Supplements

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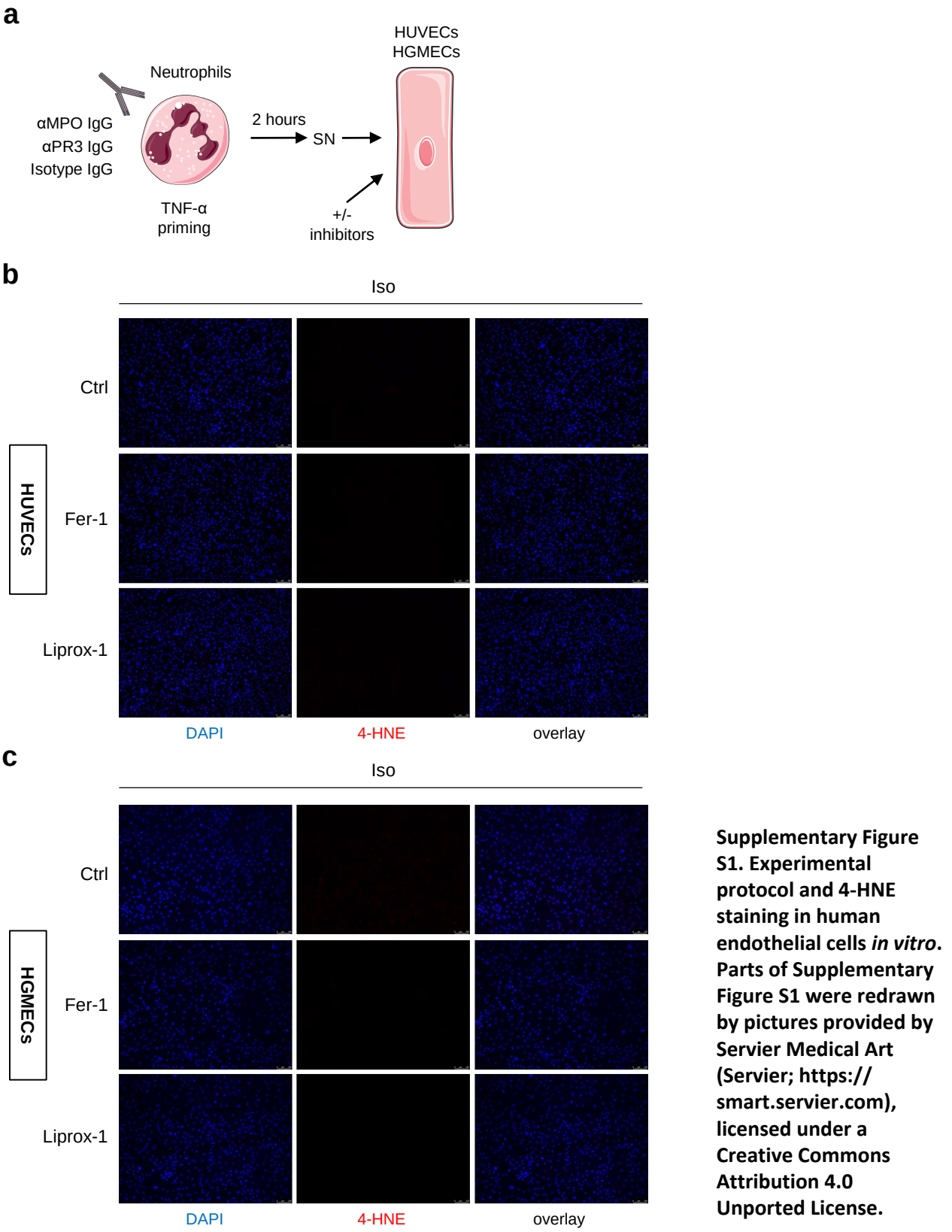
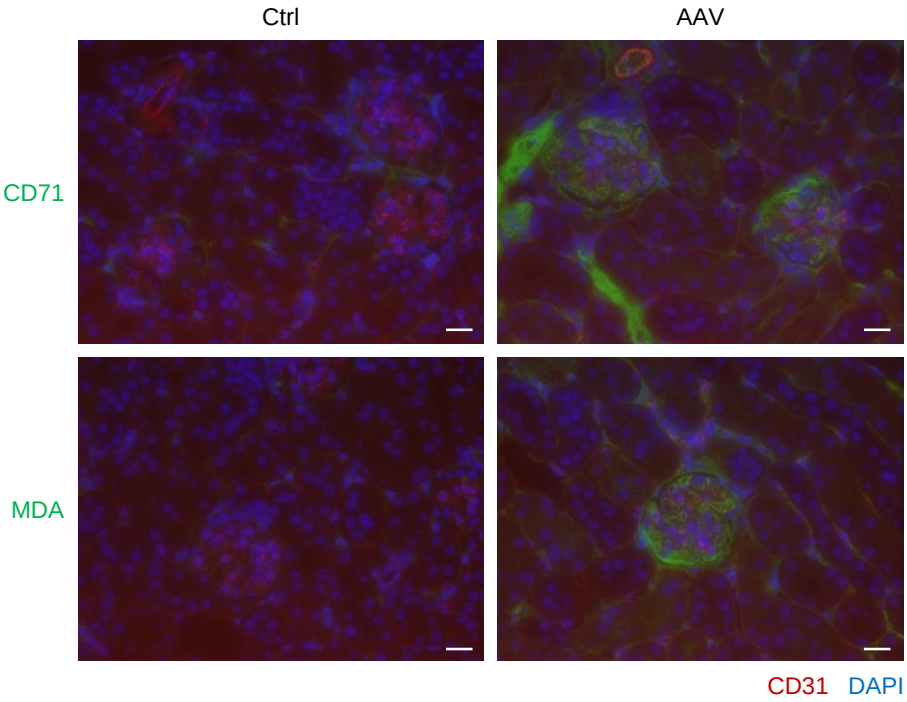
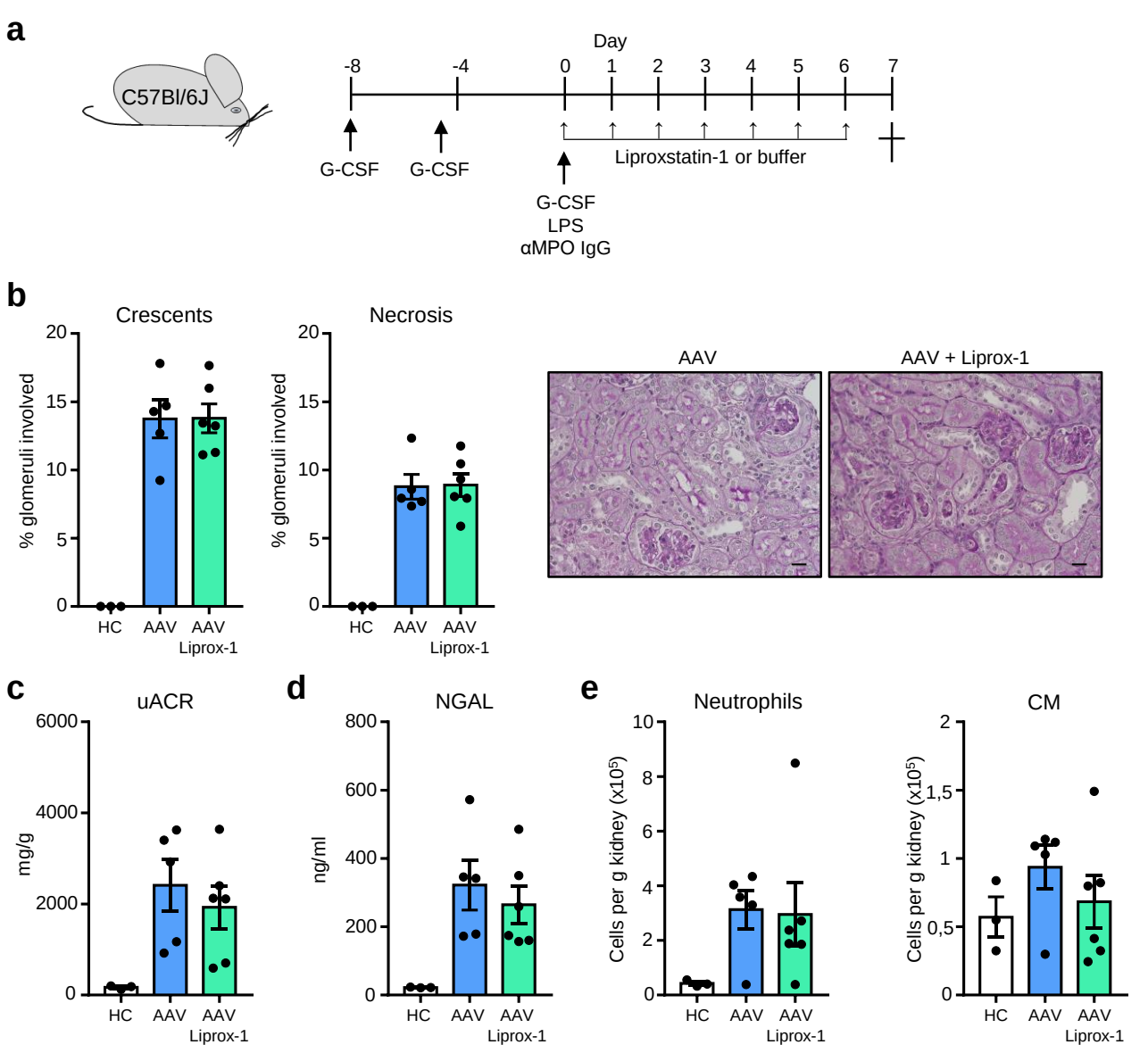


