SUPPLEMENTAL MATERIAL

Supplemental Methods

Arterial wire-induced injury

Wild-type and *Ldlr*$^{-/-}$ mice (both on the C57BL/6J background) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and *apoE*$^{-/-}$ mice (C57BL/6J background) from Charles River, Italy. At 6 to 8 weeks of age mice were placed on an atherogenic diet (21% fat, 0,15% cholesterol, Altromin) for one week before and up to three weeks after injury. Animals were treated intravenously with a bolus of 0.76 µg RNase1 (in PBS) or PBS alone immediately before wire-induced injury and continuously treated with RNase1 via Alzet® osmotic minipumps (42 µg/kg mouse per day), subcutaneously implanted one day before injury. For induction of wire-induced injury, mice were anesthetised intraperitoneally with ketamine and xylazine. After midline neck incision, the left external carotid artery was tied off distally, and via transverse arteriotomy, a 0.014-in flexible angioplasty guidewire was advanced by 1 cm. Complete and uniform endothelial denudation was achieved by 3 passes along the common carotid artery with a rotating motion. All animal studies were approved by local authorities and complied with German animal protection law.

High-fat-diet driven atherosclerosis in LDL-receptor-deficient mice

C57BL/6J and *Ldlr*$^{-/-}$ *mice* (C57BL/6J background) were obtained from Jackson Laboratory and were fed with atherogenic diet as described[1].

Immunohistochemistry

For evaluation of neointima formation, mice were sacrificed at indicated time points, *in situ* fixed with 4 % paraformaldehyde, and carotid arteries were excised and embedded in paraffin. Neointimal and medial areas were quantified in serial sections (5 µm; 10 per mouse) within 500 µm from the bifurcation by Movat's pentachrome staining and planimetry of the areas within external elastic lamina, internal elastic lamina, or lumen (Diskus software, Hilgers). Adjacent sections were used to assess plaque cellular content by immunofluorescence staining of macrophages and SMCs by mAb staining for Mac2 (rat anti-mouse, Santa Cruz) and α-smooth muscle actin (mouse anti-human, Santa Cruz), respectively. Briefly, slides were blocked with 1% bovine serum albumin (Sigma Aldrich), incubated with primary antibody overnight at 4°C, and secondary detection performed using the relevant Alexafluor 488-conjugated antibody (Molecular Probes, Life Technologies, Germany). Sections were

counterstained with DAPI (1µg/mL; Merck, Darmstadt Germany) for visualisation of cell nuclei, then coverslipped using Vectorshield mounting medium (Vector Laboratories, Burlingame, USA). Images were recorded using a Leica DM-RXE fluorescence microscope, and quantified using Diskus software.

To determine the distribution of eRNA in atherosclerotic lesions, mice were sacrificed at indicated time points and perfused via the left ventricle with 10 ml PBS containing 1 mM EDTA and 30 ml fixative (PBS, 4% paraformaldehyde and 5% sucrose), and the atherosclerotic lesions were analyzed in the heart aortic valve as described previously[1]. Immunohistochemistry was performed on 5 µm cryostat tissue sections, fixed for 15 min in acetone at -20°C. SYTO® RNASelect™ dye (Invitrogen, USA) and the primary antibody recognizing rat anti-mouse MOMA-2 monoclonal antibody (Abcam, Cambridge, UK) against monocytes/macrophages were incubated for 1 h at room temperature followed by a secondary antibody conjugated with Cy3 (Abcam), or primary antibody against TNF-α (goat anti-mouse polyclonal, Santa Cruz) or Icam-1 (rat anti-mouse monoclonal, ebioscience), detected by FITC-konjugated secondary antibody (rabbit anti-goat IgG, goat anti-rat IgG, Jackson ImmunoResearch). Tissue sections were counterstained with DAPI (1µg/ml, Sigma Aldrich) for nuclei detection. Specimens were visualized using the confocal NIKON-microscope ECLIPSE TE200-E (Nikon, Düsseldorf, Germany), sections were taken through the tissue at 0.25 µm intervals, and analyzed by image acquisition software EZ-C1 Goldversion 3.8.

