SUPPLEMENTAL MATERIAL

The endothelial GLP-1 receptor mediates cardiovascular protection by liraglutide in mice with experimental arterial hypertension

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Running title: Cardiovascular protection by GLP-1

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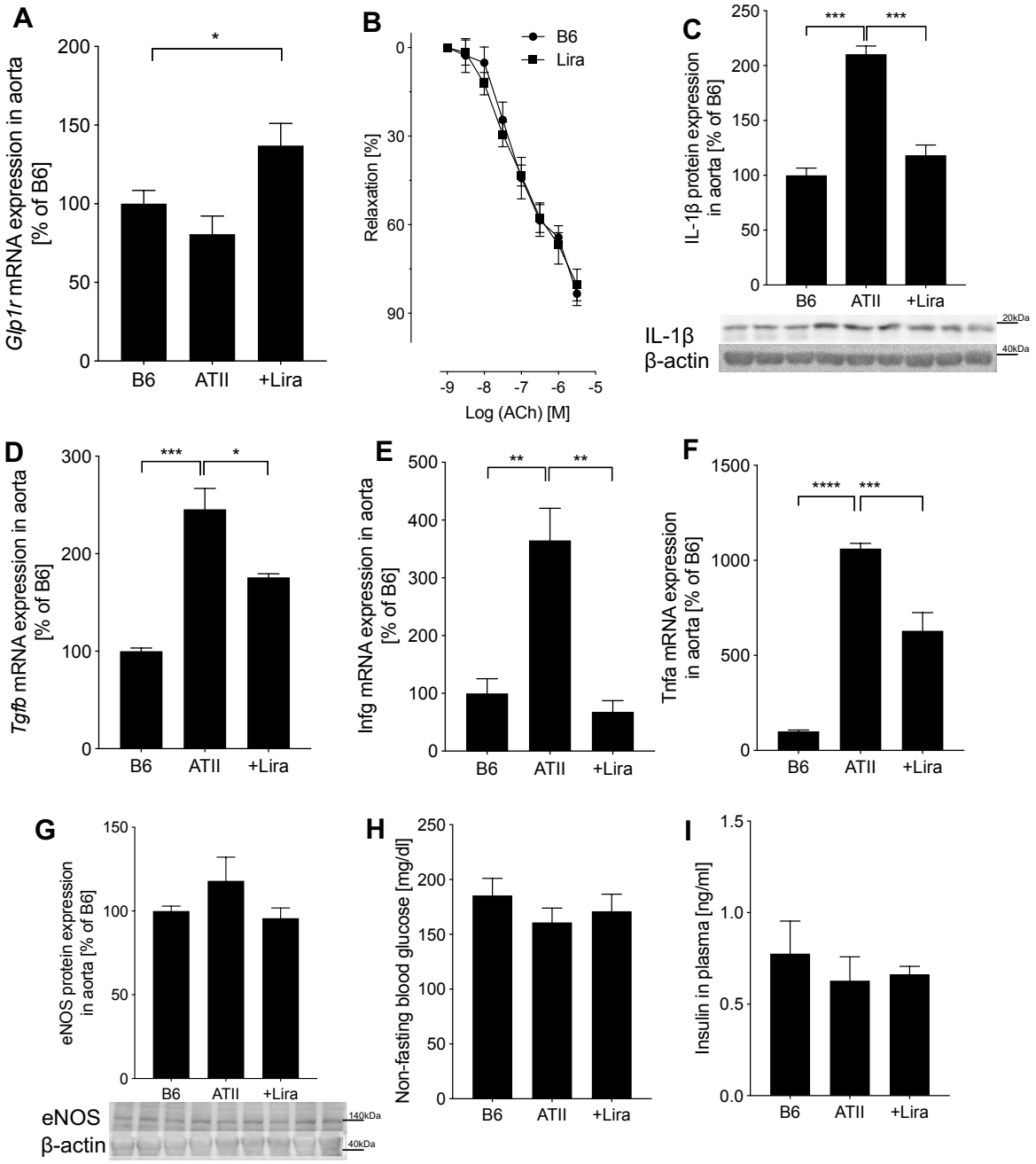
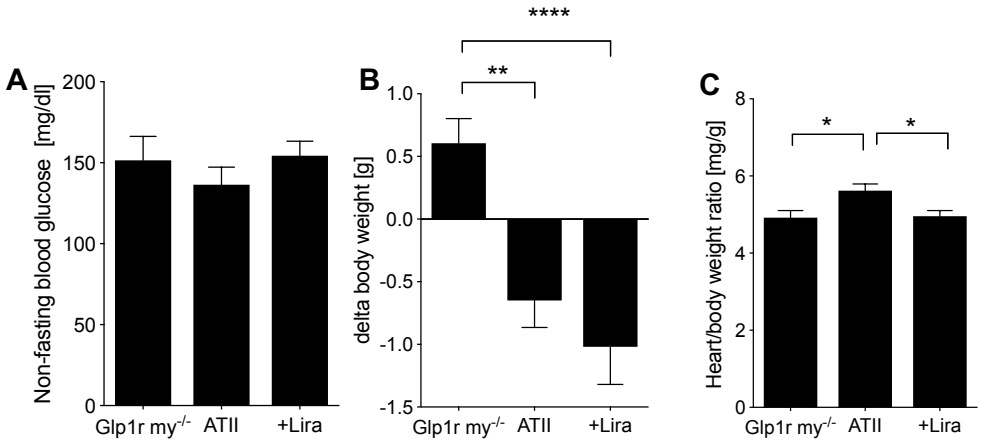
Keywords: GLP-1, GLP-1 receptor, liraglutide, angiotensin II, arterial hypertension, endothelial dysfunction, oxidative stress, vascular inflammation, nitric oxide, eNOS.

