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Arginase-II promotes tumor necrosis factor-α release from pancreatic acinar cells causing β-cell apoptosis in aging

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Supplementary Materials

Materials

All cell culture media and materials were purchased from Gibco/Thermo Fisher Scientific (Waltham, MA USA); Bio-Rad DC^{TM} protein assay kit was from Bio-Rad Laboratories (California, USA); anti-insulin (#3014), anti-glucagon (#8233), anti-caspase-3 (#9665), anti-cleaved caspase-3 (#9661), anti-PCNA (#2586), anti-Akt-T308 (#13038), anti-S6K1-T389 (#9234), anti-S6 (#2211), anti-S6-S240/244 (#5364) and anti-p38mapk-T180/Y182 (#9211) antibodies were purchased from Cell Signaling Technology (Danvers, USA); mouse anti-p38mapk antibody (612168), anti-Akt (610836), anti-S6K1 (611260) and anti-Arg-I antibody (610708) were from BD biosciences (New Jersey, USA); anti-Arg-II (sc-20151), anti-CPA1 (sc-131901) antibodies were from Santa Cruz Biotechnol (Dallas, USA); anti-F4/80 (MCA497G) was from AbD Serotec (Düsseldorf, Germany); anti-TNF-α (ab1793) was from Abcam (Cambridge, UK); IRDye800-conjugated anti-rabbit IgG (926–32211) was from LI-COR Biosciences (Lincoln, USA); Alexa fluor 488 conjugated anti-rat (A11006), Alexa fluor 488 anti-rabbit (A11008), Alexa fluor 488 conjugated anti-mouse (A11001), Alexa fluor 594 anti-rabbit IgG (A21076), Alexa fluor 594 conjugated anti-goat IgG (A11058) and Alexa fluor 680 conjugated anti-mouse IgG (A21057) were from Invitrogen/Thermo Fisher Scientific (Waltham, MA USA); Anti-tubulin (T5168), Collagenase 1A (C9891), 1077 Histopaque (10771), and 1119 Histopaque (11191) were from Sigma; CD11b MicroBeads (130-049-601) were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Normal chow (energy content: 10.6% fat, 27.6% protein, and 57% carbohydrate, fiber 4.8%) for mice feeding is purchased from Provimi Kliba NAFAG 3436, Basel, Switzerland.

Quantitative reverse transcription PCR (qRT-PCR) analyses

mRNA of *TNF-α, IL-6, MCP-1, KC* (the murine *IL-8* homologue), *F4/80, IL-1β,* and *IFN-γ* was determined by two-step qRT-PCR. Total RNA was extracted from cells or tissues with Trizol Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) following the supplier's protocol. First-strand cDNA was synthesized from 500 ng total RNA with a random primer. Real-time PCR was performed with the iQ^{TM} SYBR Green Supermix and iCycler system (Bio-Rad). mRNA expressions were normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following PCR primers of mouse origin (m) were used:

m*IL6*-F: GACAACCACGGCCTTCCCTA
m*IL6*-R: GCCTCCGACTTGTGAAGTGGT
m*KC*-F: CAATGAGCTGCGCTGTCAGTG
m*KC*-R: CTTGGGGACACCTTTTAGCATC
m*MCP1*-F: AGCACCAGCCAACTCTCAC
m*MCP1*-R: TCTGGACCCATTCCTTCTTG
m*F4/80*-F: TGGCTGCCTCCCTGACTTTC
m*F4/80*-R: CAAGATCCCTGCCCTGCACT
m*TNFα*-F: GGCAGGTCTACTTTGGAGTCATTGC

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m*TNFα*-R: ACATTCGAGGCTCCAGTGAATTCGG
mIL-1β–F: GCAACTGTTCCTGAACTCAACT
mIL-1β–R: TCTTT GGGGTCCGTCAACT
mIFN-γ–F: TGGCATAGATGTGGAAGAAAAGAG
mIFN-γ–R: TGCAGGATTTTCATGTCACCAT
mArg-II-F: TGTCCCACAAGATGATCCCT
mArg-II-R: CCAGATACAGTGGTGAGAGGT
m*GAPDH*-F: ACCCAGAAGACTGTGGATGG
m*GAPDH*-R: ACACATTGGGGGTAGGAACA

