SUPPLEMENTAL MATERIAL

Supplemental Methods

In vivo studies

Biochemical parameters

Blood glucose levels were assessed with reflectance meter (OneTouch UltraEasy, LifeScan, Milpitas, CA, USA). Plasma cholesterol and triglycerides were measured by Reflotron test (10745065202 and 10745049202, Roche Diagnostic Corporation, Indianapolis, IN, USA). Urinary albumin excretion was measured by ELISA test (E101, A90-134A and A90-134P, Bethyl Laboratories Inc, Montgomery TX, USA). Systolic blood pressure was measured with a computerized tail-cuff system in conscious mice (BP-2000, Blood Pressure Analysis System, Visitech System, Apex, NC, USA).

Renal histology

Kidney samples were fixed in Duboscq-Brazil, dehydrated and embedded in paraffin. Three-micrometer sections were stained with periodic acid-Schiff (PAS) reagent, and at least 50 glomeruli were examined for each animal. The degree of glomerular mesangial matrix expansion was quantified using a score from 0 to 3 (0=no mesangial matrix expansion; 1=minimal; 2=moderate; 3=diffuse mesangial matrix expansion). The number of glomeruli exhibiting mesangiolysis in an entire kidney section was counted and expressed as a percentage. All biopsies were reviewed by a blinded pathologist. Samples were examined using ApoTome Axio Imager Z2 (Zeiss, Jena, Germany).

Podocyte number and glomerular podocyte density

Podocytes were identified as cells positive for Wilms' tumor 1 (WT1) by immunofluorescence as

described in the Method's section. Using a confocal microscope (LSM 510 Meta, Zeiss) about 30 glomeruli for each animal were acquired (original magnification X630), and then analyzed using the LSM Image Browser. In merged images, podocytes were identified by the overlap of WT1 staining (red) with DAPI staining (blue). The outline of the minimal polygon around the glomerular tuft area was manually traced and its surface area automatically measured as pixel^2. For each glomerulus, podocytes (pink) were counted as WT-1 and DAPI positive cells and minor and major diameters were measured. The mean value of glomerular volume (VG) was then calculated using the formula:

$V_G = (\beta/k)(A_m)^{3/2}$ where k = 1.01 is a size distribution coefficient, and β = 1.38 is the shape coefficient for spheres, which is the assumed shape of glomeruli.

The estimation of the average number of podocytes per glomerulus (NP) $N_V = N_A/\overline{D}$ was determined by the stereological method of particle density proposed by Weibel (Weibel, 1979). Briefly, the volume density of the podocytes (N_V) in glomerular tuft volume is estimated as where N_A, the podocyte nuclear profile area density, is estimated by the ratio between the numbers of podocyte nuclear profiles and the glomerular profile area in each glomerulus; $\overline{D}$ is the average diameter of podocyte nuclei that are estimated from the major and minor axis of cell nuclear sections. To calculate $\overline{D}$, the average volume of podocyte nuclei ($\overline{V}$) is first calculated based on the assumption that podocyte nuclei have an ellipsoidal shape. The average diameter of an equivalent sphere having the same volume of the ellipsoid is then determined. The mean number of podocytes per glomerular tuft (NP) is calculated for each animal by multiplying podocyte volume density (NV) in the capillary tuft by the mean value of VG previously calculated.

Ultrastructural analysis

Fragments of kidney tissue were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and washed repeatedly in the same buffer. After postfixation in 1% OsO4, specimens were

dehydrated through ascending grades of alcohol and embedded in Epon resin. Ultra-thin sections were stained with uranyl acetate replacement (Electron Microscopy Sciences, Hatfield, PA) and lead citrate and examined using transmission electron microscope (Morgagni 268D, Philips, Brno, Czech Republic).

In vitro

Immunofluorescence analysis

At the end of the incubations, podocytes were fixed in 2% paraformaldehyde and 4% sucrose, then permeabilized with 0.3% Triton X-100 (Sigma Aldrich) in PBS for 3 minutes at room temperature. Then, nonspecific binding sites were blocked with 2% FBS, 2% bovine serum albumin and 0.2% bovine gelatin in PBS 1X. Cells were incubated with a rabbit anti-SOD2 (1:500; 06-984 Millipore) or anti-phosphofructokinase (PFK) (1:150; ab181064 Abcam) antibodies followed by incubation with a goat anti-rabbit Cy3-conjugated secondary antibody (1:80; 111-165-144 Jackson ImmunoResearch Laboratories). Nuclei were counterstained with DAPI (Sigma Aldrich). Negative controls were obtained by omitting primary antibodies. Samples were examined using confocal inverted laser microscope (LSM 510 Meta and LSM 880, Zeiss). The quantification of SOD2 and PFK expression was performed on 15 random fields per sample. Specifically, the areas corresponding to the stainings were measured in $pixel^2$ by using the Image J 1·40g software and normalized for the number of nuclei identified by DAPI staining.