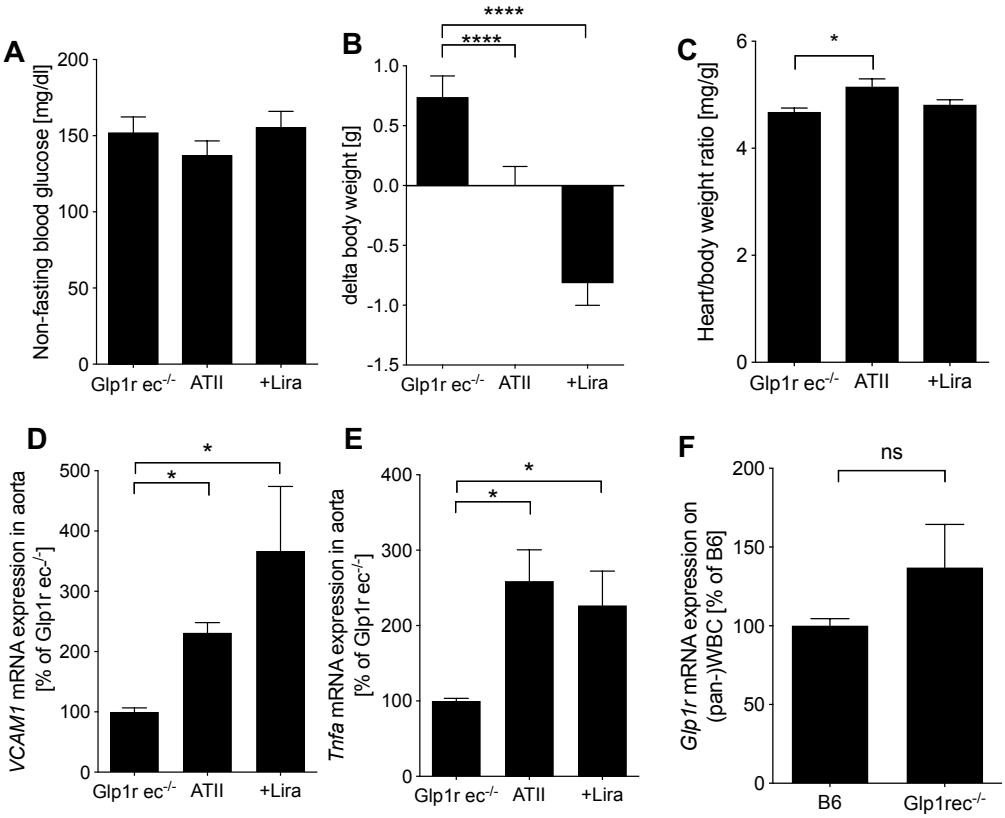
Supplemental Figures



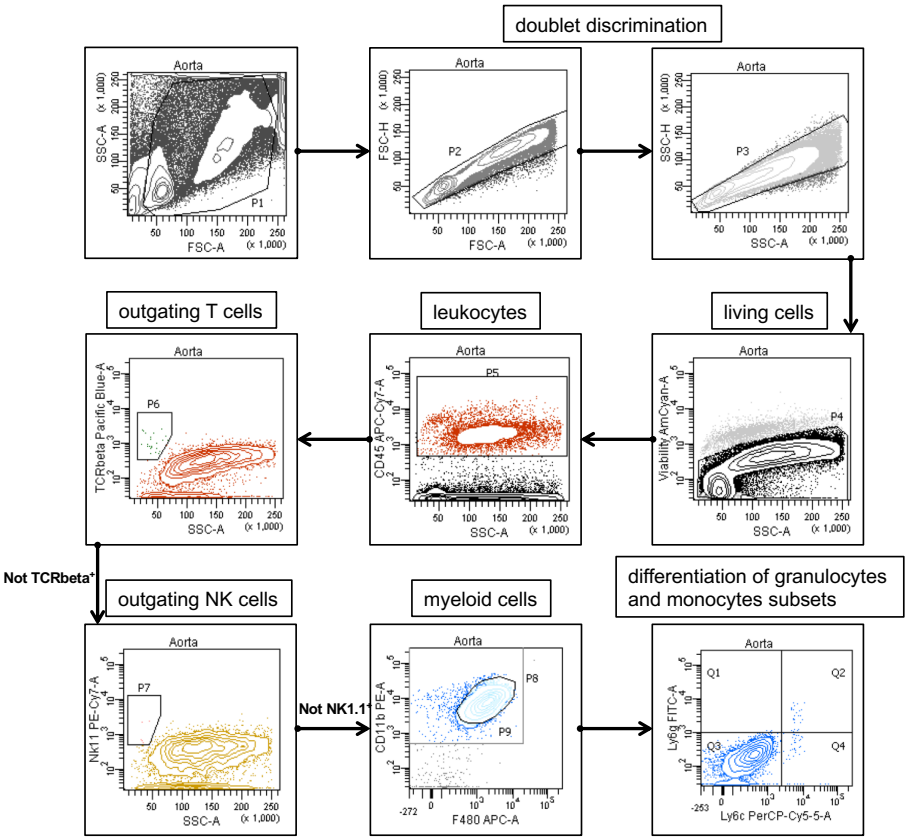
Supplemental Figure I. In wild-type mice (C57BL/6J), **(A)** GLP-1 receptor *(Glp1r)* mRNA expression was measured by quantitative rtPCR in aortic tissue. **(B)** Endothelium-dependent relaxation was evaluated after liraglutide treatment (w/o Angiotensin II). **(C)** IL-1β protein expression was measured by Western-blot. Representative blots are shown below. **(D)** *Tgfb*, **(E)** *Infg* and **(F)** *Tnfa* mRNA expression (encoding for (D) TGF-β, (E) INF-γ, (F) TNF-α) was measured by quantitative rtPCR in aortic tissue. **(G)** eNOS protein expression in aorta was determined by Western-blot **(H)** Non-fasting blood glucose and **(I)** insulin levels were evaluated in whole blood after seven days of treatment. One-way ANOVA and Bonferroni's multiple comparison test; (A) n = 4-6, (B-G) n = 3, (H, I) n = 15. Data are means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, *****P* < 0.0001



