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Animals and Tissue Preparation

In previous work, 459 F2-mice had been generated in a reciprocal intercross of atherosclerosis-resistant FVB.LDLR$^{-/-}$ mice and atherosclerosis-susceptible B6.129S7-*Ldlr*^{tmHer}/J (henceforce called B6.LDLR$^{-/-}$).[1] Livers from F1- and F2-mice had been harvested at sacrifice and stored -80°C. In addition, in the present study a total of 19 parental FVB.LDLR$^{-/-}$ and 22 parental B6.LDLR$^{-/-}$ mice were used. Congenic animals carrying the Chr12 interval (0-28 cM) from B6 on the FVB.LDLR$^{-/-}$ background were generated by backcrossing B6.LDLR$^{-/-}$ mice to FVB.LDLR$^{-/-}$. For atherosclerosis studies, these mice (designated FVB.LDLR$^{-/-}$Chr12$^{FVB/B6}$) were intercrossed to generate FVB.LDLR$^{-/-}$Chr12$^{FVB/FVB}$ (n=37), FVB.LDLR$^{-/-}$Chr12$^{FVB/B6}$ (n=38), and FVB.LDLR$^{-/-}$Chr12$^{B6/B6}$ (n=45) mice. These animals were treated and sacrificed like the F2-mice previously described.[1] In addition, whole aortas were removed and femurs were dissected and bone marrow was flushed with 10 mL PBS/heparin. The cell suspension was put through a cell strainer (BD Falcon Cell Strainer), centrifuged at 1000 rpm for 5 min, and resuspended in 1.5 mL Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany). Subsequently, cell suspension (500 µL) was transferred into 2mL cryo-tubes (Sarstedt, Nümbrecht, Germany) and cells were gradually frozen after adding 400 µL FCS and 100 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Munich, Germany). Cells were stored in liquid nitrogen. Animal care and experimental procedures involving animals were approved by

the Rockefeller University's Institutional Animal Care and Use Committee and the responsible authorities of the state of Saxony (Regierungspräsidium Sachsen, N 4/07).

Cell Culture Studies

RAW 264.7 macrophages were cultivated in DMEM containing 10% FCS, 1% penicillin/streptomycin and used for transfection studies. Cryo-preserved bone marrow cells of B6.LDLR$^{-/-}$ and FVB.LDLR$^{-/-}$ were thawed and grown in DMEM containing 20% L-cell-conditioned medium, 10% FCS, 1% penicillin/streptomycin and 1% patricin. After cultivation for 14 days, macrophages were scraped off and plated onto either 35 mm dishes (Sarstedt, Nümbrecht, Germany) or 48-well plates (Sarstedt, Nümbrecht, Germany) at a cell density of 10^6 and 1.2 x 10^5 cells per well, respectively. Macrophages on 35 mm dishes were grown for 48 h, the supernatant was removed, and cells were lysed with 750 µL of the monophasic phenol-guanidine isothiocyanate TRIzol reagent (Invitrogen, Karlsruhe, Germany) for subsequent RNA isolation. In separate experiments, bone marrow derived macrophages from FVB.LDLR$^{-/-}$ and B6.LDLR$^{-/-}$ were cultivated in 48-well plates for 24h and incubated for 1, 3, 6, and 24 hours with 100 nmol/L phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Munich, Germany). The supernatant was removed for determination of TNF-alpha and TNF-receptor I (TNFR-I) release into media. Cells were washed with PBS and 110 µL of lysis buffer (0.1% sodium dodecyl sulphate in 0.1M NaOH) were added per well. TNF-alpha and TNFR-I were determined by enzyme-linked immunosorbent assay (BioRad, Munich, Germany) and concentrations were normalized to cell protein content determined by the method of Lowry, as previously described.[2]

RNA Isolation and cDNA Synthesis

Total RNA was extracted from livers of 10 F1 and 400 F2-mice (59 from the total of 459 were not available), 9 FVB.LDLR$^{-/-}$ mice and 12 B6.LDLR$^{-/-}$ mice using TRIzol reagent. This method was also used for isolation of total RNA from cultivated bone marrow derived macrophages of FVB.LDLR$^{-/-}$ and B6.LDLR$^{-/-}$ mice. For RNA isolation from whole aortas, RNeasy Fibrous Tissue Mini (Qiagen, Hilden, Germany) was used. RNA was reverse transcribed into cDNA using SuperScript ™ II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and random hexamer primers.

