Supplemental data

MicroRNA-181b regulates NF-κB downstream signaling and vascular inflammation

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Conflict of Interest: The authors have declared no conflict of interest.

SUPPLEMENTAL METHODS

Reagents and antibodies

On-TARGET plus SMART pool mouse KPNA4 (importin-α3) siRNA (L-058423-01-0010), On-TARGET plus Non-targeting pool control siRNA (D-001810-10-20), ON-TARGETplus human KPNA4 siRNA (J-017477-07), ON-TARGETplus control non-targeting siRNA1 (D-001810-01-05) were obtained from Dharmacon, Inc.

Anti-p65 (sc-8008), anti-p50 (sc-8414), anti-IKKα (sc-7182), anti-IKKγ (sc-8330), goat anti-mouse VCAM-1 (sc-1504), rabbit anti-mouse VCAM-1 (sc-8304), mouse anti-human VCAM-1 (sc-13160), goat anti-mouse IgG-HRP (sc-2005), goat anti-rabbit IgG-HRP (sc-2004), goat anti-chicken IgG-HRP (sc-2901), donkey anti-goat IgG-HRP (sc-2020) were from Santa Cruz Biotechnology. Cy5-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch. Anti-phospho-p38 MAPK (Thr180/Tyr182) (4511), anti-phospho-SAPK/JNK (Thr183/Tyr185) (4668), anti-phospho-p44/42 MAPK (Thr202/Tyr204) (4370), anti-p38 MAPK (9212), anti-SAPK/JNK (9258), anti-IκBα (4814), anti-β-actin (4970), anti-Histone H3 (9715), anti-IKKβ (2370), anti-p44/42 (9107), anti-phospho-IκBα (2859), anti-Cox-2 (4842) were from Cell Signaling. Anti-importin-α1 (D168-3), anti-importin-α3 (D169-3), anti-importin-α5/7 (D170-3) were from MBL, Medical & Biological Laboratories. Monoclonal anti-human E-selectin (CD62E) (S9555) was from Sigma. Anti-human E-selectin monoclonal antibody (BBA16), anti-rat IgG-HRP (HAF005), anti-human ICAM-1/CD54 Clone BBIG-I1 (BBA3), anti-human PAI-1 (MAB1786) were from R&D Systems. Anti-CX3CL-1 (14-7986) was from eBioscience. Anti-Ly-6G and Ly-6C/Gr-1 (550291), anti-CD45 (550539), anti-NF-κB p65 (610868) were from BD Pharmingen. Anti-mouse CD31 Rat Monoclonal antibody from Dianova, and NF-κB p65 antibody from Abcam were used for immunostaining. Cyllindromatosis 1 (CYLD) antibody was from GeneTex (GTX100228). Anti-KPNA4 (importin-α3) (6039) from Abcam was used to detect mouse importin-α3.

Cell culture, transfection, and adenoviral transduction

HUVECs were obtained from Lonza (cc-2159) and cultured in endothelial cell growth medium EGM®-2 (cc-3162). Lipofectamine™ 2000 transfection reagent from Invitrogen was used for transfection, following manufacturer's instructions. ON-TARGETplus human KPNA4 siRNA or control non-targeting siRNA were transfected at 30 nM. In some experiments, cells were tranduced with Ad-GFP or Ad-KPNA4 (importin-α3) (SL172911, SignaGen® Laboratories) followed by transfection with miRNA negative control or miR-181b mimics at 10 nM.

ELISA

HUVECs were transfected with control miRNAs or miR-181b mimics at a final concentration of 10 nM and control miRNA inhibitors or miR-181b inhibitors at a final concentration of 50 nM. After 36 h, cells were exposed to 10 ng/ml TNF-α for 16 h. Then, the supernatants were collected for ELISA analysis by means of SearchLight Multiplex Immunoassay Kit (Aushon BioSystems, Inc).

Intimal RNA isolation from aorta tissue

Isolation of intimal RNA from aorta was modified from a previous study (1). Briefly, aorta between the heart and diaphragm was exposed, and the peri-adventitial tissues were removed carefully. The cleaned aorta was cut out and transferred to a 35 mm dish containing ice-cold Hank's Buffered Salt Solution (HBSS). The tip of an insulin syringe needle was carefully inserted into one end of the aorta to facilitate a quick flush of 150 µl QIAzol lysis buffer through it and collection of the intima eluate into a 1.5 ml tube. The aorta leftover (media + adventitia) was washed once with HBSS and snap-frozen in liquid nitrogen, for storage until total RNA extraction by TRIzol.

MiRNP immunoprecipitation

MiRNP-IP was performed as previously described (2, 3). Myc-tagged Ago-2 (a kind gift from G. Hannon, Cold Spring Harbor, NY) was co-transfected with either miR-181b or miRNA negative control in HUVECs. Cells were washed in ice cold PBS, released by scraping, and lysed in buffer (10 mM Tris-HCl

pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 100 units/ml of RNasin Plus (Promega) supplemented with 1x protease inhibitor (Roche)). The lysed cell solution was adjusted to a final NaCl concentration of 150 mM prior to centrifugation. One-twentieth of the supernatant volume was collected in TRIzol for use as an extract control. The remaining portion of the supernatant was pre-cleared with Protein A/G UltraLink Resin (Pierce), to which 2 µg anti-c-myc antibody was added and the mixture allowed to incubate overnight at 4°C; the following day Protein A/G UltraLink Resin was added. After 4 h of mechanical rotation at 4°C, the agarose beads were pelleted and washed four times in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Triton X-100). Finally, 1 ml of TRIzol was added into the beads and RNA was isolated. Total RNA was reverse transcribed into cDNA for real-time qPCR analysis.

Isolation of mouse lung endothelial cells

Isolated lungs were minced into small cubes and digested with 15 mL of pre-warmed digestion solution (1mg/ml dispase, 1 mg/ml collagenase in 1× HBSS) at 37°Cfor 40 min while shaking. Cells were collected and incubated with anti-PECAM-1 antibodies (557355, BD Pharmingen), followed by pre-washed Dynabeads (M450) containing sheep anti-rat IgG antibodies (110.35, Dynal Biotech). DynaMag™ magnets were used to separate PECAM-1 positive cells as described by the manufacturer.

Real-time quantitative PCR

Real-time qPCR was peformed using miScript primer assays Hs_RN5S1_1 (MS00007574), Hs_miR-181b_1 (MS00006699), Hs_miR-10a_1(MS00003164), Mm_miR-31_1 (MS00001407), Mm_miR-17-3p_1 (MS00005901), Mm_miR-155_1 (MS00001701), and Mm_miR-146_1 (MS00001638) from Qiagen.