Mitochondria isolation

Mitochondria were isolated from cultured human podocytes using Qproteome Mitochondria Isolation Kit (37612; Qiagen S.r.l., Milan, Italy) according to the manufacturer's protocol. Isolated mitochondria were solubilized in mitochondrial storage buffer (37612; Qiagen S.r.l.) and total protein concentration was determined using DC™ assay (5000112; Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

Isolated mitochondrial proteins were separated on 10-15% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (1620112, Bio-Rad Laboratories). After blocking with 5% BSA (A7906, Sigma Aldrich) in Tris-buffered saline (TBS) supplemented with 0.05% Tween-20, membranes were incubated with a rabbit anti-Drp1 antibody (1:1000; 5391S, Cell Signaling, Leiden, The Netherlands) and a rabbit anti-ATP5I antibody (1:1000; HPA035010, Sigma Aldrich). On the same membranes, mouse anti-VDAC (1:1000; ab14734, Abcam) was used a sample loading control. The signals were visualized on an Odyssey®FC Imaging System (LiCor, Lincoln, Nebraska, USA) by infrared (IR) fluorescence using a secondary goat anti-rabbit IRDye 680LT antibody (FE3680210, LiCor) and a goat anti-mouse IRDye 800CW (FE30926210, LiCor), as appropriate. Bands were quantified by densitometry using the Image Studio Lite (LiCor) 5.0 software.

ATP content assay

After incubations, podocytes were washed twice, enzymatically detached, centrifuged and resuspended in ATP assay buffer (K354, BioVision, Milpitas, CA, USA). Cells were lysed by sonication followed by using a blunt-ended needle and a syringe. The lysates were centrifuged 12,000 x g for 10 min at 4°C to remove detergent-insoluble material. Supernatants were harvested and total protein concentration was determined using DC™ assay (5000112, Bio-Rad Laboratories). Then supernatants were deprived of proteins by using a deproteinizing preparation kit (K808, BioVision) according to the manufacturer's protocol. ATP content assay (K354, BioVision) was then performed on deproteinized samples according to the manufacturer's protocol. The fluorescence intensities were determined by the multimode microplate reader TECAN Infinite M200® PRO (Tecan Group Ltd., Mannedorf, Schweiz) at an excitation wavelength of 535 nm and emission wavelength of 587 nm. Results were normalized for the total protein concentration of each sample.

Citrate synthase activity

After incubations, podocytes were washed twice, enzymatically detached, centrifuged and resuspended in CelLytic MT buffer (C3228, Sigma Aldrich) supplemented with protease inhibitor cocktail (P8340, Sigma Aldrich). Total cell lysate was obtained by sonication followed by a blunt-ended needle and a syringe. The cell lysates were then centrifuged 12,000 x g for 10 min at 4°C to remove detergent-insoluble material. Supernatants were collected and total protein concentration was determined using DC™ assay (5000112, Bio-Rad Laboratories). Equal amounts of proteins (10 μg) were analysed by citrate synthase activity assay (CS072, Sigma Aldrich) according to the manufacturer's protocol. The citrate synthase activity was determined by the multimode microplate reader TECAN Infinite M200® PRO (Tecan Group Ltd.) at 412 nm under temperature controlled on a kinetic program for 1.5 min every 10 sec.

Lactate dehydrogenase activity

After incubations, podocytes were washed twice, enzymatically detached, centrifuged and resuspended in LDH assay buffer (K726; BioVision). Cells were lysed by sonication followed by using a blunt-ended needle and a syringe. The sample lysates were then centrifuged 10,000 x g for 15 minutes at 4°C to remove detergent insoluble material. Supernatants were harvested and total protein concentration was determined using DC™ assay (5000112, Bio-Rad Laboratories). Lactate dehydrogenase activity content assay (K726, BioVision) was then performed on supernatants according to the manufacturer's protocol. Measurement of OD at 450 nm was performed on the multimode microplate reader TECAN Infinite M200® PRO at 37°C at the beginning of the reaction and after 30 minute incubation.