Culture of primary pancreatic acinar cells and depletion of macrophages

Isolated pancreatic acinar cells were cultured in Waymouth's medium (31220-023, Gibco) containing 2.5% FBS, 1% penicillin-streptomycin mixture, 0.25 mg/ml of trypsin inhibitor, and 25 ng/ml of recombinant human epidermal growth factor (EGF). After 48-hour culture of pancreatic acinar cells, culture medium was collected as the conditioned medium. Macrophage depletion from mixture of pancreatic acinar cells was achieved by magnetic cell sorting with CD11b microbeads (130-049-601, Miltenyi Biotec GmbH), LS Columns (130-042-401, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and QuadroMACS™ Separator (130-090-976, Miltenyi Biotec GmbH Bergisch Gladbach, Germany) according to manufacturer's instructions.

Tissue immunofluorescence staining and TUNEL assay

The detection of apoptotic cells in pancreas was carried out by staining of the 7 μm thick paraffin-embedded section of pancreas with the "In Situ Cell Death Detection Kit, TMR red" (TUNEL) (Roche Applied Science, #12156792910, Basel, Switzerland) according to manufacturer's instructions. The signals were visualized under LEICA's DIM6000 confocal microscope. Quantification of the signals was performed using NIH Image J 1.49 software. β-cell apoptosis was quantified by percentage of insulin[+] and TUNEL[+] double positive cells / insulin^{+} cells in pancreases.

Enzyme-Linked Immunosorbent Assay (ELISA)

The TNF-α protein level in the conditioned medium of isolated mouse acinar cells was measured by ELISA with the use of the ELISA MAX Deluxe from BioLegend according to manufacturer's instructions (San Diego, CA, USA). *Ex vivo* glucose-stimulated insulin secretion (GSIS) in the incubation medium was measured by ELISA according to manufacturer's instructions (EMINS, Thermo Fisher Scientific, Waltham, MA USA).
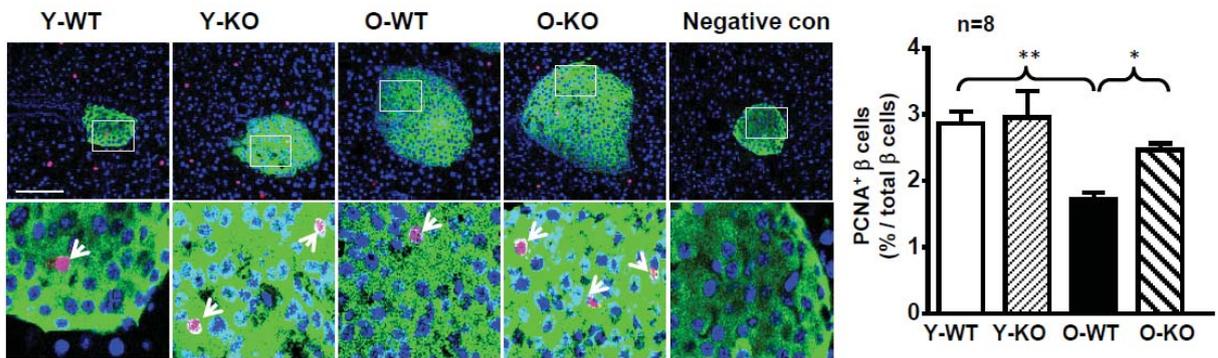
SUPPLEMENTARY DATA