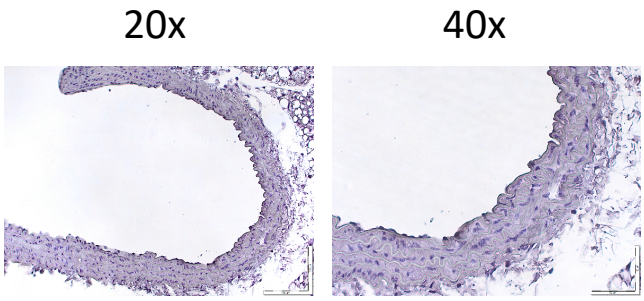
Supplemental Figure II. In myeloid cell (LysM^{+}) specific GLP-1 receptor knockout mice (Glp1r my$^{-/-}$) **(A)** non-fasting blood glucose levels were evaluated in whole blood after seven days of treatment. **(B)** Animals were weighed before and after seven days of treatment to determine Δ body weight. **(C)** Heart/body weight ratio. One-way ANOVA and Bonferroni's multiple comparison test; (A-C) n = 20-21. Data are means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, *****P* < 0.0001



Supplemental Figure III. In endothelial cell specific GLP-1 receptor knockout mice (Glp1r ec$^{-/-}$) **(A)** non-fasting blood glucose levels were evaluated in whole blood after seven days of treatment. **(B)** Animals were weighed before and after seven days of treatment to determine Δ body weight. **(C)** Heart/body weight ratio. **(D)** Vcam1 and **(E)** Tnfa mRNA expression (encoding for (D) VCAM-1 and (E) TNF-α) was measured by quantitative rtPCR in aortic tissue. **(F)** *Glp1r* mRNA expression was determined in isolated white blood cells of C57BL/6J and Glp1r ec$^{-/-}$. (A-E) One-way ANOVA and Bonferroni's multiple comparison test, (F) Student's t-test; (A-C) n = 23-25, (D-F) n = 3-4. Data are means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, *****P* < 0.0001.



Supplemental Figure IV. Gating strategy used to identify inflammatory cells in aortic tissue.



Supplemental Figure V. Isotype control for Ly6 IHC staining in B6J aorta

Supplemental Materials and Methods

Chemicals

Angiotensin II was obtained from Bachem AG (prod no. 4006473). Liraglutide was purchased as an injection pen (Victoza, 6 mg ml^{-1} from Novo Nordisk). Prostaglandin $F_{2\alpha}$ was used from Cayman Chemical. L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was purchased from Wako Pure Chemical Industries. Haematoxylin was used from Dako, sirius red from Waldeck GmbH and picric acid from AppliChem. The Bradford reagent was obtained from BioRad. The QuantiTect probe RT-PCR Kit was purchased from Qiagen and TaqMan probes from Applied Biosystems. All other chemicals were of analytical grade and obtained from Sigma-Aldrich, Fluka or Merck.

Animals and in vivo treatment

C57BL/6J mice were purchased from Charles River and *Glp1r*$^{-/-}$ were generated by Daniel Drucker [1]. Mice with the *Glp1r* allele flanked by loxP sites (Glp1r$^{flox/flox}$) (kind gift by Randy Seeley, University of Michigan) [2, 3] were bred with *Cdh5*cre^{+} (Jackson Laboratory, stock no: 006137) [4] and *LysM*cre^{+} [5] mice on a C57BL/6J background to obtain mice with an endothelial- and lysozyme M^{+} myelomonocytic-specific *Glp1r* deletion (*Glp1r ec*$^{-/-}$ and *Glp1r my*$^{-/-}$ mice, respectively). *Glp1r my*$^{-/-}$ mice were maintained in the heterozygous state for the Cre recombinase (LysM$^{cre/wt}$, LysMcre *knock-in* mouse line). Cell-specific knockdown of the GLP-1R was validated by rtPCR (Figure 6A and 7A).

Mice of each strain were randomly divided into three groups: control/sham group, ATII- and ATII+liraglutide-treated group (+Lira). One set of vascular function data was obtained in liraglutide only treated mice (w/o ATII). ATII (0.5mg kg^{-1} d^{-1} for 7days) versus sham (NaCl 0.9%) was delivered subcutaneously via miniosmotic pumps (model 1007D, ALZET), implanted under ketamine/xylazine anesthesia [5]. Mice were intraperitoneally injected with liraglutide (30µg kg^{-1} twice daily) or saline (sham and ATII group) every 12h. One week after pump implantation, animals were sacrificed under isoflurane anesthesia. Glucose levels were assessed in whole blood using the ACCU-CHEK sensor system from Roche Diagnostics

GmbH. To measure body weight gain/loss and to calculate the heart/body weight ratio as an indicator of cardiac hypertrophy, mice were weighed before pump implantation (day 0) and before euthanasia (day 7) and isolated hearts were weighed post-transection.

