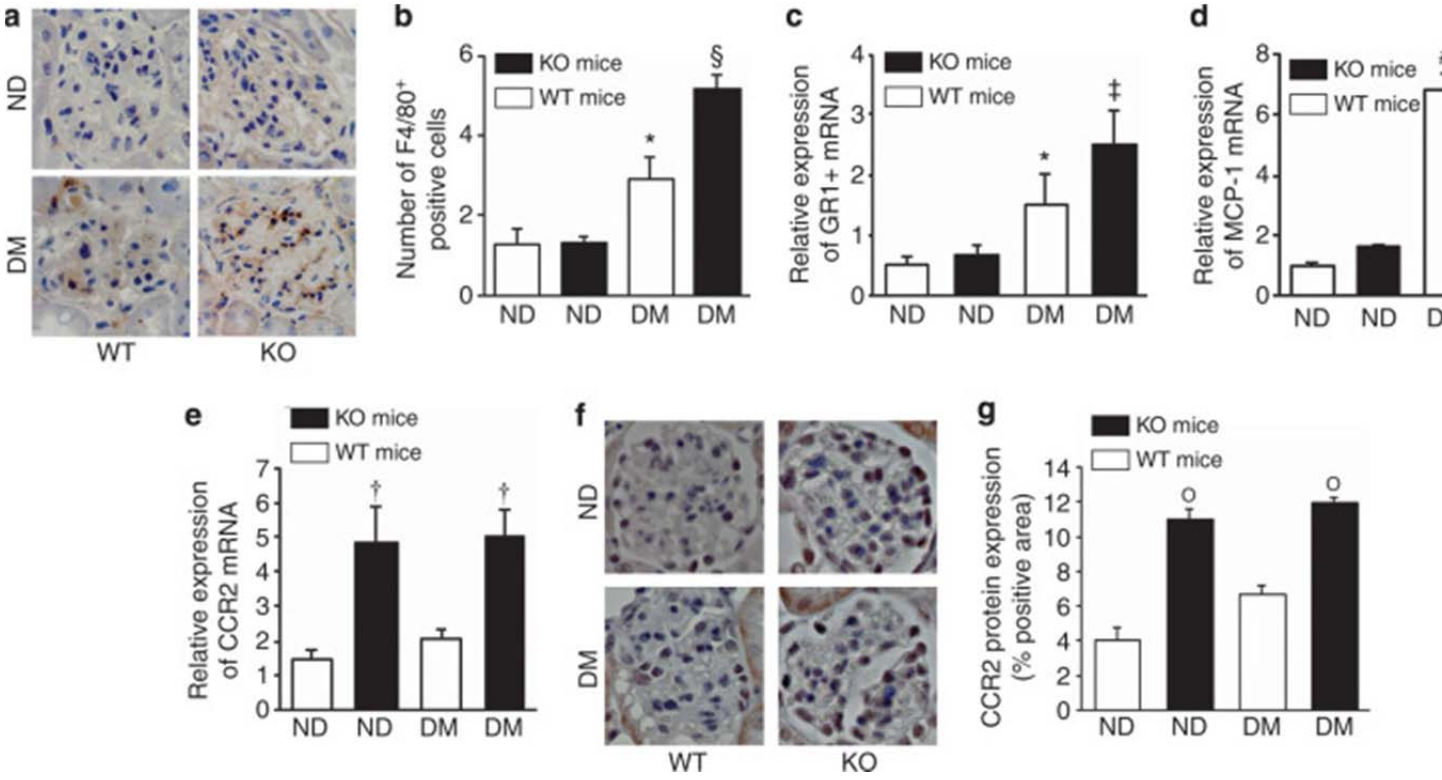

Figure 4.

Effect of CB2 deletion on markers of inflammation in diabetic (DM) and nondiabetic (ND) mice. ND-WT (n=12), ND-KO (n=6), DM-WT (n=9), and DM-KO (n=6) mice were studied 16 weeks after diabetes onset. Glomerular monocyte accrual was assessed by counting the number of F4/80-positive cells within the glomeruli. (**a**) Representative images are shown (× 400) and (**b**) the number of F4/80-positive cells are reported in the graph. (**c**) $GR1^{+}$, (**d**) monocyte chemoattractant protein 1 (MCP-1), and (**e**) C-C motif chemokine receptor 2 (CCR2) mRNA levels were measured by real-time PCR on total RNA extracts from the renal cortex, and results corrected for the expression of the housekeeping gene HRPT (hypoxanthine phosphoribosyltransferase). (**f**) Representative images of immunohistochemistry staining for CCR2 are shown (× 400) and (**g**) quantification of glomerular staining are reported in the graphs. ($^{*}P<0.05$ DM-WT vs. ND; $^{\ddagger}P<0.05$ and $^{\S}P<0.01$ DM-KO vs. DM-WT; $^{\#}P<0.001$ DM-WT and DM-KO vs. others; $^{\dagger}P<0.01$ and $^{\circ}P<0.001$ DM-KO and ND-KO vs. others.) KO, $CB2^{-/-}$ knockout; WT, wild type.

Diabetes increased MCP-1 mRNA levels without affecting the expression of the MCP-1 receptor CCR2 (Figure 4d). On the contrary, CB2 deletion did not alter MCP-1 expression but enhanced CCR2 mRNA levels in both ND and DM mice (Figure 4e). Assessment of glomerular CCR2 expression by immunohistochemistry confirmed CCR2 upregulation in mice lacking the CB2 receptor (Figure 4f and g).