Supplementary Figure 1. Body weight development of WT and Arg-II-/- mice. There is no significant difference in body weight development of **(A)** female and **(B)** male WT and Arg-II-/- mice. WT: wild type; KO: Arg-II-/-.
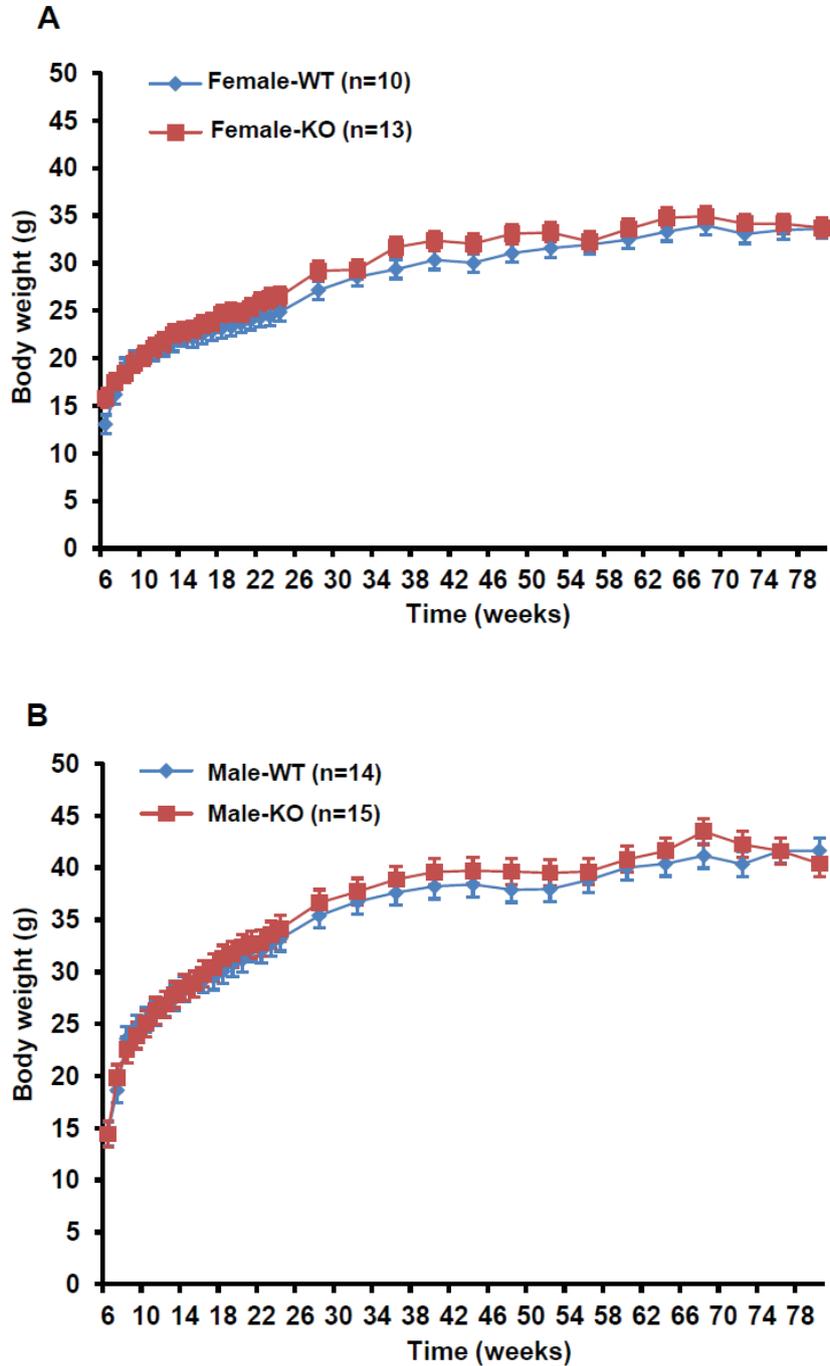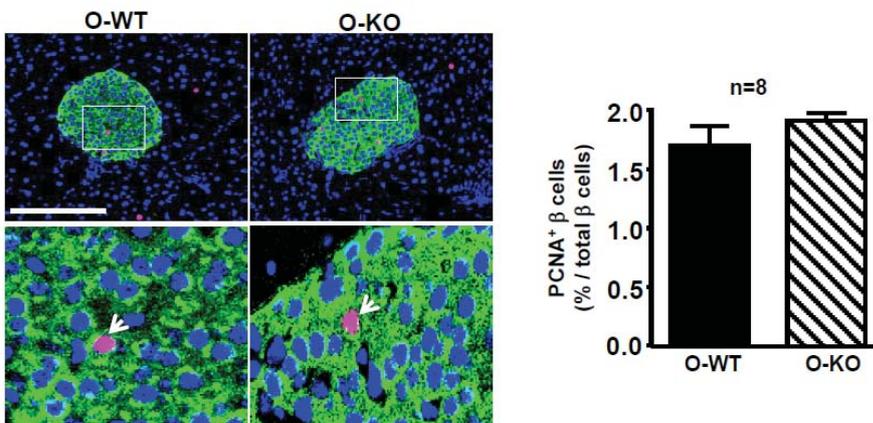
SUPPLEMENTARY DATA