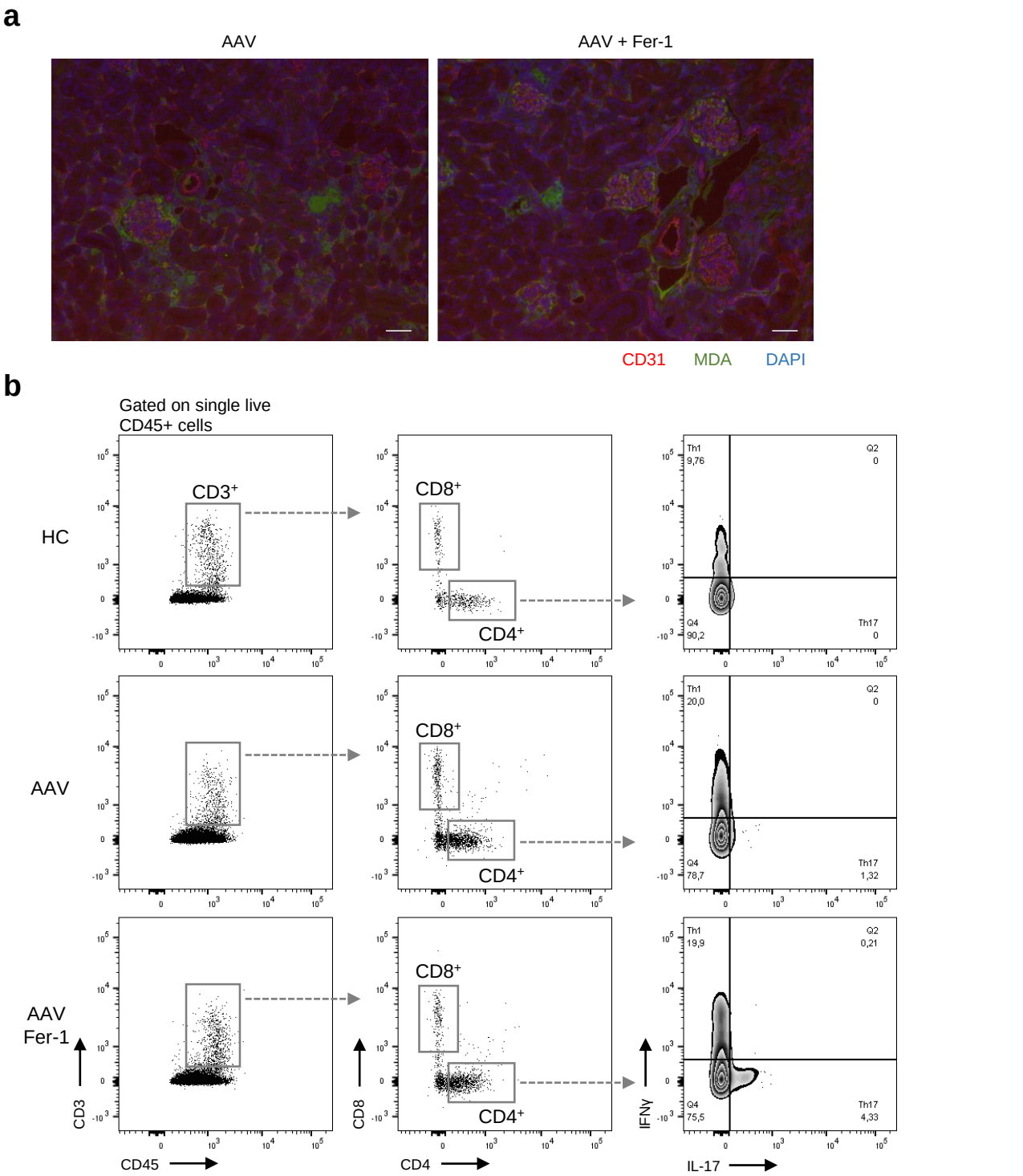
Supplementary Figure S1. Experimental protocol and 4-HNE staining in human endothelial cells *in vitro*. Parts of Supplementary Figure S1 were redrawn by pictures provided by Servier Medical Art (Servier; https://smart.servier.com), licensed under a Creative Commons Attribution 4.0 Unported License.