Detection of oxidative stress

For whole blood oxidative burst fresh citrate-anticoagulated blood (1:10) was stimulated with zymosan A (50µg/ml) and ROS formation was assessed by L-012 (100µM)-ECL using a Mithras2 chemiluminescence plate reader (Berthold Technologies). Results were expressed as counts/s after 45min.

Cardiac membrane fractions were prepared by homogenization of heart, followed by differential centrifugation (2000g, 10min; supernatant 20,000g, 20min; supernatant 100,000g, 60min; 4°C). NADPH oxidase activity of the membrane suspensions (0.2mg ml^{-1} protein) was measured by lucigenin (5µM) ECL in the presence of 200µM NADPH. ECL was detected in a single vial luminometer Lumat LB9507 (Berthold Technologies). Results were expressed as counts/min after 5min [6].

Isolated left ventricles and aortic ring segments were embedded in containing OCT (optimal cutting temperature)-resin. Cryo-sections (8µm) were incubated with DHE (1µM) for 30min at 37°C and fluorescence was detected using a Zeiss Axiovert 40 CFL microscope. Images were quantified as integrated optical density (IOD). To investigate the involvement of eNOS uncoupling in ROS production and endothelial dysfunction, selected aortic rings were pre-incubated with the NOS inhibitor L-NAME (0.5mM) [7].

Non-invasive blood pressure recordings (NIBP)

Blood pressure was recorded on day 6 of treatment at 10 am and mice were “trained” daily on the preceding 4 days before final measurement to adapt them to the procedure and to minimize stress reactions. The mean value of 5 independent measurements within a time window of 20 min was used for final data points. Accuracy of the tail cuff method was previously demonstrated in comparison to radio telemetric measurement [8].

Dot blot analysis

Cardiac protein homogenates were transferred to a nitrocellulose membrane by a Minifold I vacuum dot blot system (Schleicher&Schuell). Protein tyrosine nitration was detected using a specific antibody for 3-NT (1µg ml^{-1}, Millipore, 06-284), ADMA levels using a specific antibody detecting protein-bound asymmetric dimethylarginine (1:2,000, Covalab, mab0004-P) and inflammation using an antibody for F4/80 (1:250, eBiosciences, 14-4801-82). Positive dots were visualized by ECL with a peroxidase-coupled secondary antibody (1:10,000, Vector Lab). Densitometric quantification of the dots was performed as described below.

S-glutathionylation of endothelial nitric oxide synthase and Western blotting

eNOS was immunoprecipitated and the precipitate was subsequently immunoblotted for S-glutathionylation [9]. Magnetic beads (Dynabeads M-280 sheep anti-mouse IgG, Invitrogen, 11201D) were loaded with monoclonal mouse eNOS antibody (BD Biosciences, 610297). Precipitated proteins were separated by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) under non-reducing conditions by standard Western blot procedure using a monoclonal mouse antibody against S-glutathionylated proteins (1:1,000, Virogen, 101-A). After stripping of the membrane, the bands were immunoblotted for eNOS (1:1,000, BD Biosciences, 610297) to normalize the signals. Detection and quantification were performed by ECL with peroxidase conjugated anti–mouse secondary antibody (1:10,000, Vector Lab, PI-2000). Densitometric quantification of antibody-specific bands was performed with a ChemiLux Imager (CsX-1400M, Intas) and Gel-Pro Analyzer software (Media Cybernetics, Bethesda).

For Western Blot analysis of other proteins, protein suspension of homogenized aortic tissue was separated by 10% SDS-PAGE under reducing conditions [9]. Immunoblotting was accomplished with antibodies against IL-1β (1:1,000, abcam, ab9722) and β-actin as a loading control (1:2,500; Sigma-Aldrich, A5060). Goat anti-rabbit peroxidase-labeled secondary

antibody (1:10,000, Vector lab, PI-1000) was used and ECL development performed as described above.