Quantitative Fluorogenic RT-PCR (TaqMan)

Quantitative fluorogenic RT-PCR was performed in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Specific primers and probes for *beta-actin*, *a disintegrin and metalloproteinase domain 17 (ADAM17), aryl-hydrocarbon receptor (AHR), TNF-alpha, TNFR-I, vascular cell adhesion molecule 1 (VCAM-1),* and *cytochrome P450, family 1, subfamily a, polypeptide 1 (CYP1A1)* were selected to span two exons in order to avoid co-amplification of genomic DNA (Table I). The PCR reaction was prepared in a final volume of 12.5 µL of a reaction mixture containing 2.5 µL of cDNA (diluted 1:15), 5 mM $MgCl_2$, 1.25 µL 10 x AmpliTaq buffer A, 200 µM dNTP (each), 0.03 U/µL AmpliTaq Gold ™ (Applied Biosystems, Darmstadt, Germany), 200 nM oligonucleotide probe (*beta-actin, ADAM17, AHR, TNF-alpha, TNFR-I, VCAM-1, CYP1A1,* respectively), and 300 nM of each oligonucleotide primer. The cycling conditions were 95°C for 10 minutes and 40 two-step cycles of 95°C for 15 seconds and 60°C for 1 minute. Analysis of the data was performed with the ABI PRISM sequence detection software. Standard curves were generated for each gene using a

plasmid dilution series containing the target sequences. mRNA expression levels were normalized to 10^6 copies of *beta-actin* as a housekeeping gene.

Table I: Primers and TaqMan probes used for quantitative RT-PCR

(*: 6-FAM; # : TAMRA)

Gene		Sequence	Acc. number
Beta-actin	5´-primer	5´- GAGAAGCTGTGCTATGTTGCTC -3´	NM_007393.1
	3`-primer	5`- AGGAAGAGGATGCGGCA -3´	
	probe	5´*- AGACTTCGAGCAGGAGATGGCCA -#3'	
ADAM17	5´-primer	5´- GGAAAGGGAAGCCATGTACAGTAG -3´	NM_009615.4
	3`-primer	5`- CGCTCAATTACGTCCTGTACTCG -3´	
	probe	5´*- GCGACATGAATGGCAAATGTGAGAAACG -#3'	
AHR	5´-primer	5´- GGCAGGATTTGCAAGAAGGAG -3´	NM_013464.2
	3`-primer	5`- AGGAAGCATAGAAGACCAAGGCAT -3´	
	probe	5´*- CTTGTTACAGGCGCTGAATGGCTTTGTGC -#3'	
TNF-alpha	5´-primer	5´- CCACCACGCTCTTCTGTCTACTG -3´	NM_013693.1
	3`-primer	5`- TGGGCCATAGAACTGATGAGAGG -3´	
	probe	5´*- CGGTCCCCAAAGGGATGAGAAGTTCCC -#3'	
TNFR-I	5´-primer	5´- GCCCGAAGTCTACTCCATCATTTG -3´	NM_011609.2
	3`-primer	5`- GCTGAAGGCTGGGGAGGG -3´	
	probe	5´*- CGTGCCTGTCAAAGAGGAGAAGGCTGGA -#3'	
VCAM-1	5´-primer	5´- CTCTGGGAAGCTGGAACGAAG -3´	NM_011693.2
	3`-primer	5`- CCAGGGGGCCACTGAATTGAATC -3´	
	probe	5´*- CCACGTGGACATCTACTCTTTCCCCAAGG -#3'	
CYP1A1	5´-primer	5´- TCCGGCATTCATCCTTCGT -3´	NM_009992.2
	3`-primer	5`- TTGGGGATATAGAAGCCATTCAGAC -3´	
	probe	5´*- CACCATCCCCCACAGCACCACAAG -#3'	

cDNA and Genomic DNA Sequencing

The promoter regions (~1.5 kb and ~2.7 kb for ADAM17) and coding regions of candidate genes were amplified and sequenced using specific primers (Table II). *Histone deacetylase 9 (HDAC9), AHR* and *NF-kappa-B inhibitor alpha (IkBa)* were amplified from cDNA, *ADAM17* was also sequenced amplified from genomic DNA.