Constructs and luciferase reporter assays

The human miR-181b gene, including 96 bp upstream and 241 bp downstream flanking regions of its stem loop sequence, was amplified by PCR from human genomic DNA (Promega) using Platinum® PCR

SuperMix High Fidelity *Taq*-based enzyme mix (Invitrogen). The resultant fragment was subcloned into the pcDNA3.1(+) vector to generate the pcDNA3.1(+)-miR-181b plasmid. Primers: 5'-CCCAAGCTTTGATTGTACCCTATGGCT-3' (forward) and 5'-CGGGGTACCTGTACGTTTGATGGACAA-3' (reverse) were used to amplify human miR-181b coding sequence.

The 3'UTR of genes for importin-α1, importin-α3, importin-α4, importin-α5, and VCAM-1 were amplified and cloned into the pMIR-REPORT™ Luciferase vector, between *Sac*I and *Mlu*I restriction sites (importins) or *Mlu*I and *Hind*III restriction sites (VCAM-1). Putative miR-181b binding sites in the importin-α3 gene 3'UTR were predicted by the rna22 algorithm. Individual wild-type or mutant binding site sequence was generated by annealing the forward and reverse oligonucleotides containing *Spe*I and *Hind*III sticky ends, followed by T4 Polynucleotide Kinase (New England Biolabs) phosphorylation. The double-stranded oligonucleotides were ligated into the pMIR-REPORT™ Luciferase vector, between *Spe*I and *Hind*III restriction sites, by using T4 DNA ligase (New England Biolabs). A construct containing the open reading frame cDNA of importin-α3 was purchased from OriGene. TRAF1 3'UTR (S812938), IL1R1 3'UTR (S813850), TNFRSF11B 3'UTR (S806741) constructs were from SwitchGear Genomics. 1×LightSwitch Assay System (100) (LS010) from SwitchGear Genomics was used for 3'UTR activity of TRAF1, IL1R1 and TNFRSF11B according to the manufacturer's instructions.

Immunostaining

HUVECs grown on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma). After blocking with normal goat serum, cells were incubated with antibody against p65 followed by Cy5-conjugated goat anti-mouse IgG and DAPI (Invitrogen). Images were acquired with an Olympus Fluoview FV1000 confocal microscope equipped with Multi-Ar laser, HeNe G laser, HeNe R laser, and LD405/440 laser diode. The UPLSAPO 20× (NA: 0.75) objective lens was used. The following

parameters were set: Zoom ×2, 1024×1024[pixel] image size, C.A. 150 μm. The intensity of p65 nuclear staining was quantified by using Bitplane Imaris 6.4.2 software.

Protein extraction and Western Blot analysis

Transfected HUVECs were treated with 10 ng/ml TNF-α for 1 h, then cytoplasmic and nuclear extracts were isolated by NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). Cultured cells were harvested and lysed in RIPA buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail tablets (Roche). To extract protein, snap-frozen tissues were homogenized in RIPA buffer supplemented with protease inhibitor. Cell or tissue debris was removed by centrifugation at 12 000 rpm for 10 min. Lysates were separated by 8% or 10% SDS-PAGE gels, transferred to PVDF membranes (Bio-Rad), and incubated with the relevant antibodies as where indicated. Proteins were visualized by ECL Plus western blotting detection reagents (RPN2132; GE Healthcare). Densitometry scanning of the blots with ImageJ software was used to calculate the abundance of protein.

In vivo knockdown or rescue of importin-α3 expression

To knockdown importin-α3, 6 weeks old NF-κB transgenic mice (n=5 per condition) were intravenously injected with the mixture of lipofectamine™ 2000 and importin-α3 siRNA or control siRNA with 1 nmol siRNA per injection in 200 μl volume for 3 injections on consecutive days. Twenty-four hours after the last injection, mice were treated with LPS (20 mg/kg) for 4 h, and lungs were harvested for different assays. To rescue importin-α3 expression, NF-κB transgenic mice (n=4 per condition) were intravenously injected with the mixture of lipofectamine™ 2000 and a construct expressing importin-α3 or control vector on d 1 and d 3, with 10 μg plasmid in 200 μl volume per injection. The mice were injected with the mixture of lipofectamine™ 2000 and miR-181b mimics, or miRNA negative control on d 2 and d 4. On d 5, mice were treated with LPS (20 mg/kg) for 4 h, and lungs were harvested for analyses.

Molecular imaging of VCAM-1 in vivo

For molecular imaging of VCAM-1 in vivo, Target-Ready MicroMarker™ contrast agent from VISUALSONICS were in injected in mice in the presence or absence of systemically delivered miRNAs as described in the Methods section in the main text. Briefly, to induce a systemic inflammatory response, leading to VCAM-1 expression, an intraperitoneal injection of 200 µl of a 10 µg/ml solution of murine TNF-α was performed. MicroMarker™ ultrasound contrast agent was reconstituted with saline, and mixed with 20 µg of the biotinylated isotype control or biotinylated VCAM-1 antibody (eBioscience). After the VCAM-1 antibody-MicroMarker™ contrast mixture was delivered by tail vein injection, VCAM-1-associated MicroMarker™ contrast was detected 4 minutes later using the Vevo® 2100 Ultrasound System with non-linear contrast mode. The differential targeted enhancement value was obtained from subtracting the post-burst signal from the pre-burst signal and represents the signal detected from the Target-Ready MicroMarker™ ultrasound contrast agent bound to VCAM-1 on the vascular endothelium of the innominate artery.