Bone marrow (BM) transplantation study

To investigate the relative contributions of glomerular resident cells and BM-derived cells to the phenotype of DM-KO mice, BM transplantation experiments were performed. As shown in Table 2, BM transplantation did not affect metabolic/physiological parameters in either WT or KO animals. After 14 weeks of diabetes,

there was a significant increase in AER, serum creatinine levels, and fibronectin expression in DM-KO as compared with DM-WT animals, whereas podocin expression was significantly reduced. These abnormalities were not rescued by transplantation with BM from WT mice. Moreover, in DM-WT animals, transplantation of $CB2^{-/-}$ BM cells did not magnify albuminuria, fibronectin overexpression, renal function loss and podocin downregulation, thus failing to mimic the phenotype of DM-KO mice (Table 2 and Figure 5a–i). Collectively, these data suggest that BM-derived cells do not have a major role in mediating the deleterious effects of CB2 deprivation.

Table 2. Metabolic and physiological parameters in the bone marrow transplantation study

	ND-WT	**ND-WT-c^{ko}**	**ND-KO-c^{wt}**	**DM-WT**	**DM-KO**	**DM-WT-c^{ko}**	**DM-KO-c^{wt}**
Body weight (g)	31.1±0.8	30.3±1.3	29.9±0.4	24.9±0.7a	24.4±0.9a	23.1±1.9a	23.4±0.7a
BG (mg/dl)	98.7±6.5	100.5±2.3	102.5±3.2	360.2±23.5a	301.4±23.5a	365.7±18.5a	358.3±19.3a
Glycated Hb (%)	4.1±0.1	3.9±0.2	3.9±0.2	10.1±0.4a	9.4±0.6a	9.7± 0.8a	9.1±0.2a
SBP (mmHg)	122±2.0	118±3.5	120±1.8	120±1.0	118±2.6	117±36.3	113±2.5
KW/BW ratio	5.3±0.3	5.1±0.4	4.4±0.4	7.4±0.4b	7.3±0.94b	8.1±0.8b	7.6±0.3b
SCr (µmol/l)	5.27± 0.83	5.04±0.82	5.13±0.79	5.49±2.08	7.33±0.41d	5.17±0.70	6.85±0.56d
AER (µg/18h)	68.1 (64.6–75.3)	59.6 (56.7–65.7)	61.6 (57.4–68.9)	234.0c (200.0–285.9)	359.9d (227.9–519.4)	236.2c (211.5–257.4)	376.8d (353.2–368.5)

Abbreviatons: AER, albumin excretion rate; BG, blood glucose; c^{ko}, mice transplanted with bone marrow from $CB2^{-/-}$ mice; c^{wt}, mice transplanted with bone marrow from $CB2^{+/+}$ mice; DM, diabetic mice; KO, $CB2^{-/-}$ knockout; KW/BW, kidney weight/body weight; NAG, *N*-acetylglucosamine activity/creatinine ratio; ND, nondiabetic mice; SBP, systolic blood pressure; SCr, serum creatinine; WT, wild type. Data are shown as means±s.e.m. or geometric means (25th–75th percentile). a: $P<0.001$ all DM groups vs. all ND groups. b: $P<0.05$ all DM groups vs. all ND groups. c: $P<0.05$ DM-WT and DM-WT-c^{ko} vs. ND. d: $P<0.05$ DM-KO and DM-KO-c^{wt} vs. DM-WT and DM-WT-c^{ko}.