Confocal microscopy and quantification of RNA

Cryosections (5 µm thick) of aortic sinus from *Ldlr*$^{-/-}$ mice were air dried and fixed with 4% paraformaldehyde and then incubated with 1% bovine serum albumin for 30 min to block non-specific binding sites. After rinsing in PBS, the samples were incubated 2 h at room temperature with primary antibodies against smooth muscle α-actin TRITC-labeled (Sigma). After repeated washes with PBS, the tissue sections were incubated with SYTO® RNASelect™ dye (Invitrogen) used for RNA localization. Nuclei were visualized with DAPI (Molecular Probes). Tissue sections were examined by laser scanning confocal microscopy (Leica TCS SP2), and confocal optical sections were taken using a Leica x63/1.32 objective lens. Each recorded image was taken using multi-channel scanning and consisted of 1024 x 1024 pixels. To improve image quality and to obtain a high signal to noise ratio, each image from the series was signal-averaged and was deconvoluted using AutoQuant X2 software (Bitplane, Zürich, Switzerland). For three-dimensional image reconstructions, an Imaris 6.3.1 multichannel image processing software (Bitplane) was used.

For quantification of the eRNA, all tissue samples were simultaneously immunolabeled under identical conditions of fixation and dilutions of primary and secondary antibodies. Ten randomly chosen fields of vision were quantified using three-dimensional "Quantification" and "VoxelShop" options of Imaris 6.3.1 (Bitplane). The area of specific labeling for eRNA was calculated as arbitrary fluorescent units of positive labelling, each for the media area or neointima area, as previously described.[2]

Intravital imaging

For intravital microscopy, 0.05% rhodamine-G (Molecular Probes) was administered *i.v.* to *apoE*$^{-/-}$ mice fed an atherogenic diet and treated with RNase or vehicle (PBS) 1 day after injury. Carotid arteries were exposed in anesthetized mice (100 mg/kg bodyweight ketamine and 10 mg/kg bodyweight xylazine). Arrest of labeled leukocytes was visualized by epifluorescence microscopy (Olympus BX51, 20x water immersion), and recorded using a digital camera (Hamamatsu EM-CCD, C9100) and analysed using Cell-R software (Olympus).

Isolation of extracellular RNA and real time PCR analysis

DNA-free total RNA was extracted from plasma of mice ~~or~~, from supernatants of SMC or SMC using Zymo RNA MicroPrep kit (Zymo Research) omitting a lysis step and with an additional DNase digestion step. Total RNA (10 ng) was reverse-transcribed at 37°C for 1 h. cDNA fragments were amplified by 45 cycles of PCR (denaturing at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s); the last extension was performed at 50°C for 2 min. The following primers were used:

Mouse *Cd31* forward 5'-CTCCAACAGAGCCAGCAGTA-3',
mouse *Cd31* reverse 5'-GACCACTCCAATGACAACCA-3',
mouse *Sma* forward 5'-CTGACAGAGGCACCATGAA-3',
mouse *Sma* reverse 5'-AGAGGCATAGAGGGACAGCA-3',
mouse *Gapdh* forward 5'-CACTCAAGATTGTCAGC-3',
mouse *Gapdh* reverse 5'-CCACAGCCTTGGCAGC-3'.
mouse *Icam-1* forward 5'-GCCTTGGTAGAGGTGACTGAG-3'
mouse *Icam-1* reverse 5'-GACCGGAGCTGAAAAGTTGTA-3'
mouse *Vcam-1* forward 5'-TGCCGAGCTAAATTACACATTG-3'
mouse *Vcam-1* reverse 5'-CCTTGTGGAGGGATGTACAGA-3'
mouse *P-selectin* forward 5'-AGGGAAATGATGCCATTCAG-3'
mouse *P-selectin* reverse 5'-ACCGGAAACTCTGGACATTG-3'

mouse *Ccl2* forward 5'-GATGCAGTTAACGCCCCACT-3'
mouse *Ccl2* reverse 5'-AGCTTCTTTGGGACACCTGC-3'
mouse *Tnf-α* forward 5'-ACTGAACTTCGGGGTGATCG-3'
mouse *Tnf-α* reverse 5'-GGCTACAGGCTTGTCACTCG-3'
mouse *Il-1β* forward 5'-GGATGAGGACATGAGCACCT-3'
mouse *Il-1β* reverse 5'-GGAGCCTGTAGTGCAGTTGT-3'
mouse *Il-6* forward 5'-ATGGATGCTACCAAACTGGAT-3'
mouse *Il-6* reverse 5'-TGAAGGACTCTGGCTTTGTCT-3'