Scratch-healing assay

Migration was assessed in confluent podocytes scratched with 200-μl pipette tip to create a cell-free denuded area. Cells were exposed to control medium or C3a (1 μM) in the presence or absence of 5

μM SS31 (736992-21, BOC Sciences, Shirley, NY, USA) and monitored by phase-contrast under an 10X objective on a time-lapse microscopy (Axio Imager.z2 microscopy, Zeiss, Germany). Images were taken at different time intervals (0, 3, 6, 9, 12 and 15 hours) and the number of cells migrated into the wound track was counted at each time point (n= 5 fields/well).

Measurement of intracellular cAMP

After incubations, podocytes were washed twice, enzymatically detached, centrifuged and resuspended in HCl 0.1M. Cells were lysed by sonication and samples were then centrifuged 10,000 x g for 15 minutes at 4°C to remove detergent insoluble material. Supernatants were harvested and total protein concentration was determined using DC™ assay (5000112, Bio-Rad Laboratories). The concentration of cAMP was assessed in each sample using the cAMP direct immunoassay kit (K371, BioVision) according to the manufacturer's protocol. Measurement of OD at 450 nm was performed on the multimode microplate reader TECAN Infinite M200® PRO.

Supplemental figure legends

Supplementary Figure 1. Representative images of immunofluorescence staining for C3 (green) and C3a (red) in the liver (A-B) and in the spleen (C-D) of WT and BTBR *ob/ob* mice. Renal structures and nuclei are stained with FITC-WGA-lectin (green) and DAPI (blue), respectively. Images show increased C3 and C3a staining in hepatic sinusoidal capillaries of BTBR *ob/ob* mice (insets A and B). In BTBR *ob/ob* mice, C3 and C3a-positive cells were observed in the parenchyma and in the germinal centers of the spleen (insets C and D). (WT n=3, BTBR *ob/ob* n=3). Scale bars: 25 μm.

Supplementary Figure 2. (A) Representative images of double immunofluorescence staining for C3aR (red) and nestin (green) in C57Bl/6 mice four months after i.p. injection with a single dose of streptozotocin (STZ 200 mg/kg) or saline (control). The yellow areas (arrowheads) indicate C3aR and nestin co-localisation in podocytes. Scale bars: 25 μm. (B) Quantification of the C3aR, nestin and C3aR/nestin co-staining in the glomeruli of control and STZ-treated mice. Results are mean ± SEM (n=4 mice/group) and two-sided unpaired Student's *t* test was used, *P<0.05, **P<0.01.

Supplementary Figure 3. (A) Representative images and quantification of mesangial matrix expansion and mesangiolysis in BTBR WT, and BTBR *ob/ob* mice injected with vehicle or C3aR antagonist. Scale bars: 20 μm. Results are expressed as mean ± SEM (WT n=5, BTBR *ob/ob*+vehicle n=8, BTBR *ob/ob*+C3aR antagonist n=8) and ANOVA with Tukey multiple-comparisons test was used, *P<0.05, **P<0.01. (B) Representative images and quantification of glomerular infiltration of Mac-2 positive monocytes/macrophages. Scale bars: 20 μm. Results are

expressed as mean ± SEM (WT n=5, BTBR *ob/ob*+vehicle n=7, BTBR *ob/ob*+C3aR antagonist n=7) and ANOVA with Tukey multiple-comparisons test was used, *P<0.05, **P<0.01.

Supplementary Figure 4. Representative images and quantification of the nestin staining in BTBR WT, and BTBR *ob/ob* mice injected with vehicle or C3aR antagonist. Scale bars: 20 μm. Results are expressed as mean ± SEM (WT n=5, BTBR *ob/ob*+vehicle n=8, BTBR *ob/ob*+C3aR antagonist n=8) and ANOVA with Tukey multiple-comparisons test was used, *P<0.05, **P<0.01.

Supplementary Figure 5. (A) Representative images obtained after skeletonisation by Fiji software for quantification of mitochondrial network, individual mitochondria and mitochondrial area in control or C3a-treated podocytes (6 hours) by Mitochondrial Network Analysis (MiNA) toolset. Scale bars: 20 μm. (B) Quantification of SOD2 expression in podocytes exposed for 6 hours to control medium or C3a (1 μM), in the presence or absence of SS-31 (5 μM). Results are expressed as mean ± SEM (n=3 each group) and ANOVA with Tukey multiple-comparisons test was used, **P<0.01.