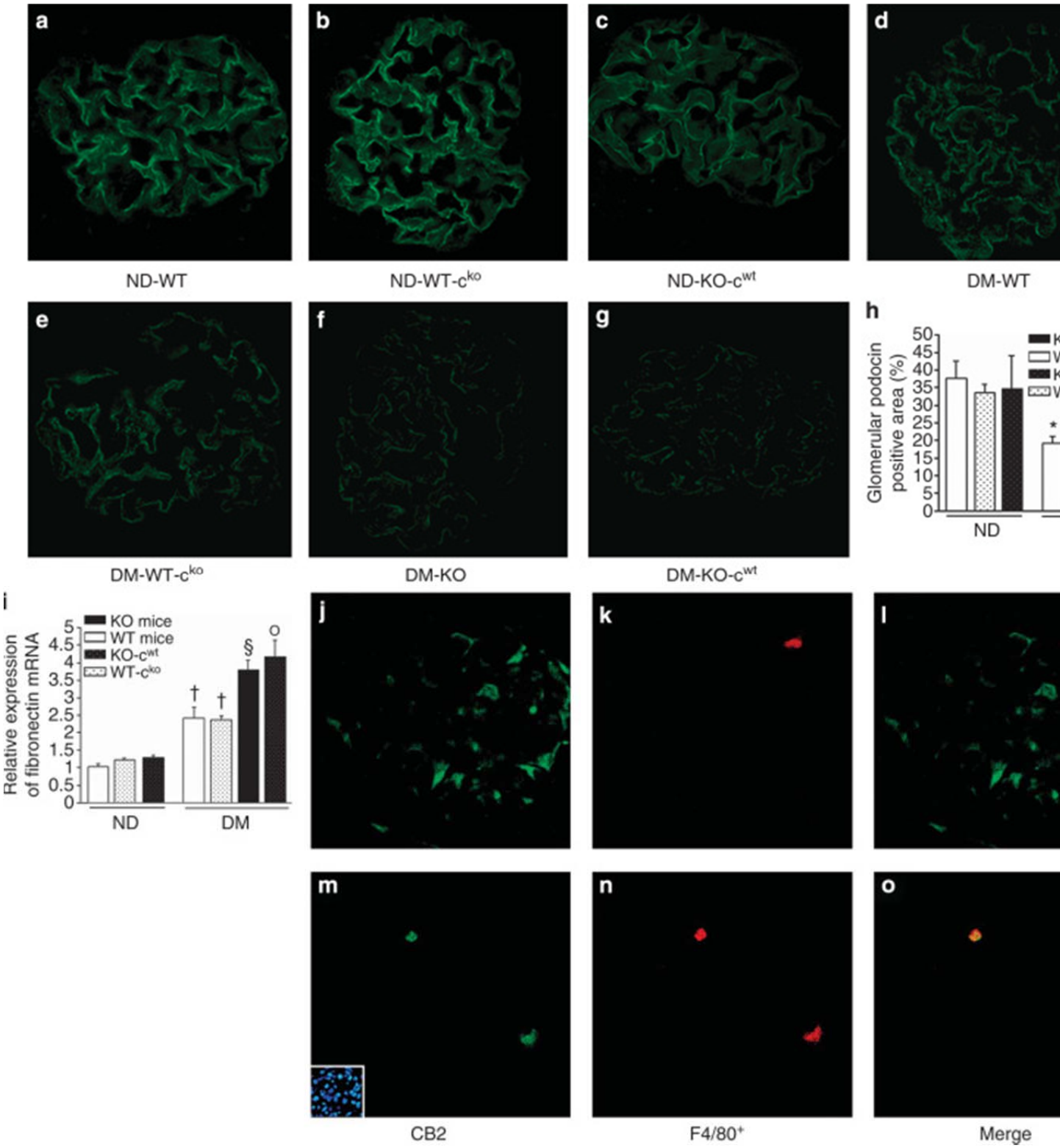

Figure 5.

Bone marrow (BM) transplantation study. Wild type, $CB2^{-/-}$ knockout (KO), and lethally irradiated recipient mice reconstituted with BM from either $CB2^{-/-}$ (chimera-KO c^{ko}) or WT (chimera-WT c^{wt}) mice were studied 14 weeks after diabetes onset (4–6 mice per group). Podocin expression was measured in renal cortex sections by immunofluorescence. (**a**–**g**) Representative immunofluorescence images are shown (× 400) and (**h**) quantification of glomerular staining is reported in the graph (*P<0.01 DM and DM-WT-c^{ko} vs. ND groups; $^{\#}P$<0.05 DM-KO and DM-KO-c^{wt} vs. DM and DM-c^{ko}). Fibronectin mRNA levels were measured by real-time PCR on total RNA extracts from the renal cortex, and (**i**) results were corrected for the expression of the housekeeping gene HRPT (hypoxantine phosphoribosyltransferase) ($^{\dagger}P$<0.05 DM and DM-WT-c^{ko} vs. ND groups, $^{\S}P$<0.05 DM-KO vs. DM-WT and DM-WT-c^{ko}; °P<0.01 DM-KO-c^{wt} vs. DM-WT and DM-WT-c^{ko}). In (**j**–**l**) DM-WT-c^{ko} and (**m**–**o**) DM-KO-c^{wt} mice, double

immunofluorescence for (**j**, **m**) CB2 and (**k**, **n**) the monocyte marker F4/80^{+} was performed and (**l**, **o**) merged images are shown. The insert in panel (**m**) shows nuclei counterstained with DAPI (4,6-diamidino-2-phenylindole).

To confirm the efficacy of BM transplantation in affecting CB2 expression on infiltrating monocytes, double staining for CB2 and F4/80 was performed in chimeric animals. As expected, in DM-WT animals transplanted with BM from KO mice (DM-WT-c^{KO}) glomerular immunostaining for CB2 showed a podocyte distribution and did not localize to infiltrating F4/80-positive cells (Figure 5j–l). On the contrary, in DM-KO animals transplanted with BM from WT mice (DM-KO-c^{WT}), the staining exclusively localized to cells expressing F4/80 (Figure 5m–o). The number of infiltrating monocytes was similar in DM-KO-c^{WT} and DM-WT mice, whereas it was enhanced in DM-WT-c^{KO} and of a comparable magnitude to that observed in DM-KO mice (ND: 0.22±0.07; DM-WT: 1.09±0.13; DM-KO: 2.34±0.11; DM-KO-c^{WT}: 1.25±0.08; DM-WT-c^{KO}: 2.53±0.17; P<0.01 DM-KO and DM-WT-c^{KO} vs. DM-WT and DM-KO-c^{WT}). This suggests that lack of CB2 on BM-derived cells drives the enhanced monocyte recruitment observed in DM-KO mice. Differences were not due to local changes in MCP-1 levels as the 2.3-fold increase in MCP-1 mRNA expression that was observed in DM as compared with ND mice was not significantly modified by either CB2 deletion or BM transplantation (DM-KO: 2.17±0.37; DM-KO-cWT: 2.16±0.15, DM-WT-cKO: 2.12±0.02; fold increase over control).

Effect of CB2 deletion on the EC system

We also investigated whether compensatory changes in the local EC system could account for the phenotype of DM-KO mice. In DM mice, expression of CB1 was enhanced (Figure 6a and b) and localized predominantly to podocytes as confirmed by co-staining with WT-1 (Figure 6c), whereas no overlap was found with the mesangial cell marker α-smooth muscle actin (Figure 6d). Similarly, CB2 had a predominant podocyte distribution and did not colocalize with mesangial cells (Supplementary Figure S1 online). Importantly, no changes in CB1 expression/distribution were observed in DM-KO mice with respect to DM-WT mice.