After incubation with or without eRNA (1 - 25 μg/ml), total cellular RNA was extracted from BMDM using TRIzol® Reagent (Invitrogen, USA). RNA samples were then subjected to RNA purification using the RNeasy minikit (Qiagen, USA). One μg of DNase-treated total RNA was reverse-transcribed using GoScript™ Reverse Transcription System (Promega, USA). Real-time quantitative RT-PCR (q-PCR) was performed at Genomics Core Facility, John A. Burns School of Medicine, Hawaii. The gene products were quantified using SYBR Green assays (Applied Biosystems). mRNA signal from GAPDH was used for normalization. The 50 pmol of each primer was used with the following sequences:
Mouse *Gapdh* forward 5'-GGCAAATTCAACGGCACAG-3',
mouse *Gapdh* reverse 5'-CGCTCCTGGAAGATGGTGA-3',
mouse *Il-1β* forward 5'-GGGCCTCAAGGAAAAGAATC-3',
mouse *Il-1β* reverse 5'-TTCTCCTTGAGAGGTGCTCA-3',
mouse *Tnf-α* forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3',
mouse *Tnf-α* reverse 5'-TGGGAGTAGACAAGGTACAACCC-3',
mouse *Il-6* forward 5'-GAGGATACCACTCCCAACAGACC-3',
mouse *Il-6* reverse 5'-AAGTGCATCATCGTTGTTCATACA-3',
mouse *Inf-γ* forward 5'-TGGCTCTGCAGGATTTTCAT-3',
mouse *Inf-γ* reverse 5'-TCAAGTGGCATAGATGTGGA-3',
mouse *Il-10* forward 5'-TGCACTACCAAAGCCACAAGG-3',
mouse *Il-10* reverse 5'-TGGGAAGTGGGTGCAGTTATTG-3',
mouse *Il-4* forward 5'-CAACGAAGAACACCACAGAGAG-3',
mouse *Il-4* reverse 5'-ATGAATCCAGGCATCGAAAAGC-3',
mouse *Arg2* forward 5'-CCTCCCTGCCAATCATGTTC-3',
mouse *Arg2* reverse 5'-CCTCCCTGCCAATCATGTTC-3',
mouse *Il-12* forward 5'-CCCTGTGCCTTGGTAGCATC-3',

mouse *Il-12* reverse 3'-CTGAAGTGCTGCGTTGATGG-3',
mouse *iNOS* forward 5'-CTCTGGTCTTGCAAGCTGATGGTCA-3',
mouse *iNOS* reverse 5'- TCCTGGAACCACTCGTACTTGGGAT-3',

Cell culture and cell adhesion assay under flow

Human coronary artery SMC (Promocell) and MonoMac6 cells were maintained as described[3, 4]. MonoMac6 cell adhesion to SMC was analyzed in parallel wall flow-chambers[3]. Confluent SMC were activated with TNF-α (50 ng/ml) in the absence or presence of RNase1 (10 µg/ml) for 16 h. Likewise, MonoMac6 cells were left untreated or pretreated with RNase1 (10 µg/ml) for 16 h and suspended (at $1x10^6$ cells/ml) in HH-medium (1x Hank's buffered saline solution, containing 10 µM HEPES and 5 mg/ml bovine serum albumin). Confluent SMC were activated with TNF-α (50 ng/ml) followed by treatment with buffer alone or RNase1 (10 µg/ml) for 16 h. Prior to the experiment, $CaCl_2$ and $MgCl_2$ (each at 1 mM) were added to MonoMac6 cells, which were perfused over SMC at 1.5 dyne/cm^2. After 5 min, firm adherence of cells was assessed by phase-contrast video microscopy and quantified in multiple fields recorded with a JVC 3CCD video camera.