Supplementary Figure 2. Arg-II deficiency enhances β-cell proliferation in old female mouse pancreas. β-cell proliferation was evaluated by co-immunostaining of PCNA (red) and insulin (green) in paraffin-section of pancreas of **(A)** female mice and **(B)** male mice. The image in the lower panel is the enlargement of the selected area in the upper pictures. The white arrows indicate PCNA positive β cells within islets. Young wild type (WT) mouse pancreatic sections stained without primary antibody were served as negative control. Quantification of percentage of PCNA positive β-cells is presented in the right bar graph. Scale bar = 100 μm. Y-WT: young wild type; Y-KO: young Arg-II-/-; O-WT: old wild type; O-KO: old Arg-II-/-. *p<0.05, **p<0.01.
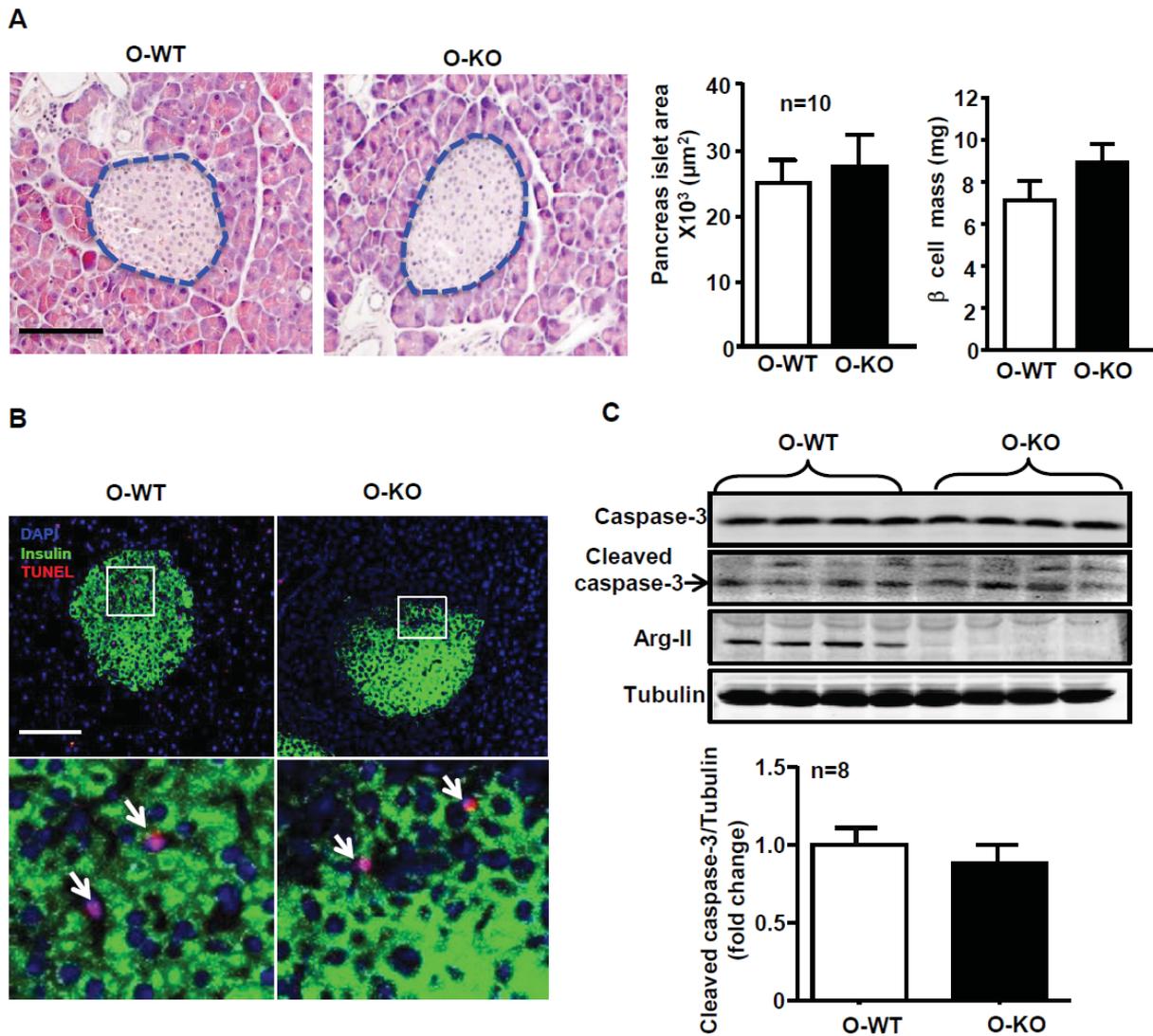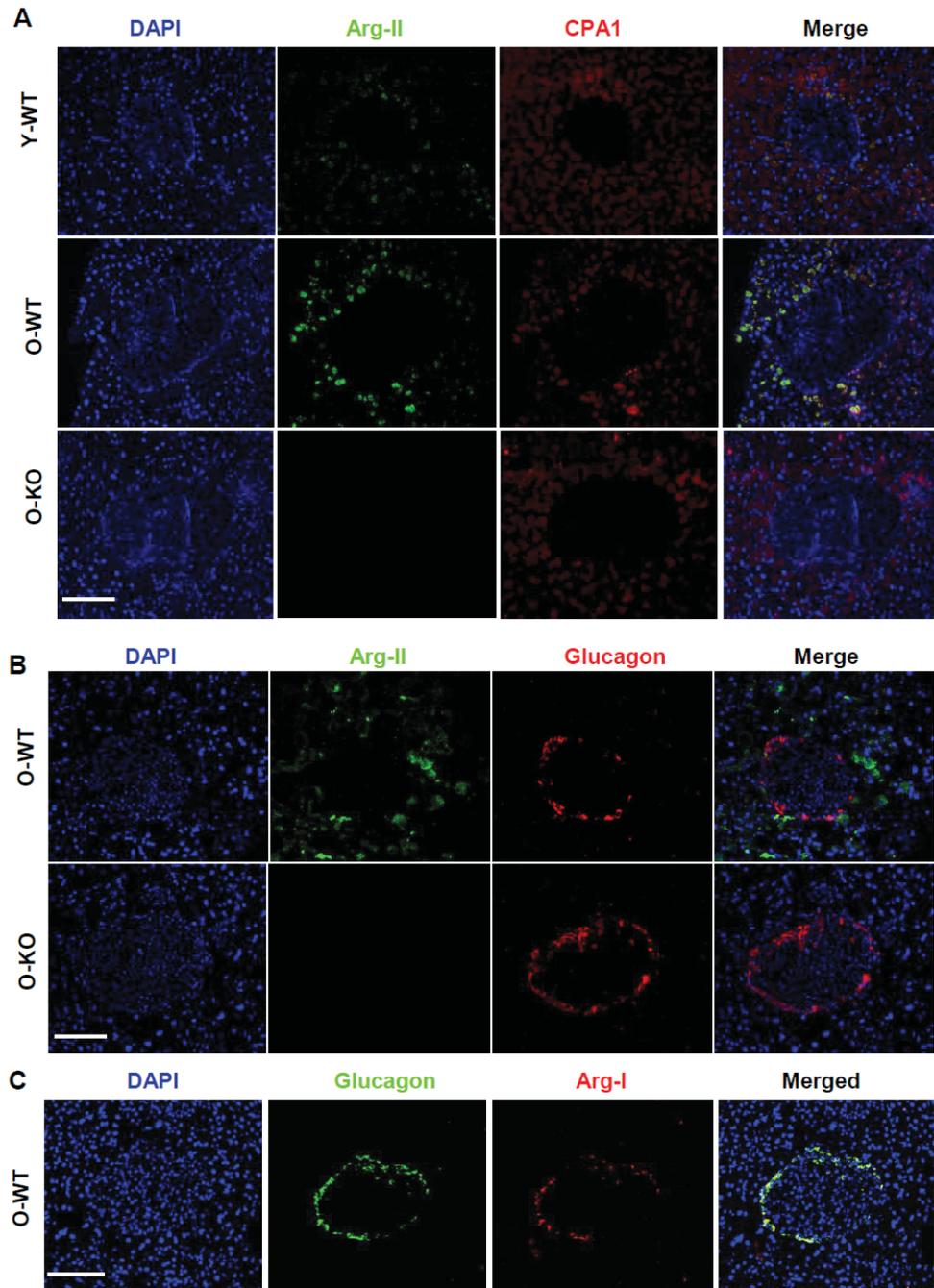
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Supplementary Figure 3. There is no significant difference in pancreatic islet size, β-cell mass or apoptosis between old male wild type (O-WT) and Arg-II-/- (O-KO) mice. Mice