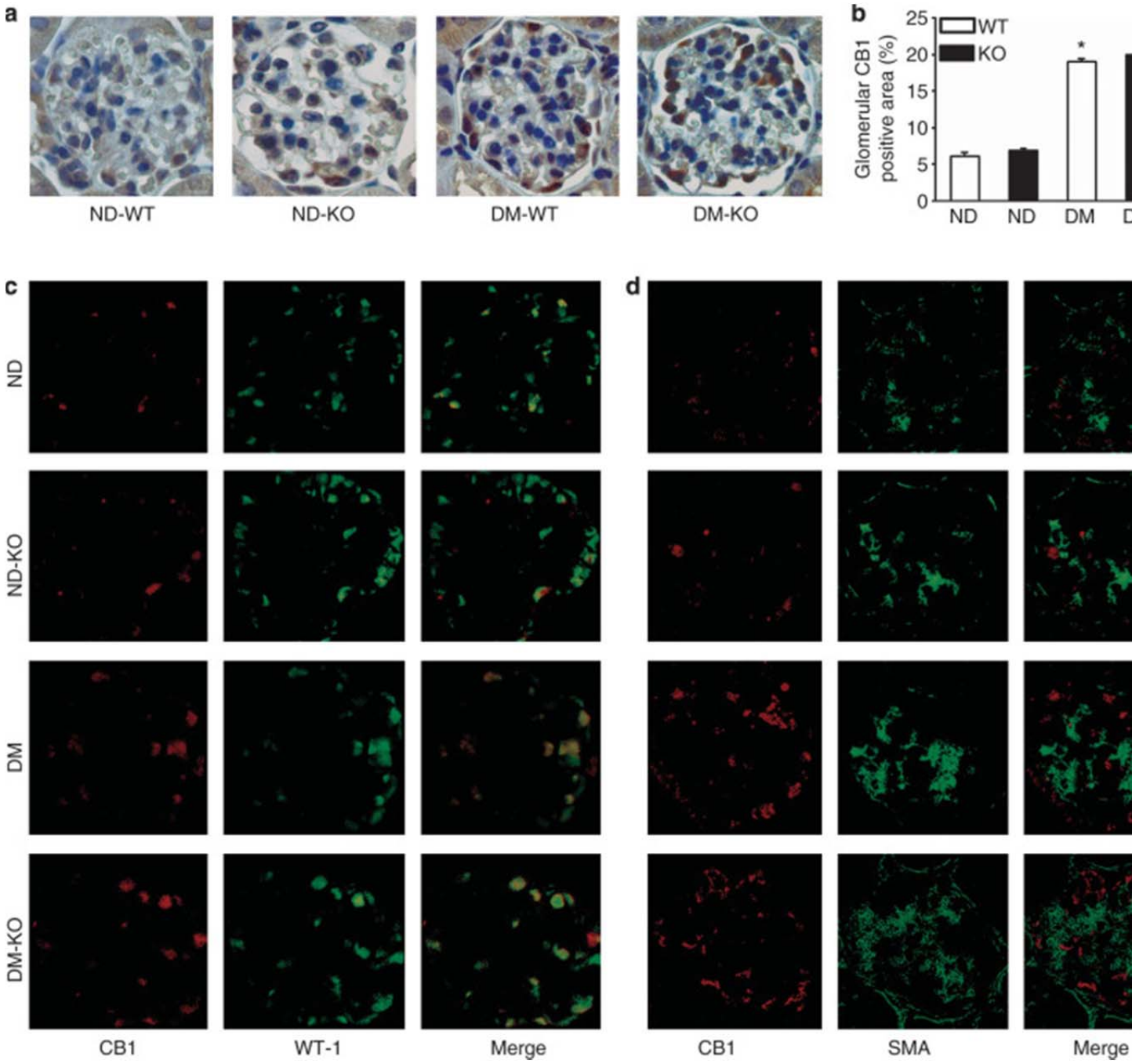

Figure 6.

Glomerular CB1 expression/distribution is not altered in CB2$^{-/-}$ knockout mice. CB1 protein expression was assessed in renal sections from ND-WT (*n*=12), ND-KO (*n*=6), DM-WT (*n*=9), and DM-KO (*n*=7) mice by (**a**) immunohistochemistry and (**b**) quantification of glomerular staining reported in the graph (*P<0.01 DM-WT and DM-KO vs. ND). Double immunofluorescence for CB1 (red) and (**c**) either the podocyte marker WT-1 (green) or (**d**) the mesangial cells' marker α-smooth muscle actin (SMA) (green) was performed in all studied groups and overlapped pictures (Merge) shown (**c**, **d**). DM, diabetic; KO, CB2$^{-/-}$ knockout; ND, nondiabetic; WT, wild type.

Figure 7 shows the levels of EC, EC-related molecules, and EC-related enzymes in the renal cortex of studied mice. 2-AG, the main CB2 ligand, was diminished in the renal cortex of DM mice in parallel with a reduced expression of the 2-AG synthetic enzyme diacylglycerol lipase-α, with no change in the levels of the degrading enzyme, monoacylglycerol lipase (Figure 7a–c). CB2 deletion did not modify these effects, but increased the levels of both AEA, the CB1 ligand, and oleoylethanolamine (OEA), an EC-related molecule (Figure 7a and b). This is was likely due to reduced degradation as levels of fatty acid amide hydrolase, which hydrolyzes both AEA and OEA, were significantly lower in DM-KO mice (Figure 7f), whereas the levels

of the AEA and OEA-biosynthesizing enzyme *N*-acyl phosphatidylethanolamine-specific phospholipase D were unaltered.

