Ambrozova & Martiskova et al., Nitro-oleic acid modulates classical and regulatory activation of macrophages and their involvement in pro-fibrotic responses

Supplementary data

Supplementary methods

Cell culture and treatment (BMDMs)

BMDMs were isolated from tibiae and femora of C57BL/6J mice (males, 25-30 g, 12-14 weeks old; Masaryk University, Brno, Czech Republic) housed in a temperature-controlled animal facility with 12 h light-dark cycle and free access to rodent chow. Prior to the isolation procedure, the mice were sacrificed by inhalation of carbon dioxide [14]. These experiments were approved by the Animal Care Committee in accordance with the EU and NIH Guides for Care and Use of Laboratory Animals. BMDMs were grown in DMEM, which was supplemented with growth factors derived from cultured CCL-1 cells (ATCC, Manassas, VA, USA), 20% of low endotoxin FBS and 1% gentamycin [14]. BMDMs were cultured no longer than 7 days before use.

BMDMs were treated with different concentrations of OA-NO_2 with or without LPS (100 ng/ml, *E. coli* serotype 026:B6) and IL-4 (20 ng/ml) for different time points. Before each experiment, BMDMs were cultured in complete media as indicated above. Two hours before the start of experiments, the complete medium was replaced with serum free DMEM. Different concentrations of OA-NO_2 (0.1, 0.25, 0.5, or 1.0 μM), based on their physiological relevance [20-24], were applied together with LPS or IL-4. Cell viability was measured by ATP Cell Viability test (BioThema, Handen, Sweden); no effect of OA-NO_2 exposure was detected (data not shown).

Immunostaining of α-SMA in heart tissues

For immunostaining of fibrotic sections in the heart tissue, α-SMA was chosen as a suitable fibrotic marker. Primary antibody against α-SMA (1:100, mouse IgG; Biomeda V1031) and goat anti-mouse IgG (H+L) secondary antibody, DyLight 488 conjugate (red color, Thermo Scientific), were used. Nuclei were stained with DAPI (blue color). Images were taken with a Retiga 1300 CCD camera mounted on Leica DMLB fluorescence microscope by iVision 4.0.

Immunostaining for $O_2^{\cdot-}$ in heart tissues

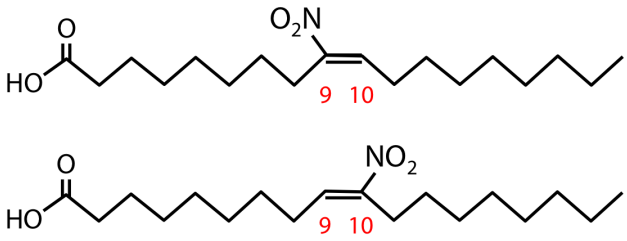
For detection of $O_2^{\cdot-}$ production in heart sections, dihydroethidium (DHE) staining was used. Frozen atrial sections were stained with DHE (5 μM, diluted in DMSO and HBSS-buffer). The slides were incubated with DHE (red color) for 30 minutes at 37°C in the dark before pictures were made. Images were taken with a Retiga 1300 CCD camera mounted on Leica DMLB fluorescence microscope by iVision 4.0.

Detection of PPARγ activity

The luciferase reporter construct PPRE pTK-LUC (kindly donated by Prof. Ronald M. Evans, Gene Expression Laboratory, Salk Institute for Biological Studies, CA, USA) was transiently

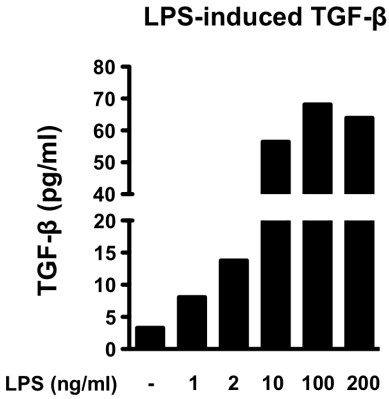
transfected into RAW 264.7 macrophages using an electroporation system (Gene Pulser II, Bio-Rad Laboratories, CA, USA) [26]. The construction of PPRE pTK-LUC was described in detail elsewhere [27]. Activation of the reporter construct was determined by luciferase activity measurement using the Luciferase Reporter Gene Assay (Roche, Basel, Switzerland). The integral value of the luminescence reaction represents the total luciferase activity in RAW 264.7 macrophages [26].