PCR reactions were prepared in a final volume of 50 µL of a reaction mixture containing either 5 µL of cDNA (1:15) or 5 µL of DNA, respectively, 2.5 mM $MgCl_2$, 5 µL 10 x PCR Buffer (Roche, Mannheim, Germany), dNTPs (200 µM each), 0.05 U/µL TaqPolymerase (Roche, Mannheim, Germany), and 400 nM of each oligonucleotide primer. Annealing temperature as well as elongation time was dependent on primer Tm and on amplicon length. All PCRs were carried out in an ABI Prism 2400 thermocycler (Applied Biosystems, Darmstadt, Germany).

The sequencing reaction mixture consisted of 1µg DNA, 1 µL Primer (5 µM), as well as 2 µL Big Dye Ready Mix 3.0 (Applied Biosystems, Darmstadt, Germany). H_2O was added to a final volume of 10 µL. Sequencing reaction was carried out in an ABI Prism 2400 thermocycler (Applied Biosystems, Darmstadt, Germany) at 95°C 5 min, followed by 60 cycles at 95°C 30s, 50°C 10s, 58°C 4 min. Sequencing was performed with an automated DNA sequencer (ABI 377, Applied Biosystems, Darmstadt, Germany) at the DNA-sequencing core facility of the Interdisciplinary Center for Clinical Research, Leipzig.

Table II: PCR and sequencing primers

Gene		Sequence	DNA/ cDNA	Length of fragment
ADAM17 Exon1	5'-primer	5'- ACGGCACCCTTCTTCCG -3'	genomic DNA	425bp
	3'-primer	5'- CCTACTCCCTCTCTCTTTCACACAC -3'		
ADAM17 Exon2	5'-primer	5'- CTTGAACTCAGAAATCCGCCTG -3'	genomic DNA	355bp
	3'-primer	5'- AGTACAAATCTTTACGAAGTATTTTAAGATTC -3'		
ADAM17 Exon3	5'-primer	5'- GCTATTGTTCATCTCCTCTCCTGTAA -3'	genomic DNA	346bp
	3'-primer	5'- CTCACAAACCACTCACTGACATAAATC -3'		
ADAM17 Exon4	5'-primer	5'- GTAACCTGCATTCTTGGTGCTAGG -3'	genomic DNA	351bp
	3'-primer	5'- AGACAACTCCAAAAAGAAGCAGAAG -3'		
ADAM17 Exon5	5'-primer	5'- AGAAACAATATTATCCAAGAACAAAGAATG -3'	genomic DNA	354bp
	3'-primer	5'- ACAAACACCTTGCCAGAAACAGT -3'		
ADAM17 Exon6	5'-primer	5'- GGGATTTGAGATCAGCCAAGG -3'	genomic DNA	395bp
	3'-primer	5'- CCTTCTCTTAATAGGATTCATTTACCCA -3'		
ADAM17 Exon7	5'-primer	5'- TTCTTGATATACCCCTCAGTGGTAAAG -3'	genomic DNA	403bp
	3'-primer	5'- TTATCAACTAGTGTTAAATACCAAAACACAAC-3'		
ADAM17 Exon8	5'-primer	5'- CTTTGGAGTTCTGGGCTAGAGG -3'	genomic DNA	343bp
	3'-primer	5'- AAGACATTAAGGTTTCTACCATGTTCC -3'		
ADAM17 Exon9/10	5'-primer	5'- GCTACACAATCCAGTTGTCTGTACATATTAC -3'	cDNA	627bp
	3'-primer	5'- CTGTCGGGACCCATGCTTAT -3'		
ADAM17 Exon11	5'-primer	5'- CTCTGACCTCACATTCATTGTGC -3'	genomic DNA	469bp
	3'-primer	5'- TGAGCTTCAGTATTTGACAGTTCCA -3'		
ADAM17 Exon12	5'-primer	5'- TCAGCAATTTCTTACTGTGGAATTTG -3'	genomic DNA	467bp
	3'-primer	5'- TTGAGTTCCAGGAGAGCCTAGTC -3'		
ADAM17 Exon13	5'-primer	5'- CCCTAGGGTCTTGCTTTAGTTTTC -3'	genomic DNA	327bp
	3'-primer	5'- AAAAAATGCCGACCAGCAAAC -3'		