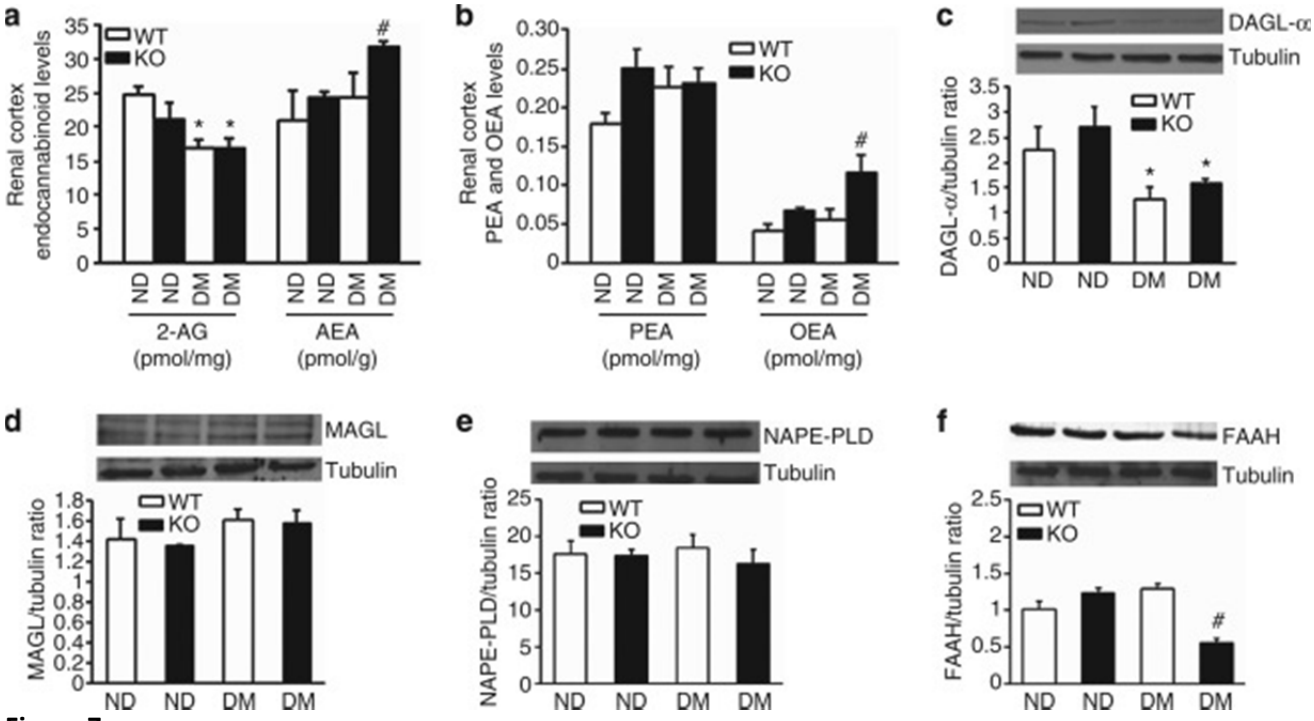

Figure 7.

Effect of CB2 deletion on the endocannabinoid system. Concentrations of (**a**) endocannabinoid (EC) (anandamide (AEA), 2-arachidonoylglycerol (2-AG)) and (**b**) endocannabinoid-related molecules (palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)) were measured in the renal cortex from ND-WT (*n*=4), ND-KO (*n*=3), DM-WT (*n*=5), and DM-KO (*n*=4) mice by isotope dilution liquid chromatography–mass spectrometry. Protein expression of (**c**) diacylglycerol lipase-α (DAGL-α), (**d**) monoacylglycerol lipase (MAGL), (**e**) fatty acid amide hydrolase (FAAH), and (**f**) *N*-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) was assessed in total renal cortex protein extracts by immunoblotting. Tubulin was used as an internal control. Representative immunoblots and results of densitometry analyses are shown. **P*<0.01 DM-WT and DM-KO vs. ND; #*P*<0.05 DM-KO vs. others. DM, diabetic; KO, $CB2^{-/-}$ knockout; ND, nondiabetic; WT, wild type.

In vitro study

Glomerular CB2 deficiency is a feature of DN in humans, and we explored in cultured podocytes the potential underlying mechanism. Exposure of podocytes to a high glucose milieu for 1, 2, and 3 days did not alter CB2 expression as assessed by immunoblotting. On the contrary, exposure to mechanical stretch induced a significant reduction in CB2 expression at 12 h, suggesting that a rise in intraglomerular pressure may contribute to CB2 downregulation in diabetes (Figure 8).