Supplementary Figures:

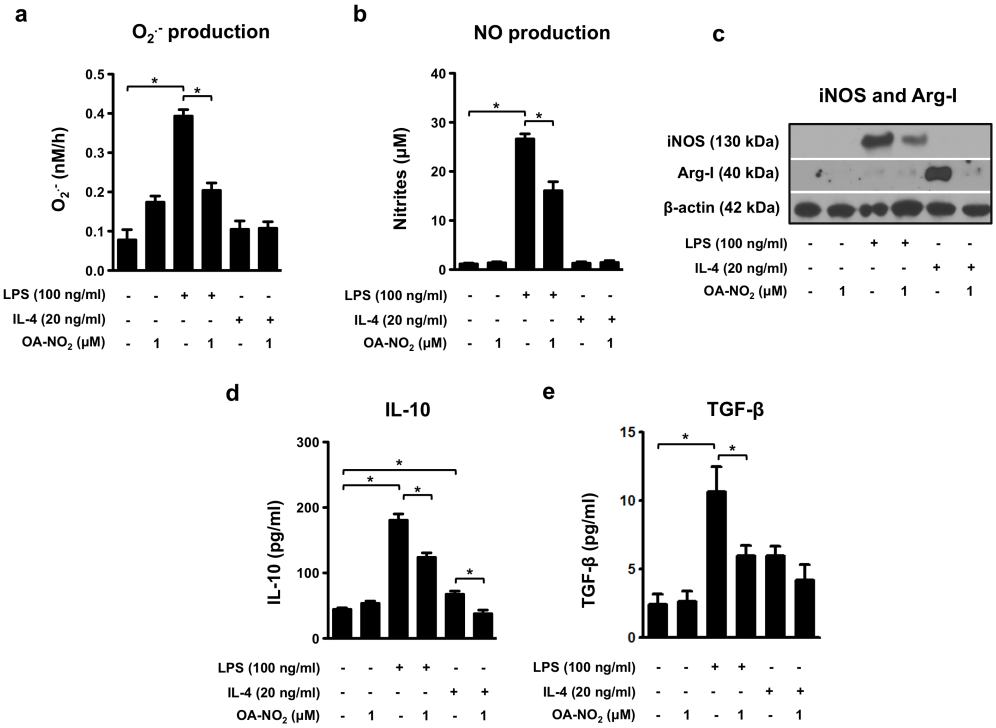


(*E*)-9- and 10-nitro-octadec-9-enoic acids

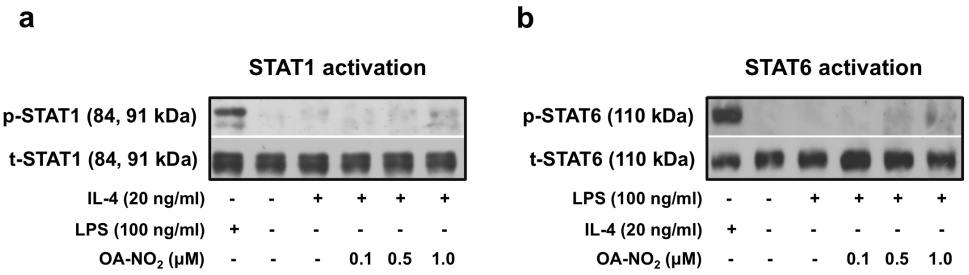
Supplement Figure 1: Chemical structure of nitro-oleic acid regioisomers.



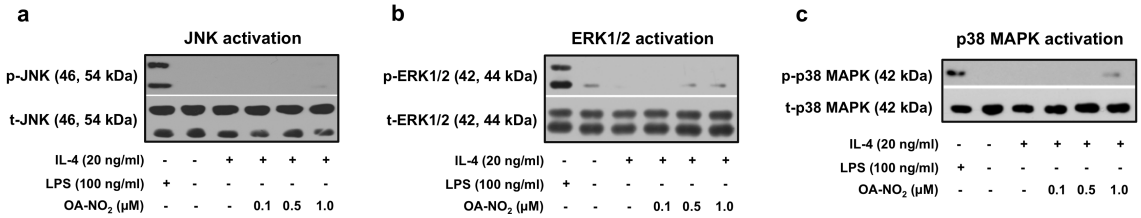
Supplement Figure 2: TGF-β production is elevated in LPS-stimulated RAW 264.7 macrophages. Cells were treated with different concentrations of LPS (1-200 ng/ml). The production of TGF-β was determined after 24 h of cell incubation and results are displayed as representative of concentration dependence.