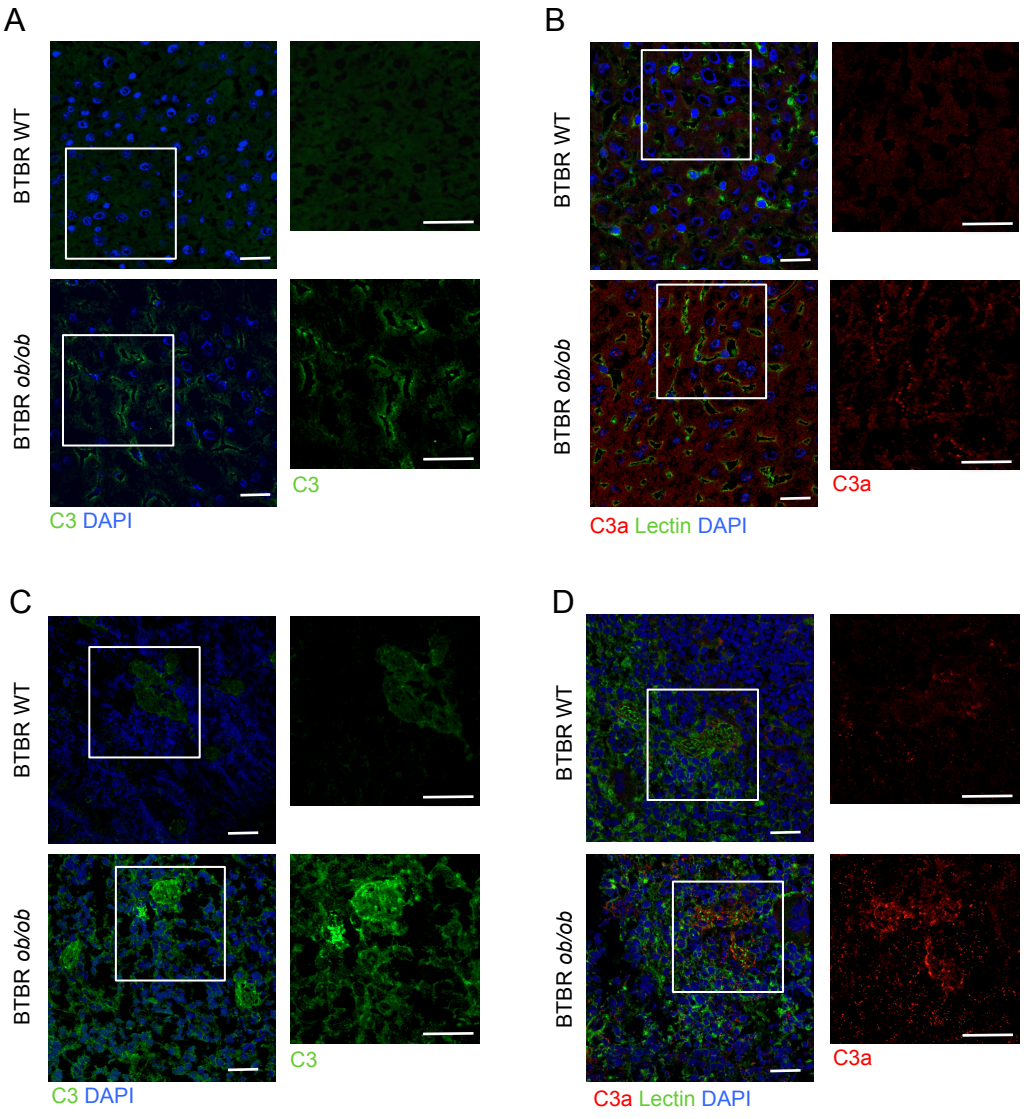
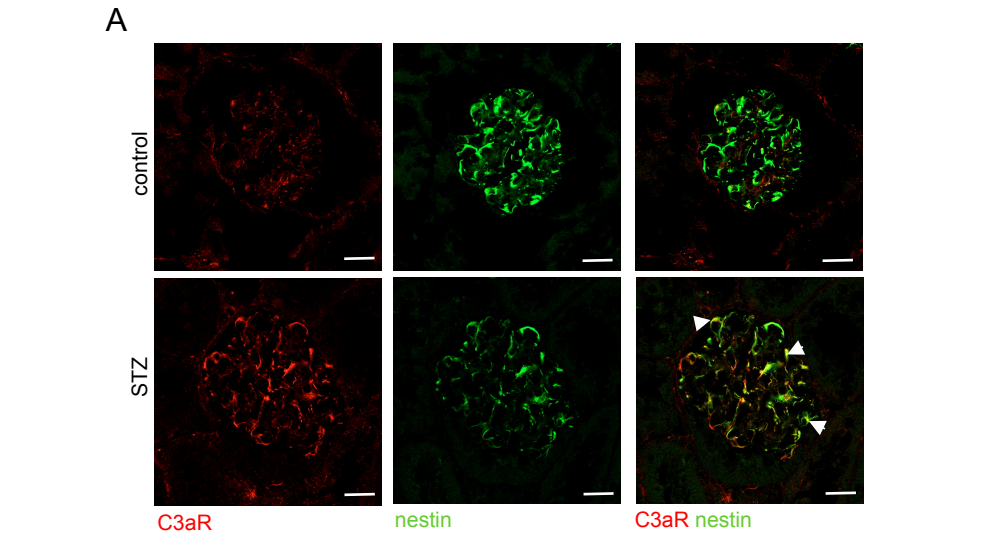