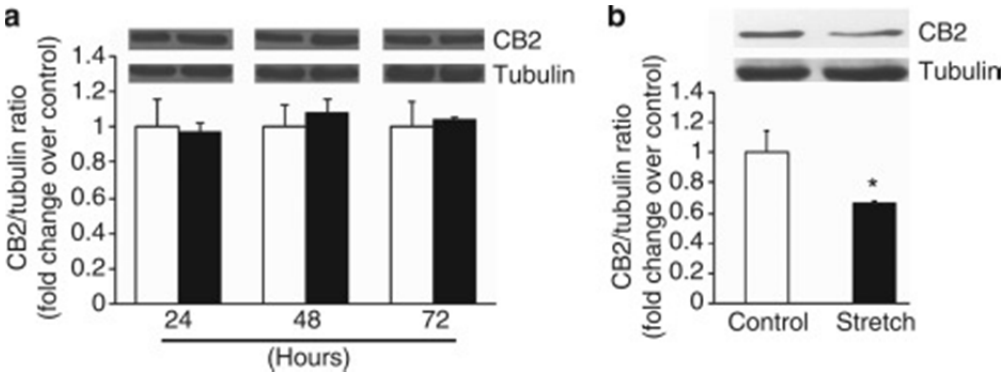

Figure 8.

Effect of high glucose and mechanical stretch on CB2 receptor expression in cultured podocytes. Serum-deprived differentiated podocytes were exposed to either (**a**) high glucose concentration (25 mmol/l) for 24, 48, and 72 h or (**b**) mechanical stretch for 12 h (black columns). Control cells in normal glucose concentration (7 mmol/l made iso-osmolar with mannitol) and in a mechanically stable environment were studied in parallel (white columns). Total proteins were extracted and CB2 expression assessed by immunoblotting. Densitometric analyses and representative immunoblots are shown. Results are expressed as fold change over control (n=3, *P<0.05 stretch vs. control).

DISCUSSION

In this study, we have provided evidence that, in experimental diabetes, deletion of the CB2 receptor worsens albuminuria, renal function, podocyte protein loss, overexpression of ECM components, and glomerular monocyte accrual.

Body weight, blood glucose levels, glycated hemoglobin, and systolic blood pressure were similar in WT and KO diabetic mice, indicating that the effects observed were independent of both metabolic and hemodynamic factors. A study has reported enhanced body weight and reduced insulin resistance in $CB2^{-/-}$ mice with aging. However, CB2 deprivation affects these parameters exclusively in mice much older that those used in our study.[17] Importantly, no differences in albuminuria were observed between WT and $CB2^{-/-}$ mice in the absence of diabetes.

In DM mice, genetic deletion of the CB2 receptor induced a significant increase in albuminuria and magnified diabetes-induced downregulation of both nephrin and podocin. These data corroborate previous results, showing that treatment with the CB2 agonist AM1241 reduces diabetes-induced albuminuria and prevents nephrin loss.[15] Taken together, our current and previous findings indicate that the CB2 is an important regulator of both slit diaphragm protein expression and glomerular permselectivity. Ultrastructural analysis showed areas of podocyte–FP fusion in DM mice; however, the extent of FP effacement and podocyte apoptosis was similar in WT and KO DM mice, suggesting that slit diaphragm protein loss has a predominant role in exacerbating proteinuria. Consistently, development of albuminuria in the presence of nephrin loss and before FP effacement and podocyte apoptosis has been reported.[18,19]

CB2 deletion also worsened diabetes-induced mesangial expansion as proven by both histological and ultrastructural analyses. Moreover, expression of fibronectin, type I collagen, and type IV α4 chain collagen, a specific component of the glomerular basement membrane,[20] was further enhanced in $CB2^{-/-}$ DM mice, possibly because of a greater rise in transforming growth factor-β1 and connective tissue growth factor expression. An increase in serum creatinine levels was exclusively observed in DM $CB2^{-/-}$ mice, indicating that exacerbation of diabetes-induced glomerular damage had a significant impact on renal function. It is unlikely that tubular damage was implicated as NAG activity was comparable in diabetic KO and WT mice.

The observation that $CB2^{-/-}$ mice display enhanced susceptibility to diabetes-induced renal fibrosis is in agreement with previous studies demonstrating that $CB2^{-/-}$ mice exhibit augmented cirrhosis when exposed to CCl4,[13,21] exacerbated cardiac fibrosis after ischemia/reperfusion,[22] and enhanced dermal fibrosis in response to bleomycin.[12] Our results were, however, relatively unexpected in experimental diabetes as in this model treatment with the CB2 agonist AM1241 fails to ameliorate renal fibrogenesis.[15] The reason of this discrepancy is unclear; however, it is possible that the extent to which the CB2 receptor was modulated pharmacologically by AM1241 was not of sufficient magnitude to elicit a protective response on renal fibrosis. In DM animals, 2-AG levels, the major CB2 ligand, are reduced, thus a greater dose of exogenous agonist may be required to achieved specific therapeutic effects.

Diabetes enhanced glomerular monocyte infiltration, and this feature was further exacerbated by CB2 deletion. Similarly, in bleomycin-induced dermal fibrosis, CB2 deletion is associated with enhanced monocyte infiltration within the skin.[12] Moreover, CB2 activation attenuates both diabetes- and cisplatin-induced renal monocyte accrual,[15,23] whereas lack of CB2 magnifies cisplatin-induced inflammation and tissue injury.[23] Binding of the chemokine MCP-1 to the CCR2 receptor controls glomerular monocyte

recruitment,[3] and our data suggest that overexpression of MCP-1 and CCR2, which are induced by diabetes and by CB2 deficiency, respectively, may enhance MCP-1 signaling and thus monocyte recruitment. Consistently, expression of GR1, a marker highly expressed by the subpopulation of inflammatory monocytes that are specifically recruited through the MCP-1/CCR2 system,[16] was significantly increased in DM $CB2^{-/-}$ mice.