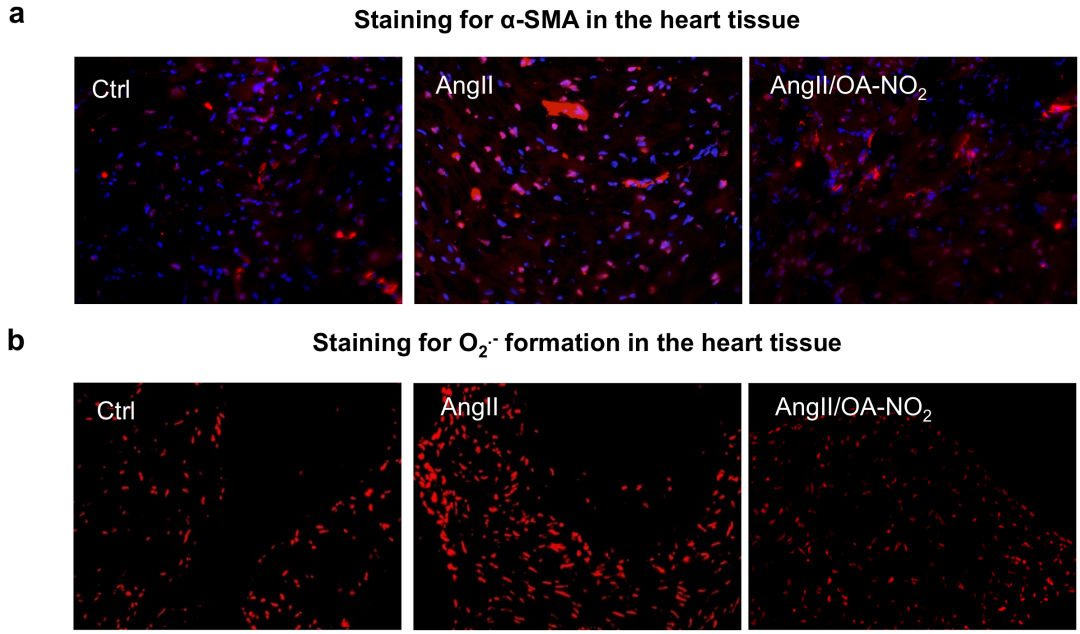
Supplement Figure 3: OA-NO_2 decreases LPS- and IL-4-induced activation of "M1-" and "M2-like" BMDMs. Cells were treated with OA-NO_2 (1.0 μM) and stimulated with LPS (100 ng/ml) or IL-4 (20 ng/ml). Formation of $O_2^{\cdot-}$ was detected in cells incubated for 4 h (n=6) (**a**). Production of NO (**b**), expression of iNOS and arginase-I (**c**), as well as accumulation of IL-10 (**d**) and TGF-β (**e**) was detected in culture medium 24 h after cell incubation (n=6). The pictures represent one of three individual experiments. A *p value of less than 0.05 was considered significant when evaluating differences between the individual bars and positive control (LPS- and IL-4-treated cells) or between two individual bars, respectively.