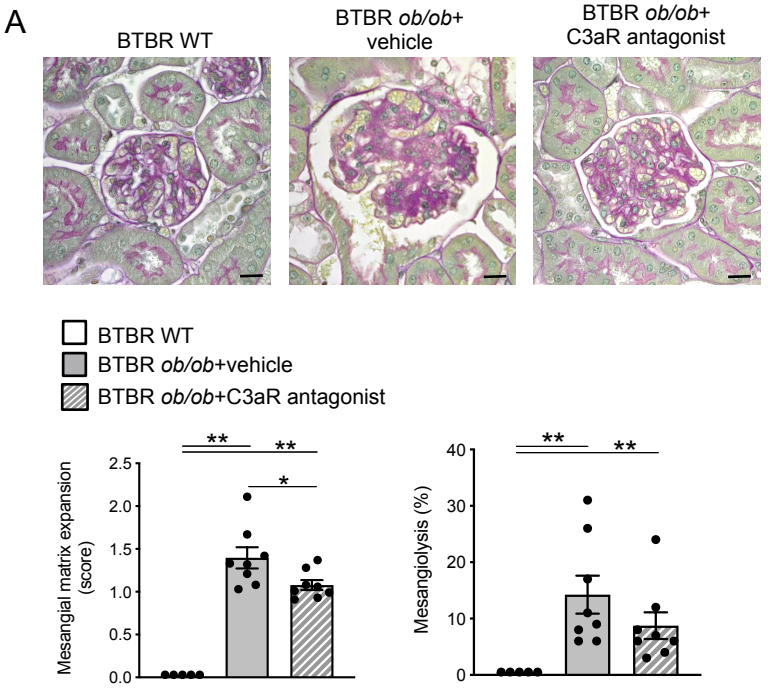