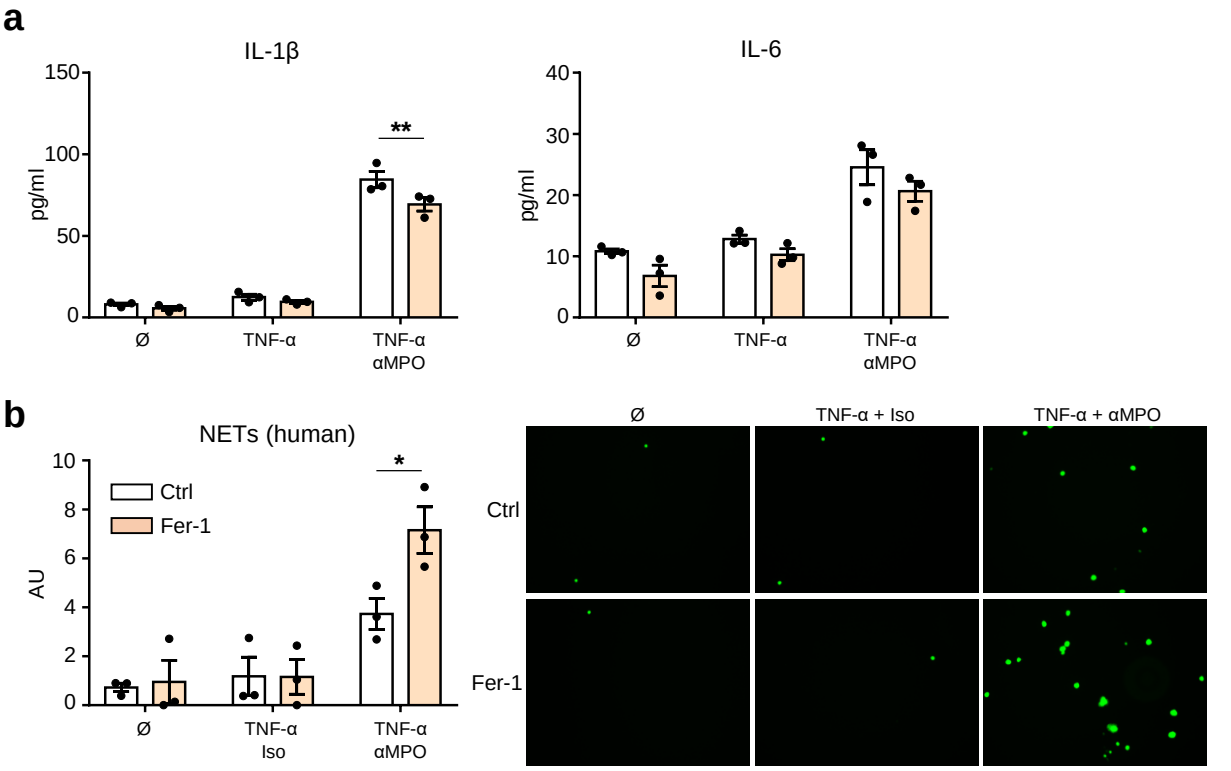
Supplementary Figure S2. Increased ferroptosis markers in kidneys from mice with AAV.



Supplementary Figure S3. Ferroptosis inhibition with Liproxstatin-1 does not protect mice from the development of ANCA-associated NCGN.



Supplementary Figure S4. Ferroptosis markers and infiltrating T cells in kidneys from mice with AAV.



Supplementary Figure S5. Murine monocyte supernatants and human NETs.

SUPPLEMENTARY MATERIAL

Supplementary methods

Histologic Examination of Renal Damage

Kidneys were collected at the time of euthanasia in cold PBS, fixed in 4% formalin overnight, and embedded in paraffin using routine procedures. Sections (3 μm) were stained with Periodic acid–Schiff and all glomeruli on each kidney section assessed by light microscopy using a Keyence BZ-9000 microscope (Osaka, Japan). Glomerular crescents and necrosis were expressed as the mean percentage of glomeruli with crescents and necrosis in each animal and scored in a blinded fashion.

Functional Measurement of Renal Damage

Urine samples were collected by housing the mice 16 hours in metabolic cages with free access to water and food before they were euthanized. Urinary albumin excretion was determined by ELISA (Bethyl Laboratories, Inc., Montgomery, TX, USA). NGAL concentration in urine was determined with the NGAL (LCN2) ELISA Kit from Dianova (Hamburg, Germany). Creatinine in urine samples was measured at the Animal Phenotyping Platform of the Max Delbrück Center for Molecular Medicine (Berlin, Germany).

Isolation of Renal Leukocytes

Kidneys were minced with scissors and digested for 50 minutes at 37°C with 0.1 mg/ml liberase and 50 U/ml DNAse in PBS without magnesium and calcium ions (PBS). Cell suspensions were washed with MACS buffer (PBS/2%BSA/2 mM EDTA), filtered through 70-μm meshes, resuspended in MACS buffer, and further processed for flow cytometry.

Flow cytometry

Renal cell suspensions were incubated 15 minutes on ice with anti-mouse CD16/32 antibody (Biolegend, San Diego, CA, USA) to block Fc receptors. Cells were then incubated with the following fluorochrome-conjugated antibodies 20 minutes at 4°C to characterize myeloid cells by flow cytometry: CD45 (30F11; BD Bioscience, Heidelberg, Germany); and CD11b (M1/70), Ly6C (HK1.4), and Ly6G (1A8), all from

Biolegend. Dead cells were excluded using the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (ThermoFisher Scientific, Rockford, IL).

To identify T_H17 T cell subset, isolated $CD4^+$ cells and renal cell suspensions were stimulated for 4 hours with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 µg/ml) in the presence of 5µg/ml Brefeldin A (Biolegend) in RPMI. Cells were the washed and incubated 15 minutes on ice with anti-mouse CD16/32 antibody cells and further stained 20 minutes with the following antibodies: anti-CD45 (30F11), anti-CD3 (17A2), anti-CD4 (GK1.5), and anti-CD8 (53-6-7) from Biolegend. After washing, cells were fixed and permeabilized with the BD cytofix/cytoperm™ fixation/permeabilization solution kit. For intracellular staining, cells were incubated 30 minutes at 4°C with anti–IL-17A (TC11-18H10.1) and anti-IFNγ (XMG12) from Biolegend. Cells were then washed, resuspended in PBS and analyzed by flow cytometry.

Flow cytometry measurements were performed using a BD CANTO II flow cytometer with BD FACSDiva software. Data were analyzed using FlowJo Software version 10 (Treestar, Ashland, OR, USA).

Immunohistochemistry

Histologic stains for 4-hydroxynonenal/4-HNE (#46545, Abcam), malondialdehyde/MDA (ab243066, Abcam), transferrin receptor protein 1/CD71 (MABC1765, Merck) and CD31 (Dia-310, Dianova, Hamburg, Germany) were performed on 3-mm thick paraffin-embedded sections. Briefly, sections were dewaxed in xylene and rehydrated through a series of decreasing concentrations of ethanol. Antigen retrieval was performed by heating sections in 10 mM sodium citrate buffer (pH 6.0) in a steamer for 20 minutes. Afterward, sections were blocked with 10% normal donkey serum and incubated with primary antibodies against 4HNE, MDA, CD71, and CD31 at 4°C overnight. After washing, sections were incubated with Alexa Fluor™ 488 anti-mouse (Invitrogen, ThermoFisher Scientific), Alexa Fluor™ 555 anti-rat (Biolegend) or Alexa Fluor™ 488 anti-rabbit (Invitrogen) secondary antibodies for 1h at room temperature. Nuclei were counterstained with DAPI and sections mounted in Faramount aqueous mounting medium from Agilent Technologies. This protocol was adapted to stain for 4-HNE and DAPI in endothelial cells in culture. Kidney sections and endothelial cells were assessed by fluorescence microscopy using a Keyence BZ-9000 microscope (Osaka, Japan).

Isolation of human neutrophils

Blood from healthy human donors was obtained after approval by Charité and after written informed consent was obtained. Polymorphonuclear neutrophils were isolated from heparinized whole blood by red blood cell sedimentation with dextran 1%, followed by Histopaque®-1077 (Sigma-Aldrich) density gradient centrifugation and hypotonic lysis of erythrocytes for 15 s using sodium chloride solution. Human neutrophil preparations contained <10% other cells, such as basophils and eosinophils.

Generation of neutrophil supernatants

Isolated human neutrophils were primed for 15 minutes with 2 ng/ml human TNF-α and further stimulated for 2 hours with either 10µg/ml IgG1 Isotype Control, anti-PR3 or anti-MPO antibodies. After centrifugation 2min at 6000 rpm, cell-free supernatants were immediately used or frozen at -80°C.

Isolation of murine neutrophils

For the isolation of bone marrow neutrophils, mice were sacrificed, femurs and tibias were dissected, and the bone marrow was flushed with ice-cold sterile PBS without calcium and magnesium. Neutrophils were further isolated by Ficoll-Hypaque (GE Healthcare, Little Chalfont, United Kingdom) density gradient centrifugation. Red blood cells in the neutrophil fraction were lysed by incubation with hypotonic saline for 15 seconds. Neutrophils were resuspended in RPMI medium and used for further experiments.