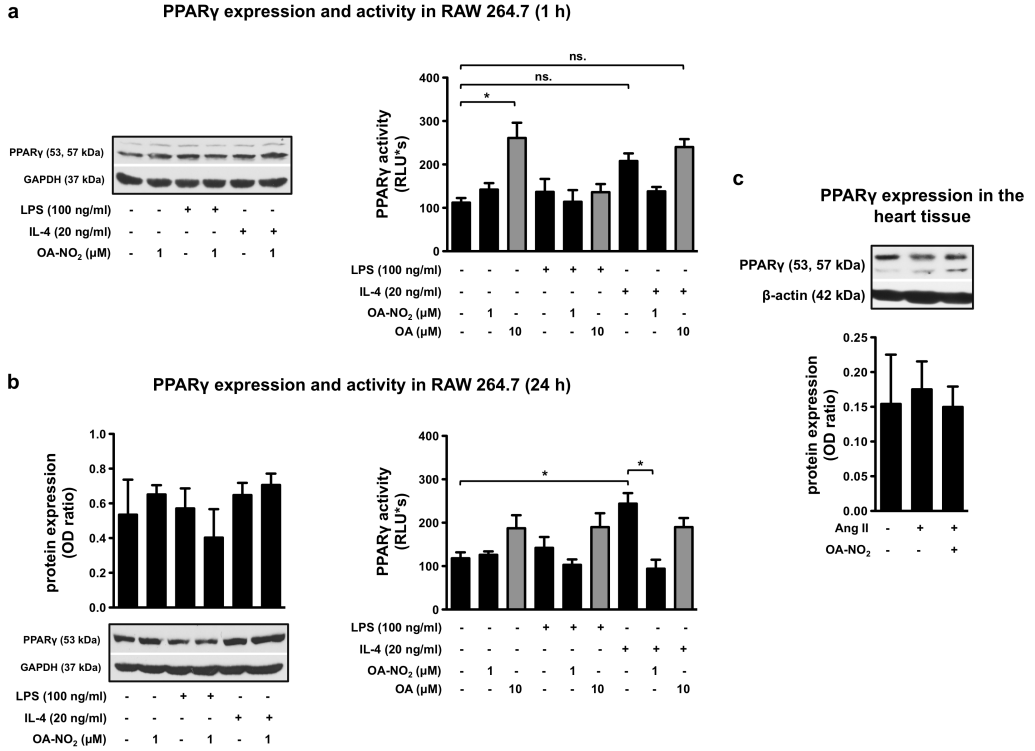
Supplement Figure 4: IL-4 does not stimulate the activation of STAT1 as well as LPS does not stimulate the activation of STAT6 in RAW 264.7 macrophages. The expression and phosphorylation of STATs was detected in RAW 264.7 cells treated with different concentrations of OA-NO_2 (0.1, 0.25, 0.5, 1.0 μM) and stimulated with LPS (100 ng/ml) or IL-4 (20 ng/ml) for 1 h. Expression of p-STAT1/t-STAT1 (**a**) and p-STAT6/t-STAT6 (**b**) was detected in both "M1-" and "M2-like" macrophages (n=3). The pictures represent one of three individual experiments.



Supplement Figure 5: IL-4 does not stimulate the activation of MAPKs in RAW 264.7 macrophages. Cells were treated with OA-NO_2 (0.1, 0.5, 1.0 μM) and exposed to LPS (100 ng/ml) or IL-4 (20 ng/ml). Phosphorylation of MAPKs (a-c) was detected 1 h after cell stimulation with LPS or IL-4. The pictures represent one of three individual experiments.



Supplement Figure 6: The OA-NO_2 reduces fibrosis in the heart tissue of Ang II-treated mice. The immunostaining for α-SMA (**a**) and $O_2^{\cdot-}$ (**b**) was proved in heart tissue of C57BL/6J mice treated for 2 weeks with Ang II (1.5 ng/g/min) and OA-NO_2 (6 mg/kg). The pictures represent one of four individual experiments.



Supplement Figure 7: The OA-NO_2 down regulates the IL-4-induced activity of PPARγ in "M2-like" RAW 264.7 macrophages. The expression and activity of PPARγ was detected in RAW 264.7 cells treated with OA-NO_2 (1 μM) and stimulated with LPS (100 ng/ml) or IL-4 (20 ng/ml) for 1 h (**a**) or 24 h (**b**). In these experiments, oleic acid (OA) was used as a positive control (n=6). The expression of PPARγ was also monitored in the heart tissue of C57BL/6J mice, treated for 2 weeks with Ang II (1.5 ng/g/min) and OA-NO_2 (6 mg/kg) (**c**). The pictures represent one of several individual experiments (n=6-9). A *p value of less than 0.05 was considered significant, when evaluating differences between the individual bars and positive control (OA-, LPS- or IL-4- treated cells and Ang II-treated mice) or between two individual bars, respectively.