SUPPLEMENTAL REFERENCES

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Table S1

	Name	Sequence (5' –> 3')
For Real time qPCR	mouse VCAM-1 forward:	GTTCCAGCGAGGGTCTACC
	mouse VCAM-1 reverse:	AACTCTTGGCAAACATTAGGTGT
	mouse E-selectin forward:	ATGCCTCGCGCTTTCTCTC
	mouse E-selectin reverse:	GTAGTCCCGCTGACAGTATGC
	mouse ICAM-1 forward:	GTGATGCTCAGGTATCCATCCA
	mouse ICAM-1 reverse:	CACAGTTCTCAAAGCACAGCG
	mouse actin forward:	GAAATCGTGCGTGACATCAAAG
	mouse actin reverse:	TGTAGTTTCATGGATGCCACAG
	human PAI-1 forward:	CATCCCCCATCCTACGTGG
	human PAI-1 reverse:	CCCCATAGGGTGAGAAAACCA
	human VCAM-1 forward:	GCTGCTCAGATTGGAGACTCA
	human VCAM-1 reverse:	CGCTCAGAGGGCTGTCTATC
	human E-selectin forward:	AATCCAGCCAATGGGTTCG
	human E-selectin reverse:	GCTCCCATTAGTTCAAATCCTTCT
	human ICAM-1 forward:	TCTGTGTCCCCCTCAAAAGTC
	human ICAM-1 reverse:	GGGGTCTCTATGCCCAACAA
	human GAPDH forward:	ATGGGGAAGGTGAAGGTCG
	human GAPDH reverse:	GGGGTCATTGATGGCAACAATA
	mouse Importin-α3 forward:	CCAGTGATCGAAATCCACCAA
	mouse Importin-α3 reverse:	CGTTTGTTCAGACGTTCCAGAT
	human Importin-α3 forward:	TCCAGTGATCGAAATCCACCA
	human Importin-α3 reverse:	CATTGGACTGAACTACTGCTTGA
	human Smad1 forward:	GATGCCAGGTAGGTTGGAATG
	human Smad1 reverse:	CGTGACACTGTGATAACACTGT
Oligonucleotides	Site1 forward:	CTAGTCTTGCTATGAAGCAGTGTGTGAAA
	Site1 reverse:	AGCTTTTCACACACTGCTTCATAGCAAGA
	Site2 forward:	CTAGTATGGACAATGTTGAATGAATGTCA
	Site2 reverse:	AGCTTGACATTCATTCAACATTGTCCATA
	Site3 forward:	CTAGTCTGTGTACGAGAGCGTGGTTGTGA
	Site3 reverse:	AGCTTCACAACCACGCTCTCGTACACAGA
	Site4 forward:	CTAGTTGGTTTACTCTGCAGCCTGTGTTA
	Site4 reverse:	AGCTTAACACAGGCTGCAGAGTAAACCAA
	Site5 forward:	CTAGTTGCATTTGCACCAGATGAATGTTA
	Site5 reverse:	AGCTTAACATTCATCTGGTGCAAATGCAA
	Site6 forward:	CTAGTTTTCCCTCAAAATAGACTGTGTTA
	Site6 reverse:	AGCTTAACACAGTCTATTTTGAGGGAAAA
	Site7 forward:	CTAGTATACCGTGCTGTGTTTAAATGTTA
	Site7 reverse:	AGCTTAACATTTAAACACAGCACGGTATA
	Site8 forward:	CTAGTCTTCCCCTTTGAGCACAAGTGTTA
	Site8 reverse:	AGCTTAACACTTGTGCTCAAAGGGGAAGA
	Site1mut forward:	CTAGTCTTGCTATGATAAAGCTTCTGAAA
	Site1mut reverse:	AGCTTTTCAGAAGCTTTATCATAGCAAGA
	Site2mut forward:	CTAGTAGGCTGAATCTTGCCAACATCACA
	Site2mut reverse:	AGCTTGTGATGTTGGCAAGATTCAGCCTA

For cloning 3'UTR	Importin-α1 forward:	ACGAGCTCATCATGTAGCTGAGACATAAATTTG
	Importin-α1 reverse:	ATAACGCGTAGAAAAGGGTGGACTTGAATGT
	Importin-α3 forward:	ACGAGCTCAAAGATGTTGTGGAAGTTAGG
	Importin-α3 reverse:	ATAACGCGTCACAGCACGGTATTCTACCAC
	Importin-α4 forward:	ACGAGCTCATTCAGTTGAGTGCAGCATC
	Importin-α4 reverse:	ATAACGCGTCCTCTACACAGATCCCTGTC
	Importin-α5 forward:	ACGAGCTCAGCAATACTCTGCTTTCACG
	Importin-α5 reverse:	ATAACGCGTGATTAGAATCGAGCTGCACC
	VCAM-1 forward:	TCGACGCGTGCAAATCCTTGATACTGC
	VCAM-1 reverse:	CCCAAGCTTATTGGGAAAGTTGCACAG