Electron paramagnetic resonance spectroscopy (EPR)

EPR was used to assess aortic NO synthesis/bioavailability using colloid Fe(II)-diethyldithiocarbamate ($Fe(DETC)_2$) as spin trap. The EPR measurements were carried out at 77K using an X-band table-top spectrometer MS400 (Magnettech) with the following settings: 10mW microwave power; 7,000mG amplitude modulation; 100kHz modulation frequency; 3,300G center field; 110G sweep width; 30s sweep time and 10 scans. The amplitude of the characteristic iron-nitrosyl triplet signal was measured to quantify total NO production [10].

Determination of nitrate and nitrite

Nitrite and nitrate were measured by ENO-20 NOx Analyzer (Eicom Corporation, Tokyo, Japan), based on the liquid chromatography method with post column derivatization with Griess reagent [11]. The plasma samples were precipitated with methanol at the ratio of 1:1 (v/v) centrifuged and supernatant was injected into the HPLC system. Nitrite and nitrate were separated on a NO-PAK column (4.6µm x 50mm; Eicom). Nitrite was detected based on the Griess reaction, with sulfanilamide and naphthylethylenediamine (both from NO-EBP, Eicom) forming a purple diazo compound (tR= 4.9min for nitrite) with UV/Vis detection at 540nm.

Intravital fluorescence microscopy

Mice were anesthetized with midazolam (5mg kg^{-1}), medetomidine (0.5mg kg^{-1}) and fentanyl (0.05mg kg^{-1}) and fixed on a custom-built stage. The femoral artery and vein was dissected. Acridine orange (50µl, 0.5mg ml^{-1} in NaCl; Sigma-Aldrich) was injected via a jugular vein catheter (inner diameter 0.28mm; outer diameter 0.61mm; Smiths Medical Deutschland GmbH). Leukocyte rolling was visualized by an upright confocal spinning disc microscope (Nikon) including a spinning disc unit (CSU-W1, Yokogawa), sCMOS 4.2 megapixels cameras (Zyla 4.2 plus, Andor), 25x water immersion objective (CFI-75 Apo 25x, numerical aperture 1.10, Nikon) and laser beamcombiner (ILE-Beamcombiner, Nikon). A real-time imaging

software (NIS Elements, Nikon) was used for image acquisition and analysis. Cell recruitment was quantified in six fields of view (100 × 150µm) per femoral vein [32].

Histological and immunohistochemical staining

Aortic ring segments (3-4mm) with intact adventitia and perivascular fat were fixed in paraformaldehyde (4%), paraffin-embedded and cut into sections of 5µm. After deparaffinization, samples were blocked with 2.5% normal horse serum blocking solution (Vector) and stained with primary antibodies against Ly-6 (1:100, BioRad, MCA771GA) or IgG isotype control (1:50, BioLegend, 400502) and anti-rat (Vector lab, BA-4001) biotinylated secondary antibody. ABC reagent (Avidin-Biotin Complex, Vector) followed by DAB (3,3'-diaminobenzidine) peroxidase substrate (Vector) was used for immunohistochemical detection. DAB was visualized using an Olympus IX71 microscope (20x objective) and an Olympus Color View II camera.

For picro-sirius red staining nuclei were pre-stained with haematoxylin for 5min, samples washed and stained in picro-sirius red solution (0.1% with 1.2% picric acid). Images were taken with an Olympus IX71 microscope (20x objective) and an Olympus Color View II camera.

Reverse transcription polymerase chain reaction (qRT-PCR)

The acid guanidinium thiocyanate–phenol–chloroform extraction method was used for RNA isolation. For RNA isolation from mouse lung endothelial cells (MLECs) and bone marrow-derived macrophages (BMDMs), cells were pelleted and resuspended in guanidinium thiocyanate (GIT) denaturing solution. For isolation of RNA from snap-frozen mouse aortas, they were placed in GIT buffer and tissue was homogenized with the TissueLyser II (Qiagen). RNA was used for real-time RT-PCR analysis with the QuantiTect Probe RT-PCR kit (Qiagen). TaqMan Gene Expression assays for *iNOS/Nos2* (Mm00440485_m1), *Nox2* (Mm00432775_m1), *Tnfa* (Mm00443259_g1), *NFkb2* (Mm00479810_g1), *Vcam1*

(Mm00449197_m1), *Icam1* (Mm00449197_m1), *Selp* (Mm01204601_m1) and *TBP* (Mm00446973_m1) were purchased as probe-and-primer sets (Applied Biosystems). Results were quantified with the relative C_t method and normalized to TATA-box binding protein (TBP)-mRNA as the endogenous control.