Viability assay

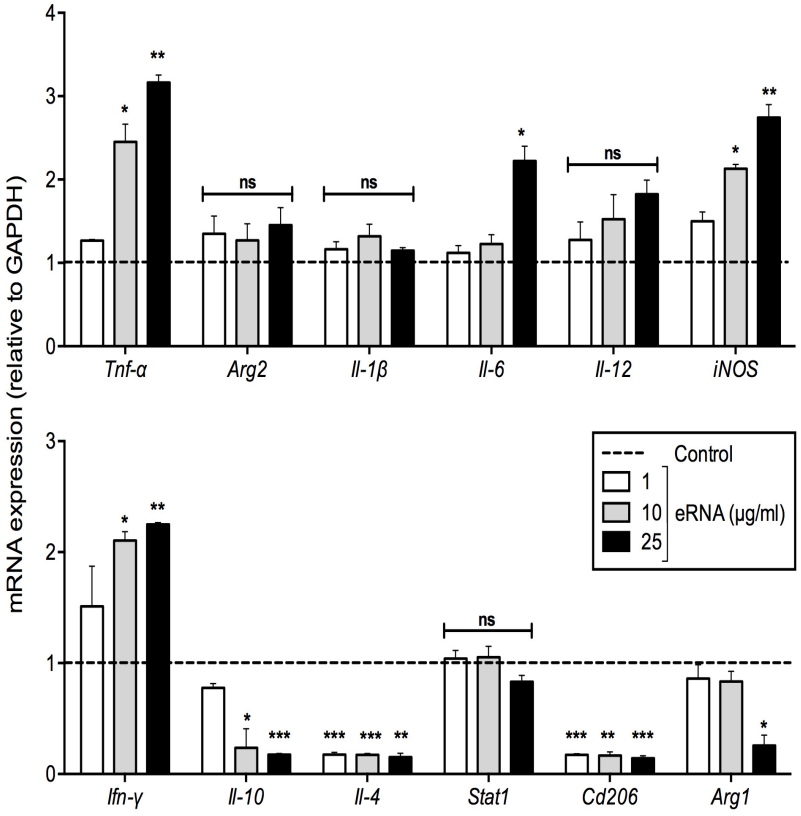
After treating SMC or MM6 with RNase1 (10 µg/ml) for 30 min or 16 h, respectively, viability assays were performed by using the fluorescent CellTiter-Blue® cell viability assay (Promega) according to the manufacturer's instructions.

Statistics

Data were analyzed by unpaired Student t tests, and if more than 2 groups were compared by ANOVA 1-way analysis of variance followed by Tukey's or Dunnett's multiple comparison post tests. Differences with $p<0.05$ were considered to be statistically significant. Analyses for Figures 5A-C, and Figure 7B were performed using two tailed t-tests. Analyses for Figure 2L and 4A were performed using a one-way ANOVA followed by Tukey multiple comparison test. Analysis for Figure 2A, 3A-D, 4B-C, 6A-G, 7B and Supplemental Figure 1 and 3 were performed using a one-way ANOVA followed by Dunnett's multiple comparison test. Statistical analyses were performed using Graph Pad Prism 6.0.