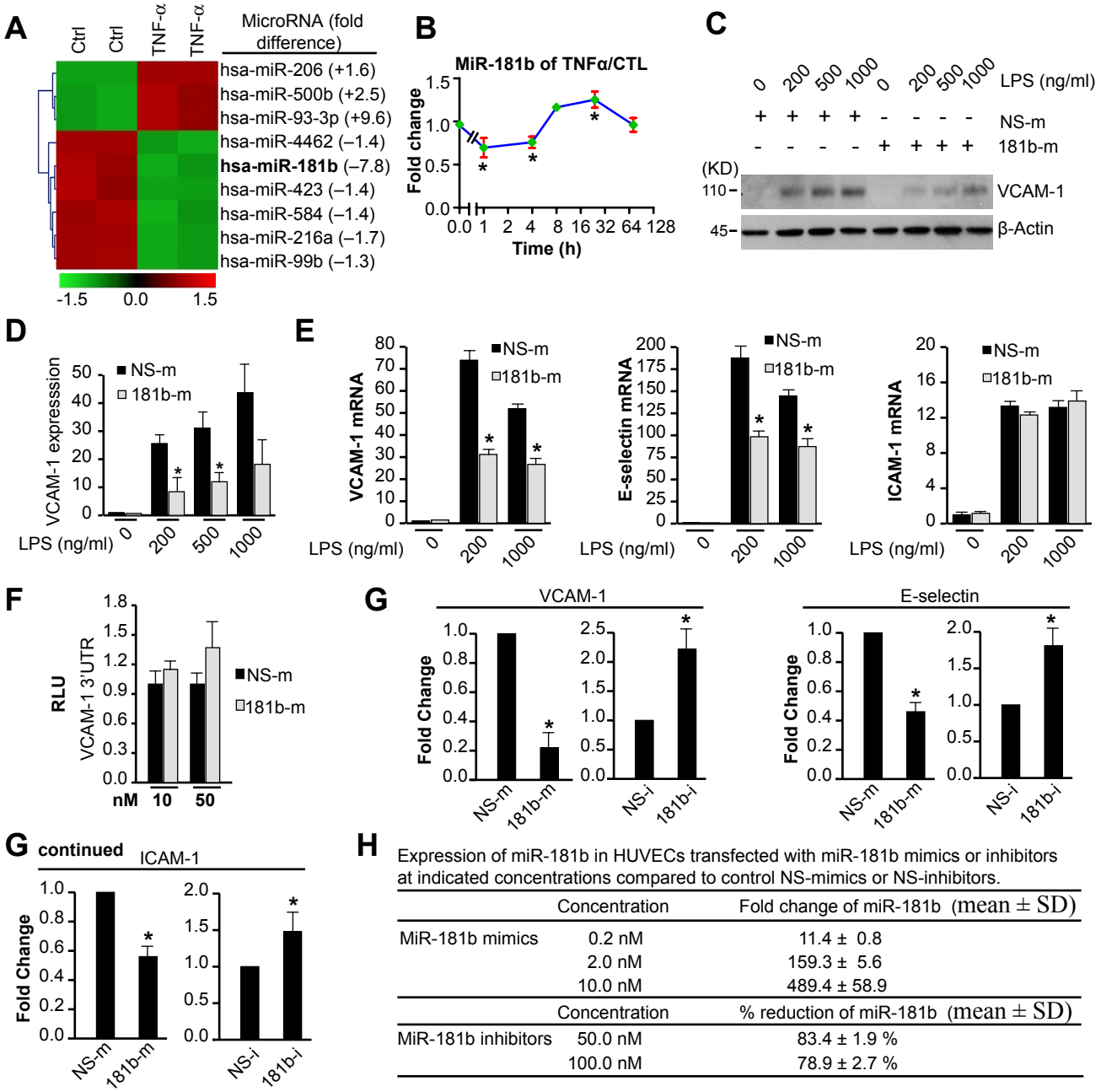
Table S2. Primer sets used in Figure 5

Name	Sequence (5' –> 3')
BCL2A1-F	TACAGGCTGGCTCAGGACTAT
BCL2A1-R	TTTTGTAGCACTCTGGACGTTT
C1QTNF1-F	CAAGGGAAATATGGCAAAACAGG
C1QTNF1-R	ATCACCGTCTGGTAGTAGTGG
CCL1-F	TCATTTGCGGAGCAAGAGATT
CCL1-R	CTGAACCCATCCAACTGTGTC
CCL7-F	CCAATGCATCCACATGCTGC
CCL7-R	GCTTCCCAGGGACACCGAC
CCR2-F	GACCAGGAAAGAATGTGAAAGTGA
CCR2-R	GCTCTGCCAATTGACTTTCCTT
CFB-F	GCGGCCCCTTGATAGTTCAC
CFB-R	CAGGGCAGCACTTGAAAGAG
CSF2-F	GGGAGCATGTGAATGCCATC
CSF2-R	GCAGTGTCTCTACTCAGGTTCAG
CX3CL1-F	ACCACGGTGTGACGAAATG
CX3CL1-R	CTCCAAGATGATTGCGCGTTT
CXCL1-F	AGGGAATTCACCCCAAGAAC
CXCL1-R	ACTATGGGGGATGCAGGATT
CXCL16-F	CAGCGTCACTGGAAGTTGTTA
CXCL16-R	CACCGATGGTAAGCTCTCAGG
CXCL3-F	CCAAACCGAAGTCATAGCCAC
CXCL3-R	TGCTCCCCTTGTTCAGTATCT
CXCL6-F	AGAGCTGCGTTGCACTTGTT
CXCL6-R	GCAGTTTACCAATCGTTTTGGGG
IL1R1-F	ACATTGTGCTTTGGTACAGGG
IL1R1-R	CCCCAACAGTCTTTGGATACAG
LIF-F	GTACCGCATAGTCGTGTACCT
LIF-R	CACAGCACGTTGCTAAGGAG
MMP11-F	GAGGCCCTAAAGGTATGGAGC
MMP11-R	CCCTTCTCGGTGAGTCTTGG
PLAU-F	GTGAGCGACTCCAAAGGCA
PLAU-R	GCAGTTGCACCAGTGAATGTT
PTGS2-F	GTGCAACACTTGAGTGGCTAT
PTGS2-R	AGCAATTTGCCTGGTGAATGAT
SDC4-F	GCTCTTCGTAGGCGGAGTC
SDC4-R	CCTCATCGTCTGGTAGGGCT
SELE-F	AATCCAGCCAATGGGTTCG
SELE-R	GCTCCCATTAGTTCAAATCCTTCT
SERPINE1-F	CATCCCCCATCCTACGTGG
SERPINE1-R	CCCCATAGGGTGAGAAAACCA
TIMP3-F	CATGTGCAGTACATCCACACG
TIMP3-R	ACATCTTGCCATCATAGACGC
TNFRSF11B-F	AAGGGCGCTACCTTGAGATAG
TNFRSF11B-R	GCAAACTGTATTTCGCTCTGGG

TRAF1-F	CCGGCCCCTGATGAGAATG
TRAF1-R	TTCCTGGGCTTATAGACTGGAG
VCAM1-F	GCTGCTCAGATTGGAGACTCA
VCAM1-R	CGCTCAGAGGGCTGTCTATC
GAPDH-F	ATGGGGAAGGTGAAGGTCG
GAPDH-R	GGGGTCATTGATGGCAACAATA

F: forward; R: reverse

Figure S1



	Concentration	Fold change of miR-181b (mean ± SD)
MiR-181b mimics	0.2 nM	11.4 ± 0.8
	2.0 nM	159.3 ± 5.6
	10.0 nM	489.4 ± 58.9
	Concentration	% reduction of miR-181b (mean ± SD)
MiR-181b inhibitors	50.0 nM	83.4 ± 1.9 %
	100.0 nM	78.9 ± 2.7 %

Figure S1. MiR-181b regulates adhesion molecule expression induced by pro-inflammatory stimuli.
(**A**) MiRNA microarray analysis identified reduced miR-181b in HUVECs treated with TNF-α for 4 h.
(**B**) Real-time qPCR analysis of miR-181b in HUVECs in response to TNF-α (10 ng/ml). **P*<0.05.
(**C**) Western blot analysis of VCAM-1 in HUVECs transfected with miRNA negative control (NS-m) or miR-181b mimics (181b-m), and treated with LPS (serotype 026:B6) for 16 h. Densitometry was performed and fold-changes of protein expression after normalization to β-actin expression are shown in (**D**). Data represent mean ± SD from two independent experiments. **P*<0.05.
(**E**) HUVECs were transfected as in (**C**), and treated with LPS for 6 h. Real-time qPCR analysis of VCAM-1, E-selectin, and ICAM-1 mRNA levels was performed. Values represent mean ± SD, n=3. **P*<0.05.
(**F**) MiR-181b does not affect the 3'UTR activity of the VCAM-1 gene. Relative luciferase activity of lysates from HUVECs transfected with a luciferase VCAM-1 3'UTR construct in the presence of miRNA negative control (NS-m) or miR-181b mimics (181b-m) at 10 nM or 50 nM respectively. Data show mean ± SD, n=3.
(G) Fold-changes of protein expression were calculated for Figure 1C. Data represent mean ± SD from at least three independent experiments. **P*<0.05. **(H)** Expression of miR-181b was detected by real-time qPCR in HUVECs at 36 h after transfection with miR-181b mimics or miR-181b inhibitors at the indicated concentrations compared to control NS-m or NS-inhibitors, respectively.