ADAM17 Exon14	5’-primer 3’-primer	5’- TAAAGACGTGCGCCACCAT -3’ 5’- TCCTCACTCTTGATGAGCAACAG -3’	genomic DNA	310bp
ADAM17 Exon15	5’-primer 3’-primer	5’- AGCCCTGGATAGTATCAGCTTTTG -3’ 5’- AACTGTGCAGGCTGTGATTCTG -3’	genomic DNA	367bp
ADAM17 Exon16	5’-primer 3’-primer	5’- CGGAATTACTACCCTGTGTCCAG -3’ 5’- AATAACACCCGCGTCATAACG -3’	genomic DNA	274bp
ADAM17 Exon17	5’-primer 3’-primer	5’- AGGCAATCAAAGCAGTGTCTTG -3’ 5’- CTGTCCACACACCACCAGTAGG -3’	genomic DNA	405bp
ADAM17 Exon18	5’-primer 3’-primer	5’- AGGCAGGTAGATTTCTGAGTTCGA -3’ 5’- AATCGGATCCTTTTAACTTCCACTAG -3’	genomic DNA	298bp
ADAM17 Exon19	5’-primer 3’-primer	5’- GTATCCAGCACCAGGTTTAGCAT -3’ 5’- CCAGATTTACCTCACCTCTTCAAAG -3’	genomic DNA	632bp
ADAM17 3’-UTR	5’-primer 3’-primer	5’- CCATAGAGCTGACCTGAATCAAAAC -3’ 5’- AAGACGACACATTGGTAAAATGG -3’	genomic DNA	618bp
ADAM17 Prom2749	5’-primer 3’-primer	5’- TTGACAATCGGTAACAAAGTCAATG -3’ 5’- ACTCTTTACCAAGTCGCGGATG -3’	genomic DNA	2855bp
ADAM17 Prom2424	5’-primer 3’-primer	5’- GCATCCAAATTAAGCTTTTATGACAGATA -3’ 5’- CGCCCCATGGTCCCGTCC -3’	genomic DNA	2432bp
ADAM17 Prom1283	5’-primer 3’-primer	5’- AAAAAGCTTGCATCTACCGGGCGGTGGT -3’ 5’- CGCCCCATGGTCCCGTCC -3’	genomic DNA	1291bp
ADAM17 Prom682	5’-primer 3’-primer	5’- CATAAGCTTATGTACAGTCCCCTGAGCAT -3’ 5’- CGCCCCATGGTCCCGTCC -3’	genomic DNA	690bp
HDAC9 cDNA1	5’-primer 3’-primer	5’- ATGCACAGTATGATCAGCTCAGTG -3’ 5’- CTTGAGCTCCTGGTAATGTGTACTTG -3’	cDNA	622bp
HDAC9 cDNA2	5’-primer 3’-primer	5’- CAAGTACACATTACCAGGAGCTCAAG -3’ 5’- GAGGCTGCTCTGTCTTCCATG -3’	cDNA	831bp