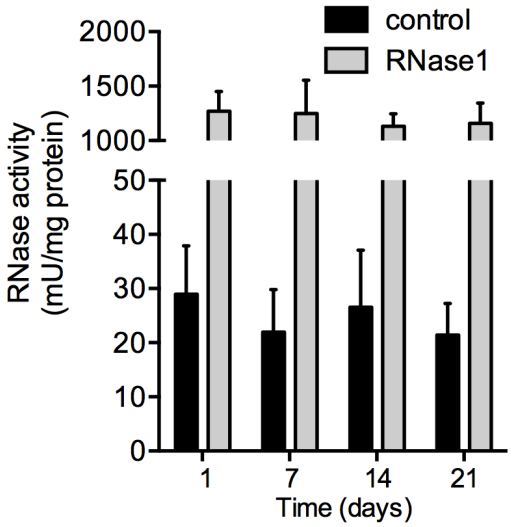
Supplemental Figures

Supplemental Figure 1



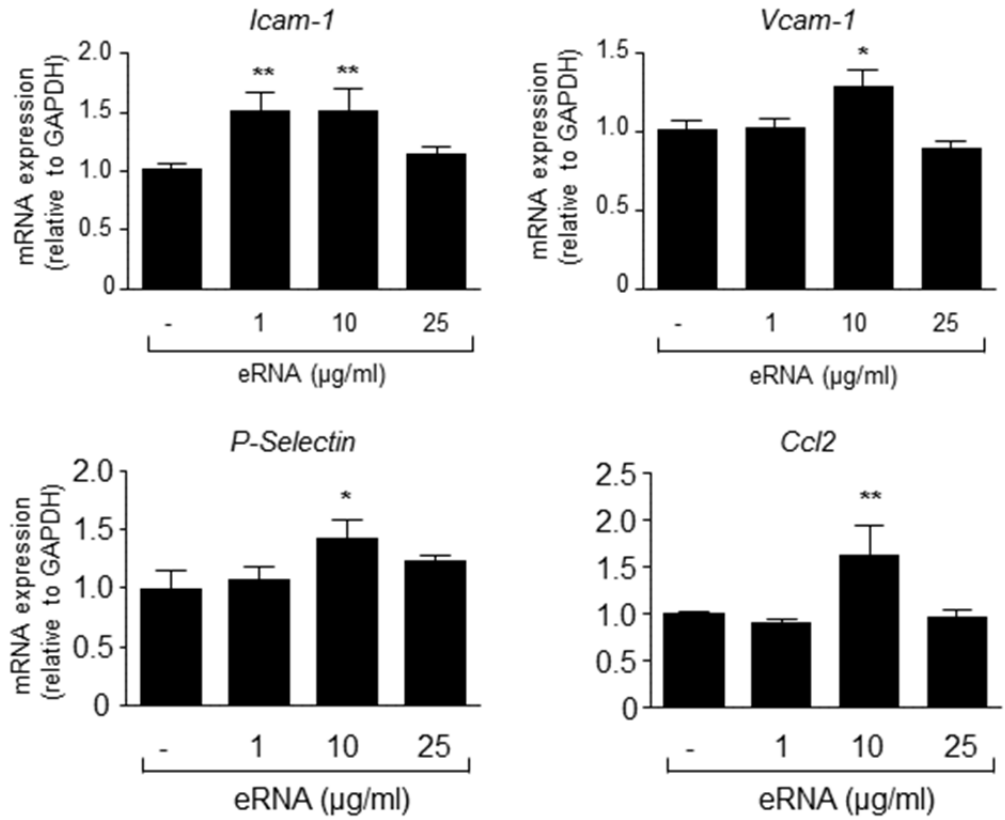
Supplemental Figure 1. mRNA expression of *Tnf-α, Arg2, Il-1β, Il-6*, *Il-12, iNOS, Ifn-γ, Il-10*, *Il-4*, *Stat1, Cd206* and *Arg1* in wild-type BMDM, differentiated in the presence of mouse recombinant GM-CSF was analyzed by real-time PCR in the absence (control, dotted line) or presence of eRNA (1, 10, or 25 µg/ml) for 24 h. Data are expressed as changes in the ratio between target gene expression and *Gapdh* mRNA. Values represent mean ± SD (n=6 per group); *p<0.05, **p<0.01, ***p<0.001 *vs*. control, ns = non-significant.

Supplemental Figure 2



Supplemental Figure 2. RNase activity in plasma of *apoE*$^{-/-}$ mice at indicated time points after arterial injury in the control and the RNase1 treatment group (n=4 per group). Values represent mean ± SEM.

Supplemental Figure 3



Supplemental Figure 3. mRNA expression of *Icam1, Vcam1, P-selectin* and *Ccl2* in SMC was analyzed by real-time PCR in the absence or presence of eRNA (1, 10, or 25 µg/ml) after 16 hours (n=9 per group). Values represent mean ± SEM; *p<0.05, **p<0.01 vs. unstimulated controls.

Supplemental References

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