Figure S2

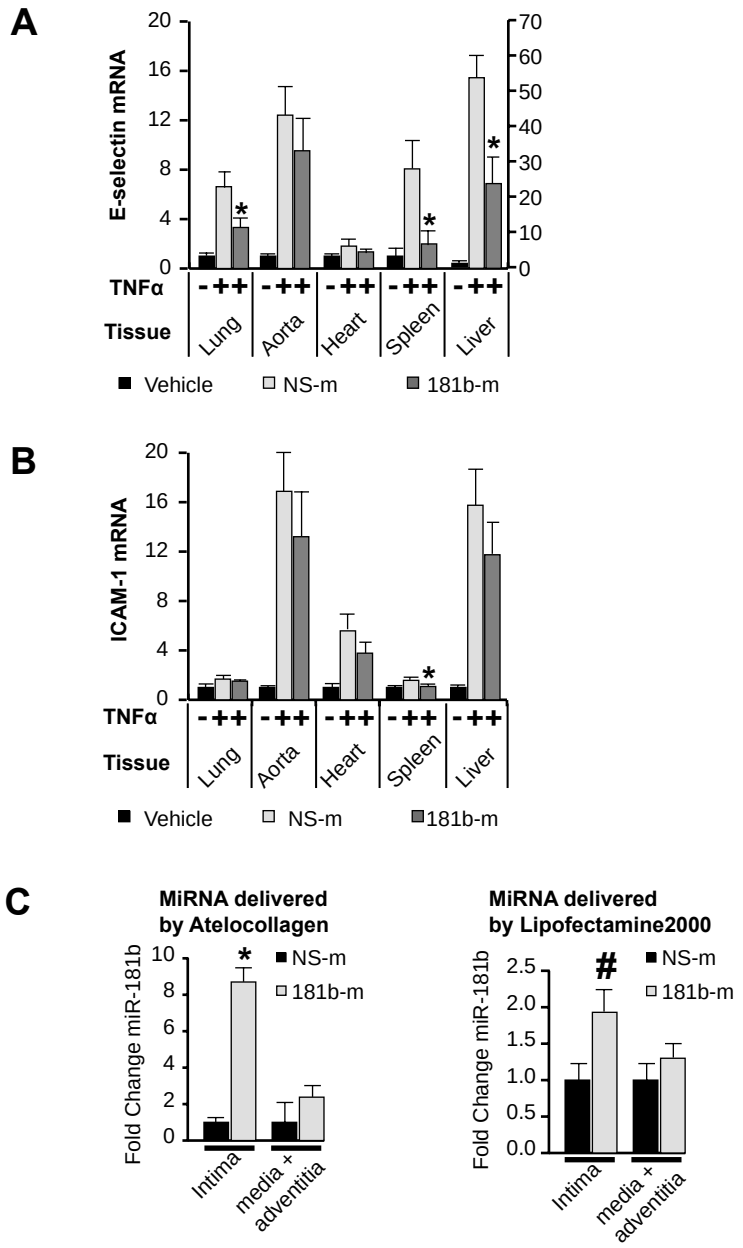


Figure S2. MiR-181b represses TNF-α-induced pro-inflammatory genes expression in vivo. Real-time qPCR analysis of E-selectin (**A**) or ICAM-1 (**B**) mRNA levels in tissues harvested from mice injected with vehicle, miRNA negative control (NS-m), or miR-181b (181b-m) with or without TNF-α treatment for 4 h. **P*<0.05. Vehicle (n=3 mice), NS-m (n=5 mice), 181b-m (n=5 mice). Y-axis on the right represents the values of E-selectin expression in liver only. Data represent mean ± SD. (**C**) Real-time qPCR analysis of miR-181b in intima or media plus adventitia of aorta from mice (n=2, Atelocollagen; n=4, Lipofectamine 2000) injected with miR negative control or miR-181b. **P*<0.01. # *P*<0.05 Data represent mean ± SD.

Figure S3

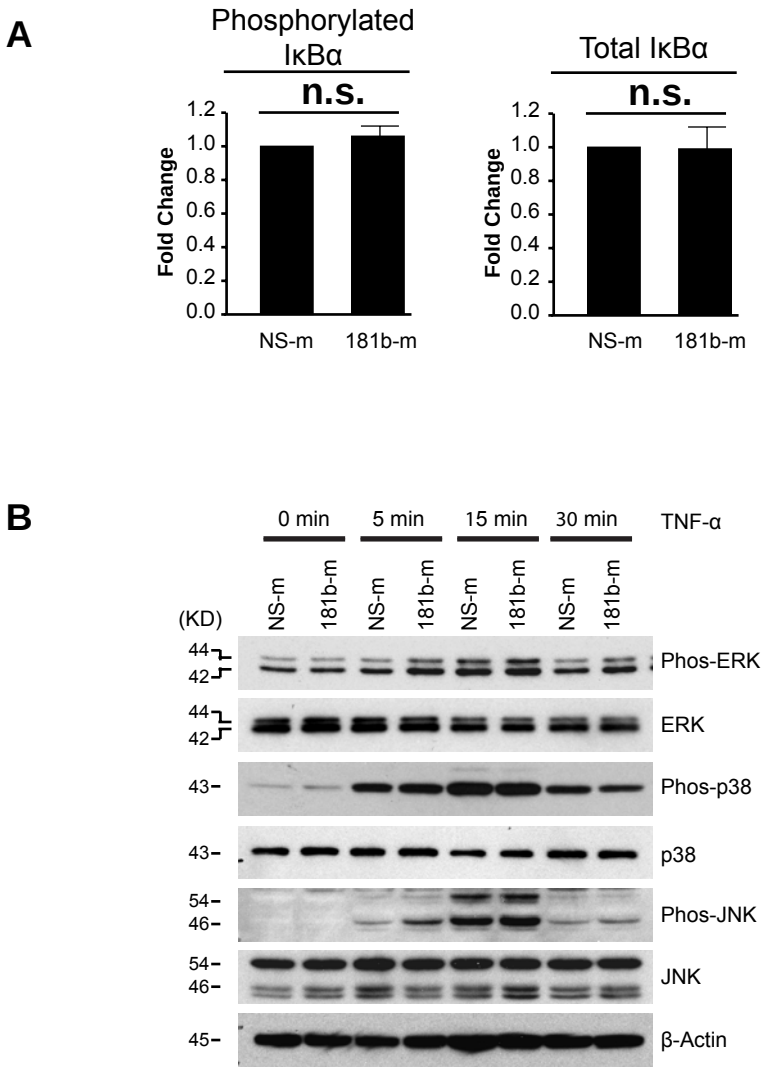


Figure S3. (**A**) Quantification of phospho-IκBα and total IκBα expression in the cytoplasmic fraction for Figure 3C. Values represent Mean ± SD. (**B**) Western blot analysis of phospho-ERK, total ERK, phospho-p38, total p38, phospho-JNK, total JNK in HUVECs transfected with either 10 nM miRNA negative control or 10 nM miR-181b mimics and treated with 10 ng/ml TNF-α for the indicated times.