Oral glucose tolerance test (OGTT) and plasma insulin

After seven days of treatment, oral glucose tolerance test was performed in for 6h (7am-1pm) fasted mice using a glucose dose of 1.5g kg^{-1}. Blood glucose levels were measured in tail vein blood samples at time 0, 15, 30, 60, 90 and 120min following glucose administration by gavage using a hand-held glucose meter (ACCU-CHEK, Roche Diagnostics GmbH). Plasma was collected from mice directly prior and 120min post-glucose stimulus during OGTT, and plasma insulin levels were quantified by ELISA according to the manufacturer's instruction (Crystal Chem, 90080).

Flow cytometric analysis

Aortic vessels were cleaned of fatty tissue, minced and digested with liberase (1mg ml^{-1}, Roche Diagnostics) for 30min at 37°C. Single-cell suspensions were obtained and treated with Fc-block, washed and surface-stained with CD45-APC-efluor 780, NK1.1-PE-Cy7, F4/80-APC (from eBiosciences), TCR-β V450, CD11b-PE, Ly6G-FITC, and Ly6C-PerCP-Cy.5.5 (from BD Biosciences). Dead cells were excluded by staining with Fixable Viability Dye eFluor506 (eBioscience). Based on a live gate, events were acquired and analyzed using a BD FACS CANTO II flow cytometer (Becton Dickinson) and FACSDiva software (Becton Dickinson), respectively [5].

Isolation of MLECs and BMDMs

Lung tissue of male mice was minced, digested for 30min at 37°C in 1.5% Collagenase A (Worthington, LS 004154) and passed through a 70µm cell strainer [4]. Cells were pelleted by 1300rpm for 15min at 4°C and resuspended in 20% FCS/DMEM. Separation was performed

by incubation with CD31 MicroBeads (Milteny Biotec, 130-097-418) for 15min on ice, followed by incubation of the $CD31^+$-cells with CD102 (BD Biosciences, 553326) pre-coated Dynabeads (Invitrogen, 11035) for 1h at 4°C. After magnetic separation, double-positive MLECs ($CD31^+$, $CD102^+$) were pelleted.

For BMDMs, femurs and tibias of male mice were flushed through 100µm cell strainers under sterile conditions. Cells were pelleted by 1500rpm for 5min at 4°C and incubated in RPMI 1640 (25mM HEPES and L-glutamine, 100U ml^{-1} penicillin-streptomycin, 20% FCS, 30% L929 cell–conditioned media) for 7d (37°C, 5% CO_2) to induce macrophage differentiation. For cell harvest, BMDMs were segregated from non-adherent cells and pelleted.

Supplemental References

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Major Resources Tables

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex
Mouse (C57BL/6J)	Charles River	C57BL/6J	Male
Mouse ($Glp1r^{-/-}$)	Bred in-house; originally from Daniel Drucker, Mt. Sinai Hospital, Toronto, Canada	C57BL/6J	Male
Mouse ($Glp1r^{flox/flox}$)	Bred in-house; originally from Randy Seeley, University of Michigan	C57BL/6J	Male
Mouse ($Cdh5cre^{+}$)	Bred in-house; originally from Jackson Laboratory	C57BL/6J	Male
Mouse ($LysMcre^{+/-}$)	Bred in-house; originally from Jackson Laboratory	C57BL/6J	Male
Mouse ($Glp1r^{flox/flox}xCdh5^{cre+}$, Glp1r $ec^{-/-}$)	Bred in-house	C57BL/6J	Male
Mouse ($Glp1r^{flox/flox}xLysM^{cre+/-}$, Glp1r $my^{-/-}$)	Bred in-house	C57BL/6J	Male