A
BTBR WT
BTBR ob/ob
C3
C3 DAPI
B
BTBR WT
BTBR ob/ob
C3a
C3a Lectin DAPI
C
BTBR WT
BTBR ob/ob
C3
C3 DAPI
D
BTBR WT
BTBR ob/ob
C3a
C3a Lectin DAPI

Figure S1


A
control
STZ
C3aR
nestin
C3aR nestin


B
Glomerular C3aR expression (% glomerular area)
**
control
STZ
nestin expression (% glomerular area)
*
control
STZ
C3aR/nestin costaining (% of glomerular C3aR area)
**
control
STZ

Figure S2


A
BTBR WT
BTBR ob/ob+ vehicle
BTBR ob/ob+ C3aR antagonist
BTBR WT
BTBR ob/ob+vehicle
BTBR ob/ob+C3aR antagonist
Mesangial matrix expansion (score)
Mesangiolysis (%)
**
**
*
**
**

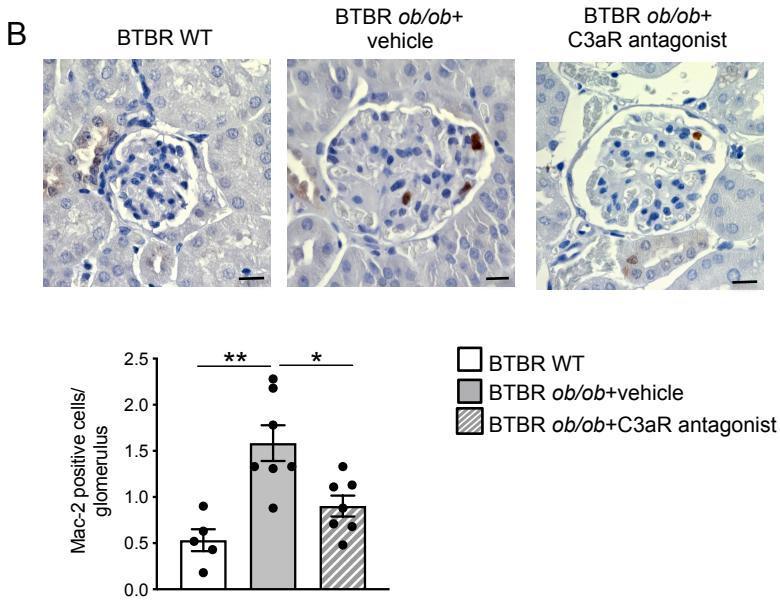

B
BTBR WT
BTBR ob/ob+ vehicle
BTBR ob/ob+ C3aR antagonist
Mac-2 positive cells/ glomerulus
**
*
BTBR WT
BTBR ob/ob+vehicle
BTBR ob/ob+C3aR antagonist

Figure S3

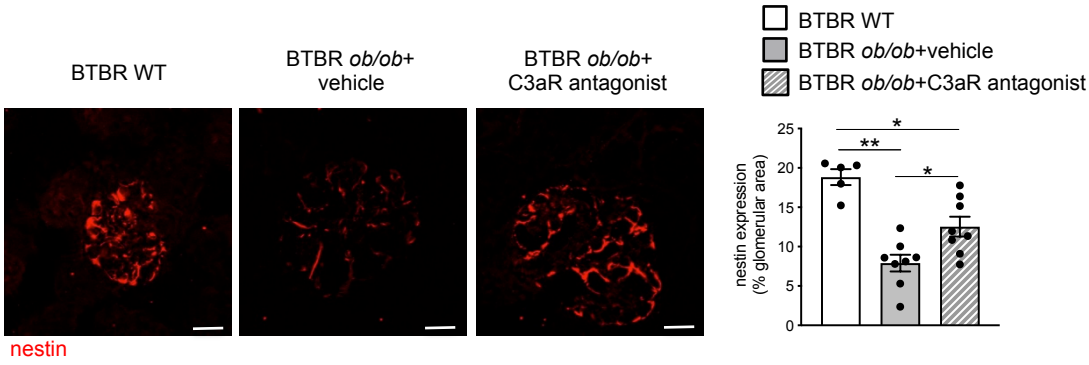
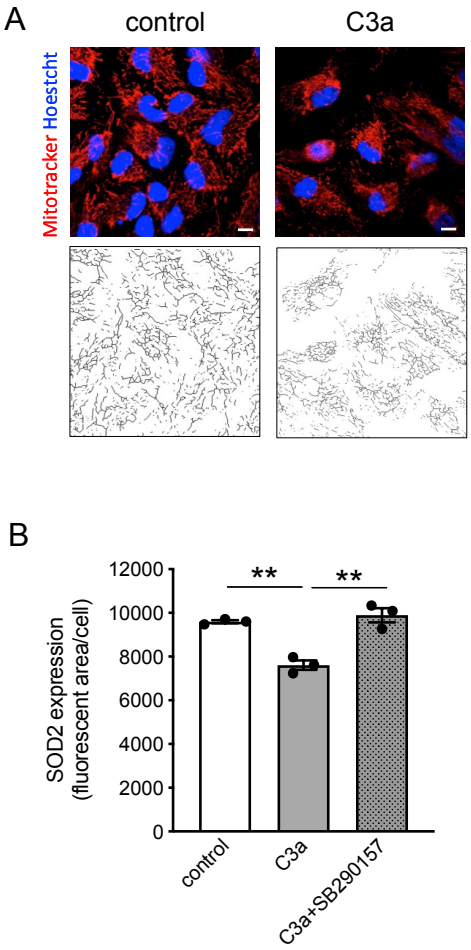

BTBR WT
BTBR ob/ob+ vehicle
BTBR ob/ob+ C3aR antagonist
nestin
BTBR WT
BTBR ob/ob+vehicle
BTBR ob/ob+C3aR antagonist
nestin expression (% glomerular area)
25
20
15
10
5
0
*
**
*

Figure S4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control
C3a
Mitotracker Hoestcht
B
SOD2 expression
(fluorescent area/cell)
12000
10000
8000
6000
4000
2000
0
**
**
control
C3a
C3a+SB290157

Figure S5