HDAC9 Prom1473	5'-primer 3'-primer	5'- ACGTTTACAGGTGAAGTGCATCTG -3' 5'- GGCTATCCTGGAAACTAAGAGCAG -3'	genomic DNA	1125bp
HDAC9 Prom684	5'-primer 3'-primer	5'- CGACCATTGTTCTATTTCTGTGC -3' 5'- TAAGAAGTAACTCTTGCTGCAGTTG -3'	genomic DNA	841bp
AHR cDNA1	5'-primer 3'-primer	5'- CCGGTGCAGAAAACAGTAAAGC -3' 5'- TGCCAGAAAACCAGATGAATTATC -3'	cDNA	642bp
AHR cDNA2	5'-primer 3'-primer	5'- CTTCTTTCATGGAGAGGTGCTTC -3' 5'- GGATCCATTATGGGAGAGAAAGG -3'	cDNA	640bp
AHR cDNA3	5'-primer 3'-primer	5'- CTACCGGAGAGGCTGTGTTGTAC -3' 5'- GCAAACGTGCCGTTGATTTG -3'	cDNA	760bp
AHR cDNA4	5'-primer 3'-primer	5'- CAGCTGTGTCAGATGGTGTGC -3' 5'- GGGTGTGATATCGGGAAGAGG -3'	cDNA	642bp
AHR Prom1467	5'-primer 3'-primer	5'- CTATCCTTTTCCTTGCTGTTGTGTATC -3' 5'- TTGTAACCCAGACATCAAGATTTACC -3'	genomic DNA	833bp
AHR Prom762	5'-primer 3'-primer	5'- TTAGAATGCTCTCCAGTCGCAG -3' 5'- TCTAACTCTGTGTTCAGCCGGTC -3'	genomic DNA	902bp
IκBα cDNA1	5'-primer 3'-primer	5'- ACAACAGTCAGACCTCGCCAG -3' 5'- CCTCCAAACACACAGTCATCATAG -3'	cDNA	995bp
IκBα cDNA2	5'-primer 3'-primer	5'- CCCCTCTACATCTTGCCTGTG -3' 5'- CGTGGTCCTTTCATTAGGTTCTG -3'	cDNA	802bp
IκBα 3'-UTR	5'-primer 3'-primer	5'- ACTGTGTGTTTGGAGGCCAGC -3' 5'- AACATTTACAAGAAGGCGACACAGAC -3'	genomic DNA	472bp
IκBα Prom1400	5'-primer 3'-primer	5'- CCCTCAGTGTCTTCACATGCATAC -3' 5'- GGATTCGCTTCTCCTAAATTTCTG -3'	genomic DNA	853bp
IκBα Prom827	5'-primer 3'-primer	5'- TGGTTCCCATACGGAGCTAGG -3' 5'- TTGCTCGTACTCCTCGTCCTTC -3'	genomic DNA	959bp

ADAM17 and Chr12 Genotyping

One single nucleotide polymorphism (SNP) in exon 19 of *ADAM17* that we found to be polymorphic between FVB.LDLR$^{-/-}$ and B6.LDLR$^{-/-}$ mice was used as an additional marker to fine-map Chr12 in the F2-mice. Five additional SNPs, rs13481285 (7Mb), rs13481363 (29Mb), rs13481428 (40Mb), rs13481505 (60Mb), rs13481569 (80Mb), spaced across Chr12 were used for generation of congenic mice. Genotyping was performed using a homogenous fluorescent method as previously described.[3] Sequences of primers and probe are given in Table III.

The PCR reaction was prepared on 384-well plates in a final volume of 12,5 µL. The reaction mixture contained 1 µL of DNA, 4 mmol/LM $MgCl_2$, 1xJumpstart buffer, 250 µM dNTP (each), 0.02 U/µL Jumpstart Taq Polymerase (Sigma-Aldrich, Munich, Germany), 250 nmol/L oligonucleotide probe, 100 nmol/L forward- and 900 nmol/L reverse-primers. Cycling conditions were 95°C for 10 minutes and 55 two-step cycles of 95°C for 25 seconds, 58°C for 20 seconds and 72°C for 30 seconds. The meting curve was generated after denaturing the reaction mixture at 95°C for 5 minutes and hybridizing probes at 35°C for 2 minutes and slowly heating to 80°C at a ramp-rate of 7% while continuously monitoring fluorescence. SNP genotyping was performed on a ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Darmstadt, Germany).

Table III: Primers and probe used for *ADAM17* genotyping

(*: 6-FAM; # : TAMRA)