Figure S4

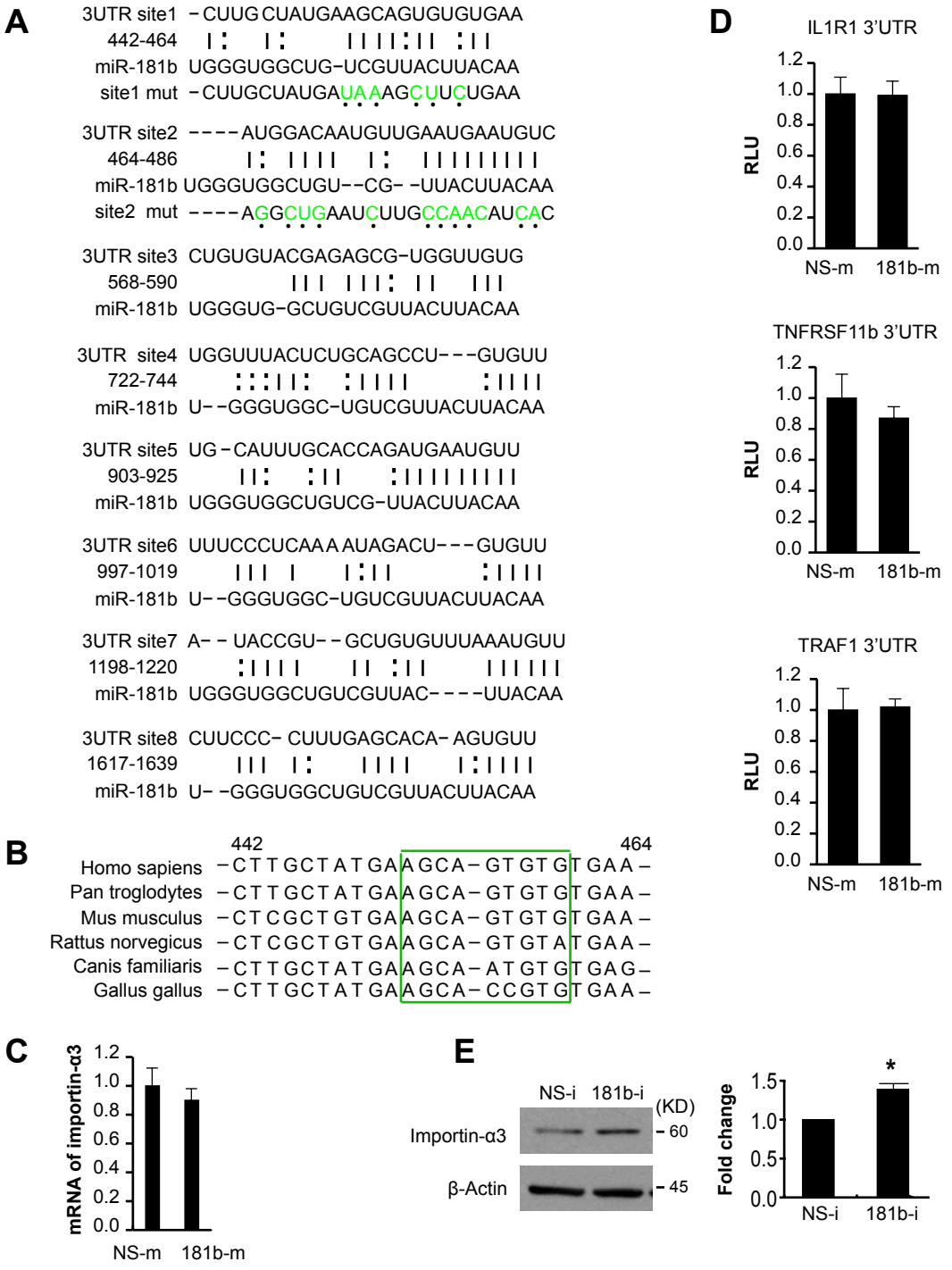


Figure S4. MiR-181b targets importin-α3 3'UTR. (**A**) Binding sites in importin-α3 3'UTR for miR-181b were predicted by rna22. The positions of binding sites are indicated as numbers in parentheses. Lines indicate perfect matches, while colons indicate G:U pairs. Nucleotides marked with dots were mutated. (**B**) MiR-181b binding site1 in the importin-α3 3'UTR is conserved across species. Mutation of boxed area of site1 impaired miR-181b's inhibtory effect on importin-α3 3'UTR. (**C**) Real-time qPCR analysis of importin-α3 mRNA levels in HUVECs transfected with miRNA negative control (NS-m) or miR-181b mimics (181b-m). (**D**) Luciferase activity of reporters containing the 3'UTRs of IL1R1, TNFRSF11b, or TRAF1 genes in cells transfected with NS-m or 181b-m. (**E**) Western blot analysis of importin-α3 in cells transfected with 50 nM miRNA control inhibitor (NS-i) or miR-181b inhibitor (181b-i). **P*<0.05. Data show mean ± SD, n=3.