Isolation of murine monocytes

Monocytes were isolated from bone marrow of C57BL/6J mice using the Monocyte Isolation Kit (BM) from Miltenyi Biotec (Bergisch Gladbach, Germany), following the manufacturer's instructions. Monocytes were primed in RPMI medium for 15 minutes with 5 ng/ml of TNFα and subsequently stimulated with or without anti-MPO IgG for 4 hours at 37°C. Unstimulated cells were used as negative control. In some conditions, monocytes were pre-incubated 30 minutes with 10 ng/ml Ferrostatin-1. At the end of the experiment, supernatants were collected after centrifugation, stored at -20°C and used for co-culture with $CD4^+$ T cells and for chemokine detections.

Endothelial cell culture

HUVECs were cultured in VascuLife® Basal Medium and supplements from the VascuLife VEGF LifeFactors Kit (Lifeline Cell Technology, Cell Systems). HGMECs were cultured in complete classic medium kit with culture boost from Cell Systems. All experiments were performed until passage 6. Cell cultures were maintained at 37°C in a humidified environment of 5% CO2. Cells were detached with 0.05% Trypsin-EDTA 1 × (Sigma Aldrich) at 37°C for approximately 2 min and split to new plates according to the experimental procedures.

Detection of endothelial cell necrosis

HUVECs and HGMECs were plated in 24-well plates until the formation of a confluent monolayer. Once confluent, cells were incubated for 16 h at 37°C with neutrophil supernatants. In some conditions, endothelial cells were pre-incubated with either 10 μM Ferrostatin-1 (Fer-1), 2μM Liproxstatin-1 (Liprox-1), 25 μM Necrostatin-1s (Nec-1s), 5μM Necrosulfonamide (NSA) or 10 μM Z-VAD-fkm for 1 hour. At the end of the experiments, both supernatants and endothelial cells (detached with Trypsin-EDTA as described above) were collected and centrifuged 5 min at 1200 rpm. Cell pellets were incubated with APC-labeled Annexin V (BD Bioscience) and propidium iodide (Sigma Aldrich) in 1x Annexin V binding buffer (BD Bioscience), and incubated at room temperature for 15 min, in the dark. After incubation, cells were washed with annexin V binding buffer and analyzed by flow cytometry.

Detection of lipid peroxidation in endothelial cells

Confluent HUVECs and HGMECs were incubated for 12 h at 37°C with neutrophil supernatants. In some conditions, endothelial cells were pre-incubated with either 10 μM Ferrostatin-1 (Fer-1), 2μM Liproxstatin-1 (Liprox-1), 25 μM Necrostatin-1s (Nec-1s), 5μM Necrosulfonamide (NSA) or 10 μM Z-VAD-fkm for 1 hour. HUVECs and HGMECs were incubated with 2 μM C11-Bodipy 581/591 (D3861, Invitrogen), for the last 20 min at 37°C in the dark. At the end of the experiments, both supernatants and HUVECs (detached with Trypsin-EDTA as described above) were collected and centrifuged 5 min at 1200 rpm. Cell pellets were then washed, resuspended in PBS and analyzed by flow cytometry. Detection of lipid peroxidation in isolated murine $CD4^+$ T cells was performed following the same procedure.

LDH detection

LDH in culture medium from HUVECs and HGMECs was detected using the CytoTox96® Non-Radioactive Cytotoxicity Assay from Promega (Walldorf, Germany) following the supplier's protocol.

Detection of endothelial cell permeability

Endothelial cell permeability was assessed as previously described.[6] In brief, HUVECs were seeded at a density of $1x10^4$ cells per well in culture medium onto fibronectin-coated transwell chamber (0.4 μM pore size, 6.5 mm diameter from Corning, Amsterdam, Netherlands) placed in 24-well plate. Confluent HUVECs were pretreated with 10 μM Fer-1, 10 μM Z-VAD-fkm or 25 μM Nec1s. After 24 h incubation, transwells were washed with prewarmed medium before BSA-FITC (Sigma Aldrich) was added for 1h at 37°C. Cells were then washed and incubated with neutrophil supernatants for 4 hours at 37°C. After washing, albumin flux to the lower chamber was measured using a SpectraMax5 plate reader (Molecular Devices, Sunnyvale, CA, USA). The basal albumin flux obtained from an EC monolayer incubated with BSA-FITC and buffer alone was subtracted.

Transfection of endothelial cells

HUVECs were transfected with 20 nM small interfering ribonucleic acid (siRNA) using Lipofectamine RNAi MAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Scramble siRNA and siRNA against ACSL4 and MLKL were purchased from OriGene Technologies GmbH (Herford, Germany). After 48 hours transfection, HUVECs were washed and used for either western blot to confirm ACSL4 knockdown or incubated with neutrophil supernatants.

$CD4^+$ T cell proliferation and polarization

$CD4^+$ T cells were isolated from spleens of WT mice after erythrocyte lysis with RBC lysing buffer Hybri-Max (Sigma-Aldrich) using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. For proliferation assays, cells were stained with carboxy-fluorescein succinimidyl ester (Thermo Fisher Scientific), washed and seeded onto 96-well plate ($2.5x10^5$ cells/well) in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol. Cells were cultivated with Dynabeads Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) at a bead/cell ratio of 1:2 together with supernatant from stimulated monocytes (to a final monocyte:T cell ratio of 5:1) or Fer-1 at day 0. After 3 days in culture, cells were labeled with anti-CD3

and anti-CD4 antibodies and cell proliferation was analyzed by flow cytometry. Dead cells were excluded using the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (ThermoFisher Scientific).

For polarization assays, isolated $CD4^+$ T cells were seeded in 96-well plate as described above with Dynabeads at a bead/cell ratio of 1:1. Cells were cultured in medium described above and supplemented with cytokines (IL-1β, IL- 6, IL-23, TGF-β) and antibodies (anti-IFNγ, anti–IL-2 and anti–IL-4) from the CytoBox T_H17 Kit (Miltenyi Biotec) following the manufacturer's instructions. Supernatants from stimulated monocytes (to a final monocyte:T cell ratio of 5:1) or Fer-1 were added at day 0 and 3. After 7 days in culture, cells were labeled with anti-CD45, anti-CD3, anti-CD4, anti–IL-17A, and anti-IFNγ antibodies and T_H cell subsets were characterized by flow cytometry. Dead cells were excluded as described above.

Neutrophil extracellular trap (NET) formation

Isolated human neutrophils were stained with SYTOX green (Invitrogen), primed for 15 minutes with 2 ng/ml human TNF-α, and stimulated with either 10 µg/ml isotype or monoclonal anti-MPO antibodies. Isolated mouse neutrophils were stained with SYTOX green (Invitrogen), primed for 15 minutes with 5 ng/ml murine TNF-α, and stimulated with 150 µg/ml polyclonal anti-MPO antibodies. For ferroptosis inhibition, cells were pre-treated 30 minutes with 10 µM Fer-1 Human and mouse NETs were observed by microscopy after 1.5- and 3-hour incubation, respectively. For NET quantification, SYTOX positive area were measured using ImageJ processing software (NIH) and expressed as arbitrary unit.

Detection of Chemokines

Chemokines were measured in monocyte supernatants using the mouse IL-1beta/IL-1F2 and IL-6 DuoSet ELISA from R&D Systems following the manufacturer's instructions.