were sacrificed and pancreas was collected. **(A)** The paraffin-sections of pancreas were subjected for hematoxylin and eosin staining (H&E staining). The size of pancreatic islets was measured and expressed as pancreatic islet area in μm2. **(B)** The paraffin sections of pancreas were subjected to TUNEL staining for apoptotic cells (red) followed by immunofluorescence staining for insulin (green) and subsequent counterstaining for nuclei with DAPI (blue). The white arrows indicate the apoptotic cells. Scale bar: 100 μm. **(C)** Immunoblotting analysis of total caspase-3, cleaved caspase-3, Arg-II, and tubulin in pancreas lysates. Quantification of cleaved caspase-3/tubulin representing apoptosis is presented in the bar graph below.
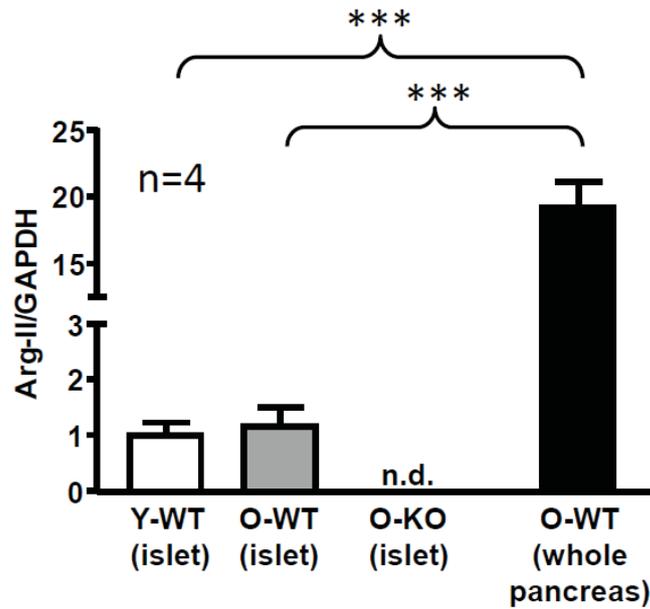
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Supplementary Figure 4. Arg-II is mainly expressed in acinar cells in pancreas, while Arg-I in α-cells. Co-immunostaining of pancreatic section for **(A)** Arg-II (green) and CPA1 (acinar cell marker, red); **(B)** Arg-II (green) and glucagon (α-cell marker, red); and **(C)** glucagon (green) and Arg-I (red), followed by counterstaining of the nuclei with DAPI (blue). Scale bar = 100 μm. Y-WT: young wild type; O-WT: old wild type; O-KO: old Arg-II-/-.

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Supplementary Figure 5. Arg-II is poorly expressed in mouse pancreatic islets. Arg-II mRNA expression in isolated islets from Y-WT, O-WT and O-KO and in whole pancreas from O-WT was analysed by qRT-PCR. GAPDH was used as reference. Y-WT: young wild type; O-WT: old wild type; O-KO: old Arg-II-/-; n.d. : not detectable; ***p<0.001.

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Supplementary Figure 6. Arg-II is expressed in macrophages in pancreas. Co-immunostaining of pancreatic sections for Arg-II (red) and F4/80 (green, macrophage marker) followed by counterstaining of the nuclei with DAPI (blue). The arrows indicate the co-stained cells. Scale bar = 100 μm. Y-WT: young wild type; Y-KO: young Arg-II-/-; O-WT: old wild type; O-KO: old Arg-II-/-.