Enhanced glomerular monocyte infiltration is a potential mechanism whereby the absence of CB2 could indirectly affect both AER and mesangial expansion, because locally recruited monocytes can release cytotoxic products, reactive oxygen species, and cytokines that can alter neighboring glomerular cell phenotypes.[3] On the other hand, as podocytes also express both the CB2 and the CCR2,[6,15] enhanced MCP-1 signaling can have direct deleterious effects in this cell type.[6,24] To clarify which of these two alternative mechanisms has a predominant role and to explore the relative contributions of glomerular cells and BM-derived monocytes to the phenotype of DM $CB2^{-/-}$ mice, we studied the effect of BM transplantation in our experimental model. The study duration was shortened, because a significant change in albuminuria between diabetic $CB2^{+/+}$ and $CB2^{-/-}$ animals was detectable at earlier time points and because sub-lethal irradiation may affect survival and enhance the risk of concomitant infections in laboratory animals, particularly in the presence of diabetes. In DM chimeric mice, the degree of glomerular monocyte infiltration was predominantly of the donor phenotype, indicating that CB2 deficiency on BM cells has a dominant role in enhancing monocyte accrual in DM-KO mice. However, neither transplantation with BM from $CB2^{+/+}$ mice in DM $CB2^{-/-}$ mice nor transplantation with BM from $CB2^{-/-}$ mice into DM $CB2^{+/+}$ animals altered the magnitude of albuminuria, renal function loss, podocin downregulation, and fibronectin overexpression. Therefore, BM-derived cells affected glomerular monocyte accrual but did not have a major role in mediating the deleterious effects of CB2 deficiency. Enhanced infiltration of inflammatory cells was by itself insufficient to magnify the glomerular damage in this model, and resident glomerular cells appeared to have a major role. The mechanism by which the absence of CB2 on resident glomerular cells contributes to the phenotype of DM $CB2^{-/-}$ mice is unknown; however, in cultured podocytes, both genetic deletion and pharmacological blockade of CB2 lead to CCR2 overexpression,[15] and MCP-1 binding to CCR2 downregulates podocyte proteins.[6] Although podocytes have a relatively low profibrotic potential and CB2 was not expressed by mesangial cells, mesangial expansion has been reported in an animal model of early podocyte-specific injury,[18] and $CB2^{-/-}$ podocytes may affect deposition of ECM components acting on mesangial cells in a paracrine manner.

At variance with our findings, previous studies on other experimental models of chronic inflammatory diseases, such as dermal fibrosis, atherosclerosis, and nerve injury,[10,12,25] have shown that enhanced monocyte infiltration is the predominant mechanism of the deleterious effects of $CB2^{-/-}$ deletion. However, in these models, CB2 and CCR2 are weakly expressed by resident cells and predominantly exposed by infiltrating monocytes, whereas the opposite occurs within the glomeruli.

There is increasing evidence of opposing effects of the two EC receptors in various organs, including the kidney,[26, 27, 28 and 29] and we have previously shown that not only CB2 activation[15] but also CB1 blockade can ameliorate experimental DN.[8] Therefore, we explored the possibility that compensatory changes in the local EC system could account for the phenotype of DM-KO mice. Levels of AEA and OEA were significantly increased in DM-KO mice likely because of fatty acid amide hydrolase downregulation. Because AEA predominantly binds to CB1 receptors,[30] the increase in AEA may result in enhanced CB1 signaling, particularly in the absence of CB2 receptors. This raises the possibility that the deleterious effects of CB2 deprivation in our model were, at least in part, mediated through the CB1 receptor. On the other hand, OEA is a PPAR-α (peroxisome proliferator-activated receptor-α) agonist, and there is evidence of a renoprotective and anti-proteinuric effect of PPAR-α activation in various animal models of kidney injury, including DN.[31, 32 and 33] Furthermore, in cultured podocytes, PPAR-α agonists reduce apoptosis and increase nephrin expression.[31,34] It is thus tempting to speculate that the increase in OEA levels may represent a protective mechanism compensating for the deleterious effects of both CB2 deletion and enhanced CB1 signaling.

Our findings may have important implications for DN in humans. Genetic deletion of CB2 in experimental animals mimics the marked downregulation of glomerular CB2 receptors reported in patients with DN.[15] The underlying mechanism of CB2 downregulation is unclear; however, in our study, podocyte exposure to stretch significantly reduced CB2 expression, while high glucose, which is known to increase CB1 expression in podocytes,[35] was ineffective. This suggests that the hemodynamic insult of glomerular hypertension, a well-established cause of DN progression,[36] is an important modulator of the glomerular response to CB2 agonists and that strategies reducing blood pressure in the glomerular capillaries may have the additional benefit of restoring CB2 receptor expression. Glomerular CB2 expression is downregulated in patients in an advanced stage of the complication, whereas a reduced CB2 signaling due to diminished 2-AG synthesis appears to be the major abnormality in early experimental diabetes.[15] This raises the possibility that treatment with CB2 agonists may be beneficial in an early stage of DN, particularly in combination with renin–angiotensin system inhibitors. In this regard, it is noteworthy that β-caryophyllene, which is a natural CB2 agonist, has been shown to ameliorate cisplatin-induced nephrotoxicity in mice.[37] Further preclinical studies are required to assess whether the addition of a CB2 agonist to current therapy with renin–angiotensin system inhibitors may provide additional benefits.