SDS-PAGE and Western Blot

Whole cell and organ lysates were prepared in RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate [SDS], 0,5% sodium deoxycholate) supplemented with complete protease and phosphatase inhibitor cocktails (Roche and Sigma, respectively). Protein lysates were loaded on SDS polyacrylamide gel and transferred to nitrocellulose membranes by electrophoresis. The

membranes were blocked in 5% non-fat dry milk for 1h and incubated overnight with the following antibodies: rabbit anti-Glutathione Peroxidase 4 (GPX4) antibody, rabbit anti-FACL4/ACSL4 (Abcam), rabbit anti-MLKL (Merck Millipore), rabbit anti–β-actin (Cell Signaling Technology, Frankfurt am Main, Germany). Horseradish peroxidase–labeled donkey anti-rabbit IgG (GE Healthcare) was used as secondary antibody. Thermo Scientific SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific) was used for detection.

RNA isolation and real-time PCR

Whole-kidney RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and reverse transcription was performed with the

SuperScript III One-Step RT-PCR System (Thermo Fisher Scientific). Renal Gpx4 and Acsl4 gene expression was determined by quantitative RT-PCR using the QuantStudio 3 instrument

(Thermo Fisher Scientific) and primers synthetized by BioTeZ Berlin-Buch GmbH (Berlin, Germany).

Statistical analysis

Results were expressed as means ± SEM. All statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). A t-test was used for comparison between two groups, and one-way or two-way analysis of variance with *post hoc* analysis was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Supplementary figures

Supplementary Figure S1. Experimental protocol and 4-HNE staining in human endothelial cells *in vitro*. (**a**) Experimental protocol describing the generation of cell-free neutrophil supernatants (cf-SN) and their incubation together with HUVECs and HGMECs in culture pre-treated with different cell death inhibitors. (**b**) Following incubation with supernatants from isotype (Iso)-stimulated neutrophils, HUVECs and (**c**) HGMECs were washed, stained for lipid peroxidation (4-HNE) and nuclei (DAPI) and observed by microscopy. Ferrostatin (Fer-1) and Liproxstatin-1 (Liprox-1) pre-treatment were used

for ferroptosis inhibition. Representative images of 3 independent experiments are presented. Scale bar represent 100µm.

Supplementary Figure S2. Increased ferroptosis markers in kidneys from mice with AAV. Representative immunofluorescence images (40x magnification) for CD71 (green, upper panel) and MDA (green, lower panel) show increased ferroptosis in kidney from mice with αMPO-induced NCGN (AAV) compared to control mice (Ctrl). CD31 (red) was used to stain endothelial cells and DAPI (blue) to stain nuclei. Scale bar represent 20µm.

Supplementary Figure S3. Ferroptosis inhibition with Liproxstatin-1 does not protect mice from the development of ANCA-associated NCGN. (**a**) Experimental settings describing the induction of NCGN in WT mice induced by G-CSF (30µg/mouse), LPS (5µg/g body weight) and αMPO IgG (50µg/g body weight) injection. Ferroptosis inhibition was mediated by daily injection of Liproxstatin-1 (Liprox-1, 10µg/g body weight) from day 0 until sacrifice at day 7. (**b**) Liprox-1 treatment led to similar renal damage compared to mice without treatment. Representative images of kidney sections stained with PAS at high (40x) magnification and quantitative analyses of glomeruli with crescents and necrosis are shown for each group. Scale bar represent 20µm. Data from 3 healthy mice are provided for comparison. (**c**) The urine albumin-creatinine ratio (uACR) and (**d**) NGAL urine levels determined by ELISA were similar in both groups. (**e**) Myeloid cell infiltration was analyzed by flow cytometry. Infiltration of $CD11b^{+}Ly6G^{+}$ neutrophils and $CD11b^{+}Ly6G^{-}Ly6C^{+}$ classical monocytes (CM) was similar in both groups. Error bars indicate means ± SEM.

Supplementary Figure S4. Ferroptosis markers and infiltrating T cells in kidneys from mice with AAV. (**a**) Representative immunofluorescence images (20x magnification) for MDA (green, lower panel) show similar ferroptosis in kidney from mice with αMPO-induced NCGN (AAV) compared to control mice (Ctrl). CD31 (red) was used to stain endothelial cells and DAPI (blue) to stain nuclei. Scale bar represent 50µm. (**b**) Gating strategy for the characterization of renal T cell subsets per flow cytometry.

Supplementary Figure S5. Murine monocyte supernatants and human NETs. (**a**) Murine monocytes were isolated from the bone marrow, primed with TNF-α and stimulated with anti-MPO IgG.

In some conditions, cells were pre-treated 30 min with Ferrostatin-1 (Fer-1). Unstimulated cells were used as negative controls. IL-1β and IL-6 levels in culture medium were determined by ELISA. (**b**) Isolated human neutrophils were primed with TNFα and subsequently stimulated with either isotype or anti-MPO IgG for 90 min. In some conditions, cells were pre-treated 30 min withFerrostatin-1 (Fer-1). Viable cells were stained with SYTOX green to detect neutrophil extracellular traps (NETs) by microscopy (20X magnification). The corresponding statistical analysis of the amount of SYTOX green positive area (NET-producing neutrophils) expressed as arbitrary unit (AU) from three independent experiments with different neutrophil donors is shown. Error bars indicate means ± SEM. *P<0.05; **P<0.01.

SUPPLEMENTARY MATERIAL

Supplementary methods

Histologic Examination of Renal Damage

Kidneys were collected at the time of euthanasia in cold PBS, fixed in 4% formalin overnight, and embedded in paraffin using routine procedures. Sections (3 µm) were stained with Periodic acid–Schiff and all glomeruli on each kidney section assessed by light microscopy using a Keyence BZ-9000 microscope (Osaka, Japan). Glomerular crescents and necrosis were expressed as the mean percentage of glomeruli with crescents and necrosis in each animal and scored in a blinded fashion.

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Kidneys were minced with scissors and digested for 50 minutes at 37°C with 0,12 mg/ml liberase and 1050 U/ml DNAse in PBS without magnesium and calcium ions (PBS). Cell suspensions were washed with MACS buffer (PBS/2%BSA/2 mM EDTA), filtered through 70-µm meshes, resuspended in MACS buffer, and further processed for flow cytometry.

Flow cytometry

Renal cell suspensions were incubated 15 minutes on ice with anti-mouse CD16/32 antibody (Biolegend, San Diego, CA, USA) to block Fc receptors. Cells were then incubated with the following fluorochrome-

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conjugated antibodies 20 minutes at 4°C to characterize myeloid cells by flow cytometry: CD45 (30F11; BD Bioscience, Heidelberg, Germany); and CD11b (M1/70), Ly6C (HK1.4), and Ly6G (1A8), all from Biolegend. Dead cells were excluded using the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (ThermoFisher Scientific, Rockford, IL).

To identify T_H17 T cell subset, isolated $CD4^+$ cells and renal cell suspensions were stimulated for 4 hours with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 µm~~m~~g/ml) in the presence of 5µg/ml Brefeldin A (Biolegend) in RPMI. Cells were the washed and incubated 15 minutes on ice with anti-mouse CD16/32 antibody cells and further stained 20 minutes with the following antibodies: anti-CD45 (30F11), anti-CD3 (17A2), anti-CD4 (GK1.5), and anti-CD8 (53-6-7) from Biolegend. After washing, cells were fixed and permeabilized with the BD cytofix/cytoperm™ fixation/permeabilization solution kit. For intracellular staining, cells were incubated 30~~25~~ minutes at 4°C with anti–IL-17A (TC11-18H10.1) and anti-IFNγ (XMG12) from Biolegend. Cells were then washed, resuspended in PBS and analyzed by flow cytometry.

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Flow cytometry measurements were performed using a BD CANTO II flow cytometer with BD FACSDiva software. Data were analyzed using FlowJo Software version 10 (Treestar, Ashland, OR, USA).