Animal breeding

	Species	Vendor or Source	Background Strain	Other Information
Parent - Male	$Glp1r^{-/-}$	Bred in-house	C57BL/6J	
Parent - Female	$Glp1r^{-/-}$	Bred in-house	C57BL/6J	
Parent - Male	$Glp1r^{flox/flox}$	Bred in-house	C57BL/6J	Endothelial cell-specific GLP-1R knockout mice were generated by crossing $Glp1r^{flox/flox}$ with $Cdh5cre^{+}$ mice and mating the resulting $Glp1r^{flox/wt}xCdh5cre^{+}$ mice back with $Glp1r^{flox/flox}$ to obtain $Glp1r^{flox/flox}xCdh5cre^{+}$ mice. The colony was maintained by crossing $Glp1r^{flox/flox}xCdh5cre^{+}$ with $Glp1r^{flox/flox}$mice. Pubs were checked for the presence of the Cdh5cre transgene.
Parent - Female	$Glp1r^{flox/flox}xCdh5cre^{+}$	Bred in-house	C57BL/6J	

Parent - Male	$Glp1r^{flox/flox}$	Bred in-house	C57BL/6J	Myelomonocytic cell-specific GLP-1R knockout mice were generated by crossing $Glp1r^{flox/flox}$ with $LysMcre^{+/+}$ mice and mating the resulting $Glp1r^{flox/wt}xLysMcre^{+/-}$ mice back with $Glp1r^{flox/flox}$ to obtain $Glp1r^{flox/flox}xLysMcre^{+/-}$ mice. The colony was maintained by crossing $Glp1r^{flox/flox}xLysMcre^{+/+}$ with $Glp1r^{flox/flox}$mice to only obtain heterozygous $Glp1r^{flox/flox}xLysMcre^{+/-}$ mice to prevent altered immune cell function due to compromised LysM gene expression in homozygous knock-in mice.
Parent - Female	$Glp1r^{flox/flox}xLysMcre^{+/+}$	Bred in-house	C57BL/6J	

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
Flow Cytometry				
CD45-APC-efluor 780	eBioscience	47-0451-82	2 µg/ml	
NK1.1-PE-Cy7	eBioscience	25-5941-81	2 µg/ml	
F4/80-APC	eBioscience	17-4801-82	4 µg/ml	
TCR-β V450	BD Biosciences	560706	2 µg/ml	
CD11b-PE	BD Biosciences	553311	2 µg/ml	
Ly6G-FITC	BD Biosciences	551460	5 µg/ml	
Ly6C-PerCP-Cy.5.5	BD Biosciences	560525	2 µg/ml	
Fixable Viability Dye eFluor506	eBioscience	65-0866-14	n.a. (1:1000)	
Coating of Dynabeads				
CD102	BD Biosciences	553326	0.5 µg/µl	
Western and dot blot				
Anti-Nitrotyrosine (3-NT)	Millipore	06-284	1 µg/ml	
Asymmetric dimethylarginine (ADMA)	Covalab	mab0004-P	0.5 µg/ml	
F4/80	eBiosciences	14-4801-82	2 µg/ml	
Anti-Glutathione	Virogen	101-A	1 µg/ml	
eNOS	BD Biosciences	610297	0.25 µg/ml	
IL-1β	abcam	ab9722	0.1 µg/ml	
β-actin	Sigma-Aldrich	A5060	n.a. (1:2500)	
Goat anti-rabbit peroxidase-labeled secondary antibody	Vector lab	PI-1000	0.1 µg/ml	
Horse anti–mouse peroxidase-labeled secondary antibody	Vector lab	PI-2000	0.1 µg/ml	
Goat anti-rat peroxidase-labeled secondary antibody	Santa Cruz	sc-2006	0.04 µg/ml	
Immunohistochemistry				
Ly-6	BioRad	MCA771GA	10 µg/ml	
IgG isotype control	BioLegend	400502	10 µg/ml	
Rabbit anti-rat biotinylated secondary antibody	Vector lab	BA-4001	5 µg/ml	

Isolated / Cultured Cells

Name	Source	Sex (F, M or unknown)
Mouse lung endothelial cells (MLECs)	- $Cdh5^{cre+}$ - $Glp1r^{flox/flox}xCdh5^{cre+}$	Male
Bone marrow-derived macrophages (BMDMs)	- $LysM^{cre+/-}$ - $Glp1r^{flox/flox}xLysM^{cre+/-}$	Male