MATERIALS AND METHODS

All materials were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

Animals

CB2-intact C57BL6/J and CB2-deficient B6.129P2-Cnr2tm1Dgen/J mice, backcrossed at least five generations to C57BL/6 mice, were from Jackson Laboratories (Bar Harbor, ME). Animals were maintained on a normal diet under standard animal house conditions. Principles of laboratory animal care (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov.offcampus.dam.unito.it/grants/olaw/references/phspol.htm) were followed. Both housing and care of laboratory animals were in accordance with Italian law (D.L.116/1992), and the study was approved by the local Ethical Committee. Diabetes was induced in mice aged 8 weeks by streptozotocin intraperitoneal injection as we have previously described.[15]

KO study

Groups of DM-WT (*n*=9) and DM-KO (*n*=7) mice with equivalent blood glucose levels and ND-WT (*n*=12) and ND-KO (*n*=6) mice were studied in parallel. Before euthanasia, blood samples were collected via saphenous vein puncture on alert 4-h-fasted animals. Glucose levels were measured using a glucometer (Accu-chek; Roche, Milan, Italy) and the glycated hemoglobin by quantitative immunoturbidimetric latex determination (Sentinel Diagnostic, Milan, Italy). Systolic blood pressure was assessed by tail-cuff plethysmography. Urinary albumin was measured by enzyme-linked immunosorbent assay (Bethyl Laboratories, Milan, Italy) in 18-h urine collections. Serum creatinine levels were determined by high-performance liquid chromatography and urinary NAG levels using a colorimetric assay (Roche). Sixteen weeks after diabetes onset, mice were killed by decapitation. The kidneys were rapidly dissected, weighed, and processed for subsequent analyses.

BM transplantation study

In this study, 6–8-week-old female $CB2^{-/-}$ and $CB2^{+/+}$ mice were used as BM donors. BM cells were flushed from tibial and femur cavities under sterile conditions, filtered through 70-μm nylon meshes (BD Biosciences, Milan, Italy), and then transplanted without further purification or *in vitro* expansion. Before transplantation, recipient male mice, aged 8 weeks, underwent whole-body irradiation with 8 Gy. After 24 h, postirradiated mice were injected with 2.0×10^6 BM cells via the tail vein. $CB2^{-/-}$ mice received a BM transplant from $CB2^{+/+}$ mice (KO-c^{wt}; *n*=15) and $CB2^{+/+}$ mice from $CB2^{-/-}$ mice (WT-c^{ko}; *n*=15). Diabetes was

induced as described above, and 14 weeks thereafter mice were killed. DNA was isolated (DNeasy Blood and Tissue Kit, Qiagen, Milan, Italy), and chimerism was confirmed by PCR. Only BM-chimeric mice that showed >80% reconstitution efficiency with donor cells were used.

In vitro experiments

Conditionally immortalized mouse podocytes were kindly provided by P. Mundel (Harvard Medical School, Boston, MA) and cultured as previously described.[38] After serum deprivation (0.5% FCS) for 24 h, differentiated podocytes were exposed to either a high (25 mmol/l) or normal (7 mmol/l made iso-osmolar with mannitol) glucose concentration for 24, 48, and 72 h. Cells, seeded on type I collagen-coated silicon elastomer-base culture plates (Flex I plates) were subjected for 6 and 12 h to repeated stretch–relaxation cycles by mechanical deformation using a Stress Unit as described previously.[7,39,40] Cells grown in a mechanically stable environment (Flex II plates) were used as controls.

EC measurement

EC and EC-related molecules were measured by isotope-dilution liquid chromatography–mass spectrometric analysis in lipid extracts of frozen kidney tissue samples from ND-WT (*n*=4), ND-KO (*n*=3), DM-WT (*n*=5), and DM-KO (*n*=4) mice as we described previously [15] and detailed in the Supplementary Information online.

Immunohistochemistry and immunofluorescence

Glomerular expression of CB1, CB2, CCR2, F4/80, cleaved caspase-3, nephrin, podocin, and fibronectin was assessed by either immunohistochemistry or immunofluorescence.[6,8,15] Co-staining for either CB1 or CB2 and specific markers of podocytes (WT-1), mesangial cells (α smooth muscle actin), and monocytes (F4/80) was performed by double immunofluorescence (Supplementary Information online).

Immunoblotting

Expression of nephrin, podocin, CB2, diacylglycerol lipase-α, monoacylglycerol lipase, fatty acid amide hydrolase, and *N*-acyl phosphatidylethanolamine-specific phospholipase D was measured in total protein extracts from either renal cortex or cultured podocytes by immunoblotting as detailed in the Supplementary Information online.

mRNA analysis

mRNA expression was analyzed by real-time PCR using pre-developed TaqMan reagents (Applied Biosystems, Monza, Italy) as detailed in the Supplementary Information online.

Histological and ultrastructural analyses

Paraffin-embedded tissue sections were stained with PAS. Mesangial area was analyzed (percentage of glomerular area) from digital pictures of 15–20 glomeruli per kidney per animal using the Axiovision 4.7 software (Milan, Italy).[41] Glomerular collagen was assessed by picrosirius red staining. The glomerular cross-sectional area was measured in 20 glomerular profiles per mouse using the Axiovision software.[42] Electron microscopy was performed as detailed in the Supplementary Information online.

Statistical analysis

Data, presented as mean±s.e.m. or geometric mean (25–75% percentile), were analyzed by analysis of variance. Least significant difference test was used for *post hoc* comparisons. Values for $P<0.05$ were considered significant.

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