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Histologic stains for 4-hydroxynonenal/4-HNE (#46545, Abcam), malondialdehyde/MDA (ab243066, Abcam), transferrin receptor protein 1/CD71 (MABC1765, Merck) and CD31 (Dia-310, Dianova, Hamburg, Germany) were performed on 3-mm thick paraffin-embedded sections. Briefly, sections were dewaxed in xylene and rehydrated through a series of decreasing concentrations of ethanol. Antigen retrieval was performed by heating sections in 10 mM sodium citrate buffer (pH 6.0) in a steamer for 20 minutes. Afterward, sections were blocked with 10% normal donkey serum and incubated with primary antibodies against 4HNE, MDA, CD71, and CD31 at 4°C overnight. After washing, sections were incubated with Alexa Fluor™ 488 anti-mouse (Invitrogen, ThermoFisher Scientific), Alexa Fluor™ 555 anti-rat (Biolegend) or Alexa Fluor™ 488 anti-rabbit (Invitrogen) secondary antibodies for 1h at room temperature. Nuclei were counterstained with DAPI and sections mounted in Faramount

aqueous mounting medium from Agilent Technologies. This protocol was adapted to stain for 4-HNE and DAPI in endothelial cells in culture. Kidney sections and endothelial cells were assessed by fluorescence microscopy using a Keyence BZ-9000 microscope (Osaka, Japan).

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Blood from healthy human donors was obtained after approval by Charité and after written informed consent was obtained. Polymorphonuclear neutrophils were isolated from heparinized whole blood by red blood cell sedimentation with dextran 1%, followed by Histopaque®-1077 (Sigma-Aldrich) density gradient centrifugation and hypotonic lysis of erythrocytes for 15 s using sodium chloride solution. Human neutrophil preparations contained <10% other cells, such as basophils and eosinophils.

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Isolated human neutrophils were primed for 15 minutes with 2 ng/ml human TNF-α and further stimulated for 2 hours with either 10µg/ml IgG1 Isotype Control, anti-PR3 or anti-MPO antibodies. After centrifugation 2min at 6000 rpm, cell-free supernatants were immediately used or frozen at -80°C.

Isolation of murine neutrophils

For the isolation of bone marrow neutrophils, mice were sacrificed, femurs and tibias were dissected, and the bone marrow was flushed with ice-cold sterile PBS without calcium and magnesium. Neutrophils were further isolated by Ficoll-Hypaque (GE Healthcare, Little Chalfont, United Kingdom) density gradient centrifugation. Red blood cells in the neutrophil fraction were lysed by incubation with hypotonic saline for 15 seconds. Neutrophils were resuspended in RPMI medium and used for further experiments.

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Isolation of murine monocytes

Monocytes were isolated from bone marrow of C57BL/6J mice using the Monocyte Isolation Kit (BM) from Miltenyi Biotec (Bergisch Gladbach, Germany), following the manufacturer's instructions. Monocytes were primed in RPMI medium for 15 minutes with 5 ng/ml of TNFα and subsequently stimulated with or without anti-MPO IgG for 4 hours at 37°C. Unstimulated cells were used as negative control. In some conditions, monocytes were pre-incubated 30 minutes with 10 ng/ml Ferrostatin-1. At

the end of the experiment, supernatants were collected after centrifugation, stored at -20°C and used for co-culture with $CD4^+$ T cells and for chemokine detections.

Endothelial cell culture

HUVECs were cultured in VascuLife® Basal Medium ~~VascuLife® Basal Medium Endothelial Cell Growth Basal Medium 2 (Lonza, Bioscience) supplemented with EGM 2 SingleQuots Supplements (Lonza, Bioscience)~~and supplements from the VascuLife VEGF LifeFactors Kit (Lifeline Cell Technology, Cell Systems). HGMECs were cultured in complete classic medium kit with culture boost from Cell Systems. All experiments were performed until passage 6. Cell cultures were maintained at 37°C in a humidified environment of 5% CO2. Cells were detached with 0.05% Trypsin-EDTA 1 × (Sigma Aldrich) at 37°C for approximately 2 min and split to new plates according to the experimental procedures.

Detection of endothelial cell necrosis

HUVECs and HGMECs were plated in 24-well plates ~~(1×10^5 cells/well)~~ until the formation of a confluent monolayer. Once confluent, ~~HUVECs~~ cells were incubated for 16 h at 37°C with neutrophil supernatants. In some conditions, endothelial cells ~~HUVECs~~ were pre-incubated with either 10 µM Ferrostatin-1 (Fer-1), 2µM Liproxstatin-1 (Liprox-1), 25 µM Necrostatin-1s (Nec-1s), 5µM Necrosulfonamide (NSA) or 10 µM Z-VAD-fkm for 1 hour. At the end of the experiments, both supernatants and ~~HUVECs~~endothelial cells ~~(~~detached with Trypsin-EDTA as described above) were collected and centrifuged 5 min at 1200 rpm. Cell pellets were incubated with APC-labeled Annexin V (BD Bioscience) and propidium iodide (Sigma Aldrich) in 1x Annexin V binding buffer (BD Bioscience), and incubated at room temperature for 15 min, in the dark. After incubation, cells were washed with annexin V binding buffer and analyzed by flow cytometry.

Detection of lipid peroxidation in endothelial cells

Confluent HUVECs and HGMECs were incubated for 4,12 h at 37°C with neutrophil supernatants. In some conditions, ~~HUVECs~~ endothelial cells were pre-incubated with either 10 µM Ferrostatin-1 (Fer-1), 2µM Liproxstatin-1 (Liprox-1), 25 µM Necrostatin-1s (Nec-1s), 5µM Necrosulfonamide (NSA) or 10 µM Z-VAD-fkm for 1 hour. HUVECs and HGMECs were incubated with 2 µM C11-Bodipy 581/591

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(D3861, Invitrogen), for the last 20 min at 37°C in the dark. At the end of the experiments, both supernatants and HUVECs (detached with Trypsin-EDTA as described above) were collected and centrifuged 5 min at 1200 rpm. Cell pellets were then washed, resuspended in PBS and analyzed by flow cytometry. Detection of lipid peroxidation in isolated murine $CD4^+$ T cells was performed following the same procedure.

LDH detection

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LDH in culture medium from HUVECs and HGMECs was detected using the CytoTox96® Non-Radioactive Cytotoxicity Assay from Promega (Walldorf, Germany) following the supplier's protocol.

Detection of endothelial cell permeability

Endothelial cell permeability was assessed as previously described.[6] In brief, HUVECs were seeded at a density of $1x10^4$ cells per well in ~~Endothelial Cell Growth Basal Medium-2 (Lonza, Bioscience)~~ culture medium onto fibronectin-coated transwell chamber (0.4 µM pore size, 6.5 mm diameter from Corning, Amsterdam, Netherlands) placed in 24-well plate. Confluent HUVECs were pretreated with 10 µM Fer-1, 10 µM Z-VAD-fkm or 25 µM Nec1s. After 24 h incubation, transwells were washed with prewarmed medium before BSA-FITC (Sigma Aldrich) was added for 1h at 37°C. Cells were then washed and incubated with neutrophil supernatants for 4 hours at 37°C. After washing, albumin flux to the lower chamber was measured using a SpectraMax5 plate reader (Molecular Devices, Sunnyvale, CA, USA). The basal albumin flux obtained from an EC monolayer incubated with BSA-FITC and buffer alone was subtracted.

Transfection of endothelial cells

HUVECs were transfected with 20 nM small interfering ribonucleic acid (siRNA) using Lipofectamine RNAi MAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Scramble siRNA and siRNA against ~~for~~ ACSL4 and MLKL were purchased from OriGene Technologies GmbH (Herford, Germany). After 48 hours transfection, HUVECs were washed and used for either western blot to confirm ACSL4 knockdown or incubated with neutrophil supernatants.