Figure S5

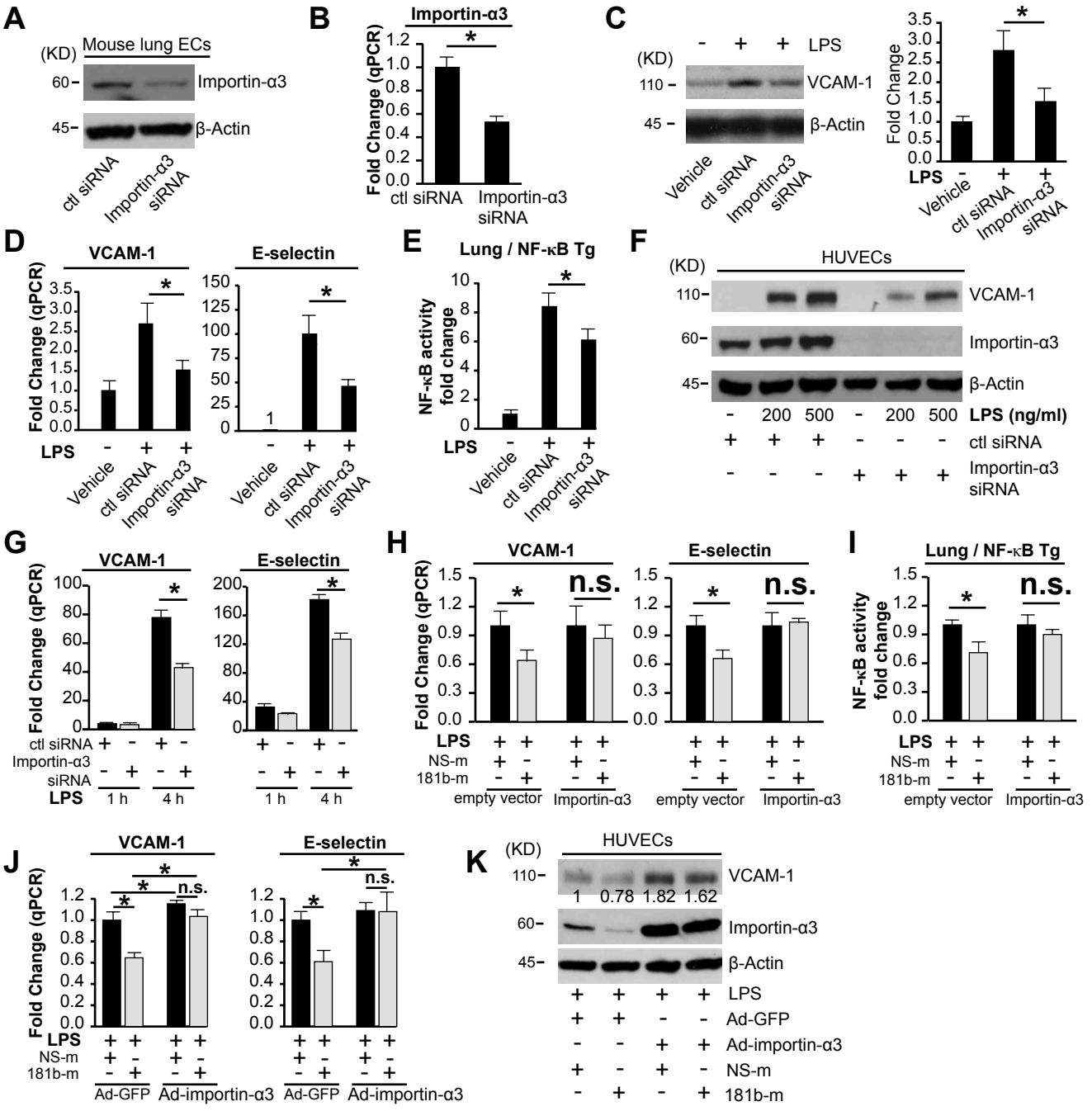


Figure S5. Knockdown of importin-α3 inhibits, and exogenously expressed importin-α3 rescues, LPS-induced pro-inflammatory gene expression in vivo and in vitro. (**A-E**) NF-κB transgenic mice (n=5 per condition) were intravenously injected with vehicle, control siRNA, or importin-α3 siRNA. Mouse lungs were harvested 4 h after LPS (20 mg/kg) for analyses. (**A** and **B**) Western blot and real-time qPCR analysis of importin-α3 of lung ECs freshly isolated from treated mice. Mean ± SEM. **P*<0.05. (**C**) Western blot analysis of VCAM-1 expression in lung. Quantification represents mean ± SEM. **P*<0.05. (**D**) Real-time qPCR analysis of VCAM-1 and E-selectin mRNA level. Mean ± SEM. **P*<0.05. (**E**) Luciferase activity assay from lung tissues. Mean ± SD. *, $P < 0.05$. (**F** and **G**) HUVECs transfected with control siRNA or importin-α3 siRNA, and treated with LPS. (**F**) Western blot analysis of VCAM-1 and importin-α3. (**G**) Real-time qPCR analysis of VCAM-1 and E-selectin mRNA. Mean ± SD. *, $P < 0.05$. (**H** and **I**) NF-κB transgenic mice (n=4 per condition) were injected with importin-α3-expressing plasmid and miRNA negative control or miR-181b mimics. Lung tissues were harvested after LPS for real-time qPCR analysis of VCAM-1 and E-selectin expression (**H**) and luciferase assay (**I**). Mean ± SEM.**P*<0.05. (**J** and **K**) HUVECs were transduced with Ad-GFP or Ad-importin-α3, transfected with NS-m or miR-181b mimics (181b-m), and treated with 200 ng/ml LPS for 4 h (**J**) or 24 h (**K**). Cells were harvested for real-time qPCR (**J**), or western blot (**K**). Mean ± SD. *, $P < 0.05$.

Figure S6

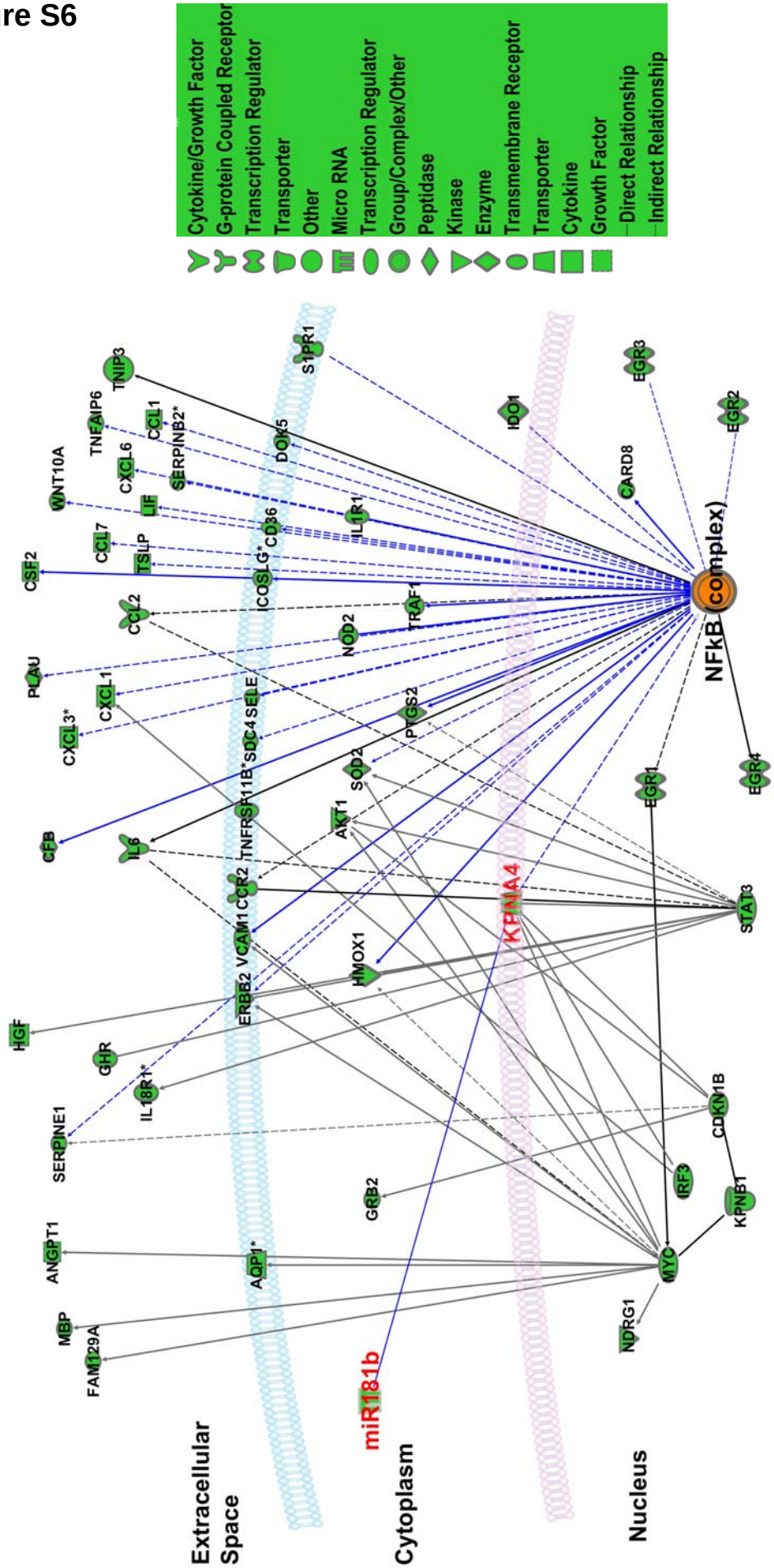


Figure S6. Bioinformatic analysis of 841 genes repressed by miR-181b using the Ingenuity Pathway Analysis (IPA) software suggests that miR-181b regulates inflammatory responses in endothelial cells by modulating the NF-κB signaling pathway via reduced expression of importin-α3 (gene symbol KPNA4). The major connected network of miR-181b downregulated genes is shown here. The key node of the major network is the NF-κB complex.