$CD4^+$ T cell proliferation and polarization

$CD4^+$ T cells were isolated from spleens of WT mice after erythrocyte lysis with RBC lysing buffer Hybri-Max (Sigma-Aldrich) using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. For proliferation assays, cells were stained with carboxy-fluorescein succinimidyl ester (Thermo Fisher Scientific), washed and seeded onto 96-well plate ($2.5x10^5$ cells/well) in RPMI 1640 medium supplemented with 10% FBS, 50 µM 2-mercaptoethanol. Cells were cultivated with Dynabeads Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) at a bead/cell ratio of 1:2 together with supernatant from stimulated monocytes (to a final monocyte:T cell ratio of 5:1) or Fer-1 at day 0. After 3 days in culture, cells were labeled with anti-CD3 and anti-CD4 antibodies and cell proliferation was analyzed by flow cytometry. Dead cells were excluded using the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (ThermoFisher Scientific).

For polarization assays, isolated $CD4^+$ T cells were seeded in 96-well plate as described above with Dynabeads at a bead/cell ratio of 1:1. Cells were cultured in medium described above and supplemented with cytokines (IL-1β, IL- 6, IL-23, TGF-β) and antibodies (anti-IFNγ, anti–IL-2 and anti–IL-4) from the CytoBox T_H17 Kit (Miltenyi Biotec) following the manufacturer's instructions. Supernatants from stimulated monocytes (to a final monocyte:T cell ratio of 5:1) or Fer-1 were added at day 0 and 3. After 7 days in culture, cells were labeled with anti-CD45, anti-CD3, anti-CD4, anti–IL-17A, and anti-IFNγ antibodies and T_H cell subsets were characterized by flow cytometry. Dead cells were excluded as described above.

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Neutrophil extracellular trap (NET) formation

Isolated human neutrophils were stained with SYTOX green (Invitrogen), primed for 15 minutes with 2 ng/ml ~~murine~~ human TNF-α, and stimulated with either 10 µg/ml isotype or monoclonal anti-MPO antibodies. Isolated mouse neutrophils were stained with SYTOX green (Invitrogen), primed for 15 minutes with 5 ng/ml murine TNF-α, and stimulated with 150 µg/ml polyclonal anti-MPO antibodies. For ferroptosis inhibition, cells were pre-treated 30 minutes with 10 µM Fer-1 ~~After 1.5 hours, neutrophil extracellular traps (~~Human and mouse NETs~~)~~ were observed by microscopy after 1.5- and 3-hour incubation, respectively. For NET quantification, SYTOX positive area were measured using ImageJ processing software (NIH) and expressed as arbitrary unit.

Detection of Chemokines

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Chemokines were measured in monocyte supernatants using the mouse IL-1beta/IL-1F2 and IL-6 DuoSet ELISA from R&D Systems following the manufacturer's instructions.

SDS-PAGE and Western Blot

Whole cell and organ lysates were prepared in RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate [SDS], 0,5% sodium deoxycholate) supplemented with complete protease and phosphatase inhibitor cocktails (Roche and Sigma, respectively). Protein lysates were loaded on SDS polyacrylamide gel and transferred to nitrocellulose membranes by electrophoresis. The membranes were blocked in 5% non-fat dry milk for 1h and incubated overnight with the following antibodies: rabbit anti-Glutathione Peroxidase 4 (GPX4) antibody, rabbit anti-FACL4/ACSL4 (Abcam), rabbit anti-MLKL (Merck Millipore), rabbit anti–β-actin (Cell Signaling Technology, Frankfurt am Main, Germany). Horseradish peroxidase–labeled donkey anti-rabbit IgG (GE Healthcare~~), Little Chalfont, United Kingdom)~~ was used as secondary antibody. Thermo Scientific SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific) was used for detection.

RNA isolation and real-time PCR

Whole-kidney RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and reverse transcription was performed with the

SuperScript III One-Step RT-PCR System (Thermo Fisher Scientific). Renal Gpx4 and Acsl4 gene expression was determined by quantitative RT-PCR using the QuantStudio 3 instrument

(Thermo Fisher Scientific) and primers synthetized by BioTeZ Berlin-Buch GmbH (Berlin, Germany).

Statistical analysis

Results were expressed as means ± SEM. All statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). A t-test was used for comparison between two groups, and one-way or two-way analysis of variance with *post hoc* analysis was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Supplementary figures

Supplementary Figure S1. Experimental protocol and 4-HNE staining in human endothelial cells *in vitro*. (**a**) Experimental protocol describing the generation of cell-free neutrophil supernatants (cf-SN) and their incubation together with HUVECs and HGMECs in culture pre-treated with different cell death inhibitors. (**b**) Following incubation with supernatants from isotype (Iso)-stimulated neutrophils, HUVECs and (**c**) HGMECs were washed, stained for lipid peroxidation (4-HNE) and nuclei (DAPI) and observed by microscopy. Ferrostatin (Fer-1) and Liproxstatin-1 (Liprox-1) pre-treatment were used for ferroptosis inhibition. Representative images of 3 independent experiments are presented. Scale bar represent 100µm.

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Supplementary ~~F~~figure S2~~1~~. Increased ferroptosis markers in kidneys from mice with AAV. Representative immunofluorescence images (40x magnification) for CD71 (green, upper panel) and MDA (green, lower panel) show increased ferroptosis in kidney from mice with αMPO-induced NCGN (AAV) compared to control mice (Ctrl). CD31 (red) was used to stain endothelial cells and DAPI (blue) to stain nuclei. Scale bar represent 20µm.

Supplementary Figure S3. Ferroptosis inhibition with Liproxstatin-1 does not protect mice from the development of ANCA-associated NCGN. (**a**) Experimental settings describing the induction of NCGN in WT mice induced by G-CSF (30µg/mouse), LPS (5µg/g body weight) and αMPO IgG (50µg/g body weight) injection. Ferroptosis inhibition was mediated by daily injection of Liproxstatin-1 (Liprox-1, 10µg/g body weight) from day 0 until sacrifice at day 7. (**b**) Liprox-1 treatment led to similar renal damage compared to mice without treatment. Representative images of kidney sections stained with PAS at high (40x) magnification and quantitative analyses of glomeruli with crescents and necrosis are shown for each group. Scale bar represent 20µm. Data from 3 healthy mice are provided for comparison. (**c**) The urine albumin-creatinine ratio (uACR) and (**d**) NGAL urine levels determined by ELISA were

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similar in both groups. (**c**) Myeloid cell infiltration was analyzed by flow cytometry. Infiltration of $CD11b^+Ly6G^+$ neutrophils and $CD11b^+Ly6G^-Ly6C^+$ classical monocytes (CM) was similar in both groups. Error bars indicate means ± SEM.

Supplementary Figure S42. Ferroptosis markers and infiltrating T cells in kidneys from mice with AAV. (**a**) Representative immunofluorescence images (20x magnification) for MDA (green, lower panel) show similar ferroptosis in kidney from mice with αMPO-induced NCGN (AAV) compared to control mice (Ctrl). CD31 (red) was used to stain endothelial cells and DAPI (blue) to stain nuclei. Scale bar represent 50µm. (**b**) Gating strategy for the characterization of renal T cell subsets per flow cytometry.

Supplementary Figure S5. Murine monocyte supernatants and human NETs. (**a**) Murine monocytes were isolated from the bone marrow, primed with TNF-α and stimulated with anti-MPO IgG. In some conditions, cells were pre-treated 30 min with Ferrostatin-1 (Fer-1). Unstimulated cells were used as negative controls. IL-1β and IL-6 levels in culture medium were determined by ELISA. (**b**) Isolated human neutrophils were primed with TNFα and subsequently stimulated with either isotype or anti-MPO IgG for 90 min. In some conditions, cells were pre-treated 30 min withFerrostatin-1 (Fer-1). Viable cells were stained with SYTOX green to detect neutrophil extracellular traps (NETs) by microscopy (20X magnification). The corresponding statistical analysis of the amount of SYTOX green positive area (NET-producing neutrophils) expressed as arbitrary unit (AU) from three independent experiments with different neutrophil donors is shown. Error bars indicate means ± SEM. *P<0.05; **P<0.01.

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Figure 3a

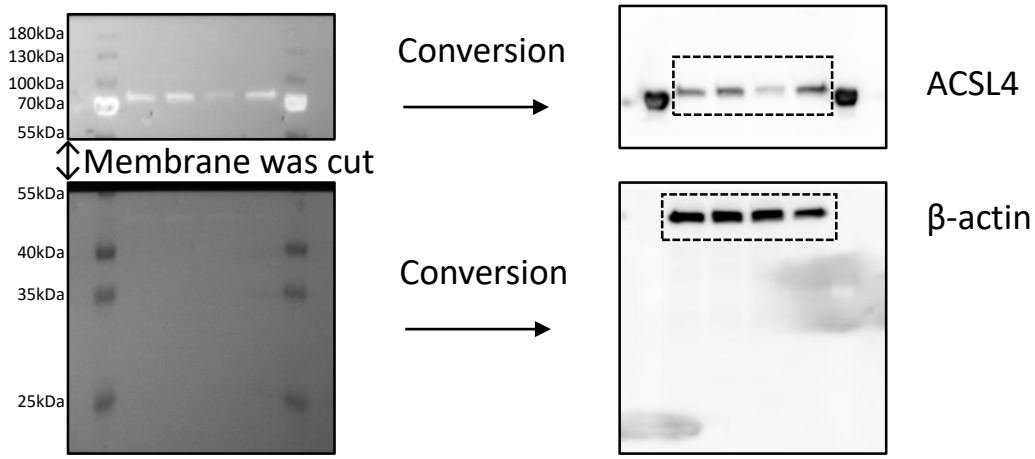


Figure 3d

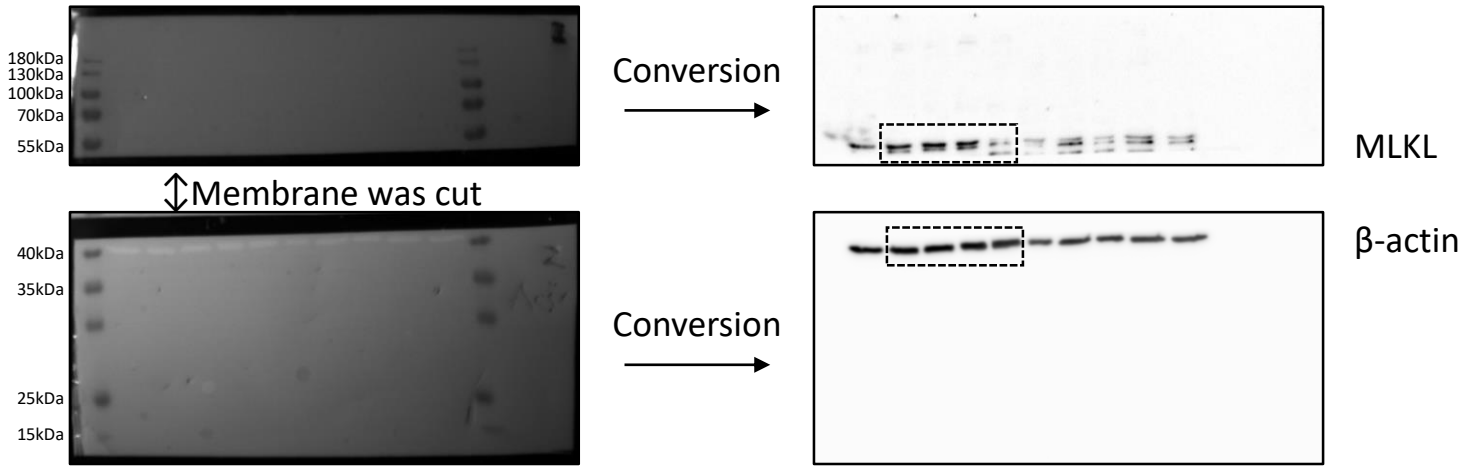
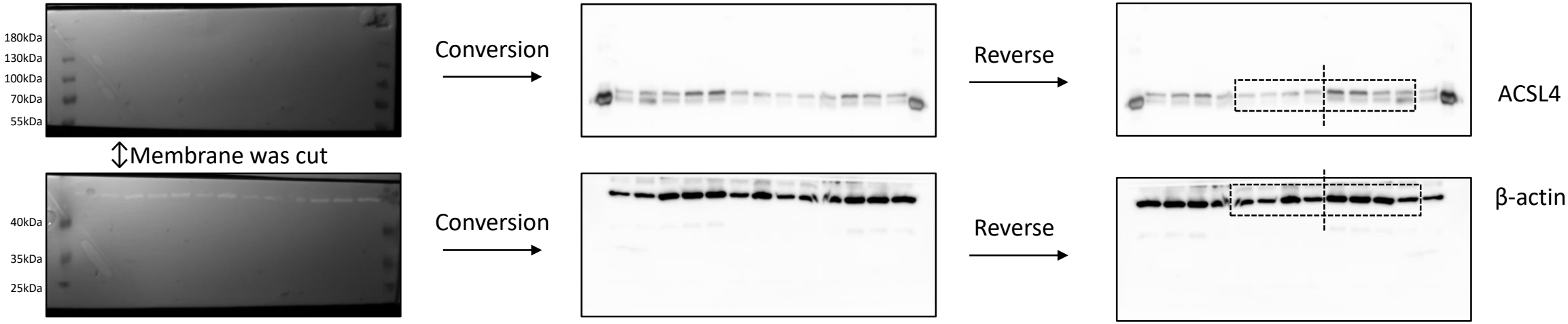


Figure 3b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70kDa
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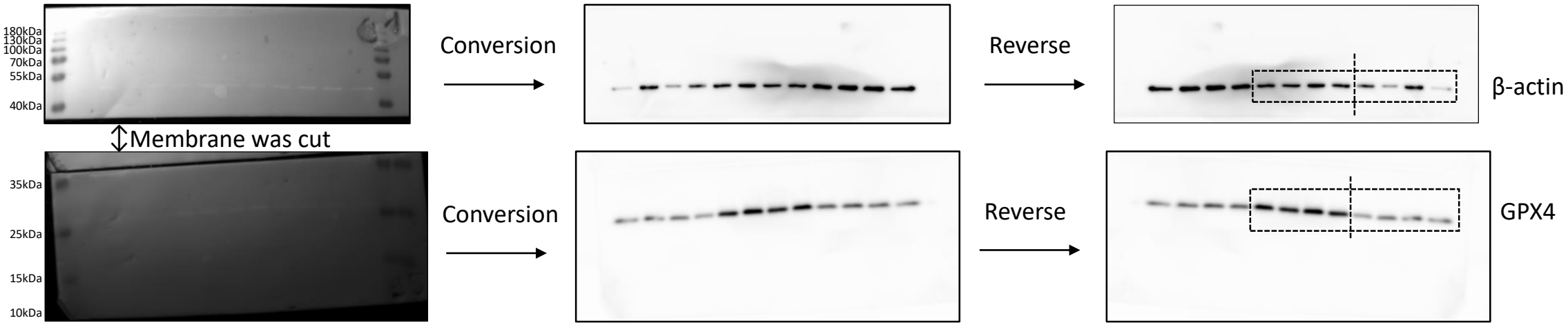
β-actin

Figure 3c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