Figure S7

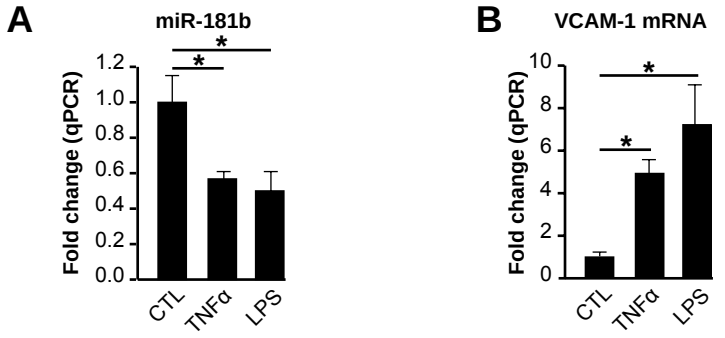


Figure S7. MiR-181b expression in the aortic intima in response to TNF-α or LPS. Mice were treated with TNF-α (2μg/mouse) i.p., LPS i.p. (40 mg/kg, serotype 026:B6), or saline for 4 h, and aortic intima (endothelium) were isolated for total RNA extraction, followed by reverse transcription and real-time qPCR analysis of miR-181b (**A**), and VCAM-1 (**B**). n=3-4 mice per group, values represent mean ± SEM. **P*<0.05.

Figure S8

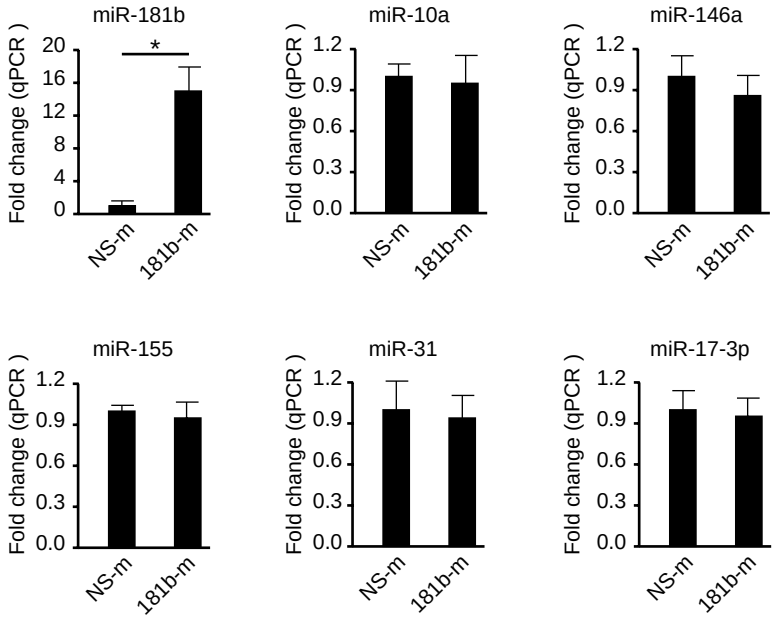


Figure S8. Systemic delivery of miR-181b does not affect the expression of miRNAs implicated in inflammation or NF-κB activation. Mice were intravenously injected with miRNA negative control (NS-m) or miR-181b (181b-m). Mouse lungs were harvested for real-time PCR analyses of miR-10a, miR-146a, miR-155, miR-31, miR-17-3p. Data show mean ± SD, n=4. **P*<0.05.

Figure S9

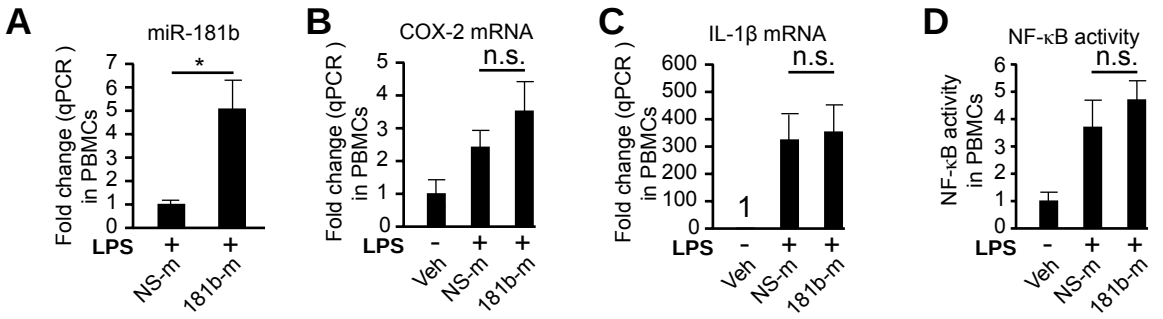


Figure S9. Systemic delivery of miR-181b has no effect on NF-κB activation and downstream NF-κB gene expression in mouse peripheral blood mononuclear cells (PBMCs) in response to LPS. NF-κB transgenic mice were intravenously injected with miRNA negative control (NS-m) or miR-181b (181b-m). PBMCs were harvested from LPS (026:B6, 40 mg/kg) treated mice after 4 h for analyses: (**A**) Real-time PCR analysis of miR-181b. (**B**) Real-time qPCR analysis of COX-2. (**C**) Real-time PCR analyses of IL-1β. (**D**) NF-κB activity in PBMCs was examined by luciferase assay. All values show mean ± SD, n = 4-6. **P*<0.05.

Figure S10

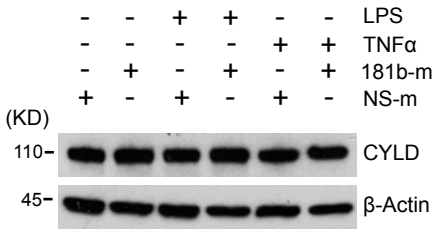


Figure S10. MiR-181b does not reduce cylindromatosis (CYLD) expression in HUVECs. Western blot analysis of CYLD in HUVECs transfected with miRNA negative control (NS-m) or miR-181b mimics (181b-m) after treatment with 10 ng/ml TNF-α for 8 h, or 200 ng/ml LPS for 24 h.