Gene		Sequence
rs13481285 (7Mb)	5´-primer	5´- CAAAACTGTCACTTACATTCAGACCCTAG -3´
	3`-primer	5`- TTTTCATGACTTCAACTACCTGGTAACTG -3´
	probe	5´*- TTGGTAAAAGTTGCCCAGGAAGATGG -#3'
ADAM17-SNP (Exon 19)	5´-primer	5´- CCATAGAGCTGACCTGAATCAAAAC -3´
	3`-primer	5`- AAGACGACACATTGGTAAAATGG -3´
	probe	5´*- TTTTTCTTTTCTAATCTAAAATCTC -#3'
rs13481363 (29Mb)	5´-primer	5´- GCTTTCCAGAACAATGAGGTTACCAGC -3´
	3`-primer	5`- TCTGATAATGGCCAGAGAGCATCAGC -3´
	probe	5´*- CAGAATTGGCCTCTCATGGT -#3'
rs13481428 (40Mb)	5´-primer	5´- GTCTAGTGTACAGTGAGGGAAACAGACAACTTA -3´
	3`-primer	5`- GAACTTGTATATTCTCCAACTCTCACATGTGG -3´
	probe	5´*- TAGAAGTAcGACTTGCTG -#3'
rs13481505 (60Mb)	5´-primer	5´- CTGAGCTGGAGACAGACTTGATTTGC -3´
	3`-primer	5`- TCAGCCTGATGATGAAGTGAAATTGTG -3´
	probe	5´*- ACAGCTTTGACAAGTCCAT -#3'
rs13481569 (80Mb)	5´-primer	5´- TGTTGCAGCGTCGTTCTTCCTG -3´
	3`-primer	5`- GTTGGTGGCGCCCATACACAC -3´
	probe	5´*- TTTTGTGTAGGCCTCACAGC -#3'

Allele-Specific Transcript Quantification (Cis-Trans Test)

Allele-specific *ADAM17* transcript quantification was performed in livers from 10 F1-mice using quantitative sequence analysis of cDNA of the A1098G sequence variant in exon 9 between FVB and B6. cDNA concentrations were adjusted to 10,000 copies of *ADAM17* per sample as determined by quantitative RT-PCR. Serial mixtures containing 10,000 copies *ADAM17* cDNA from FVB and B6 (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100) were used for standardization and amplified in the same run with samples containing F1 cDNA. PCR conditions and oligonucleotide primers for amplification of exons 9 and 10 are given in Table II. DNA concentrations of the resultant PCR products were determined and 1 µg was used for DNA sequencing. The allelic ratio in the F1 was determined by comparison of the peak heights for the G-allele (FVB) and A-allele (B6) with peak heights for these alleles obtained in the standard mixtures.

Transfection Study of ADAM17 Promoter Fragments

ADAM17 promoter fragments of FVB and B6 starting at -681 bp upstream of the start codon were amplified and cloned into the luciferase reporter vector pGl4.11[luc2CP] (Promega, Mannheim, Germany). Four sequence variations were identified in the 681 bp promoter fragment between FVB and B6. For sequential insertion of the B6 variants into the FVB promoter fragment, the following primers were used: AC-640DEL 5'- and 3'-primer, G-457T-primer, A-216C-primer, G-25T-primer. For insertion of the FVB variant into the C57 promoter vector (T-25G) the following primer was used: T-25G-primer. Primer sequences are given in Table IV.

Mutagenesis of the sequence variants was carried out with QuickChange II Site Directed Mutagenesis Kit (for AC-640DEL, Invitrogen, Karlsruhe, Germany) and QuickChange Multi-Site Directed Mutagenesis Kit (for G-457T, A-216C, G-25T, and T-25G, Invitrogen, Karlsruhe) according to the manufacturers instructions.

Table IV: Primers used for mutagenesis

Primers		Sequence
AC-640DEL	5'-primer	5'- CCCCTGAGCATTTTCAGTGACAAGAGACCTAGTCTCCCGC -3'
	3'-primer	5'- GCGGGAGACTAGGTCTCTTGTCACTGAAAATGCTCAGGGG -3'
G-457T	5'-primer	5'- TCACGAAGAAGGTCGCACCTGTCTAAGGACCTGCCC -3'
A-216C	5'-primer	5'- CGGCTGTGGGCCGCGGGGTGGTCTC -3'
G-25T	5'-primer	5'- CGCTCTTTCGGAGAAGGTTTCCCAGAGAGGTGGTGG -3'
T-25G	5'-primer	5'- CGCTCTTTCGGAGAAGGTTGCCCAGAGAGGTGGTGG -3'

For cell transfection experiments, 0.5×10^6 murine RAW 264.7 cells were plated on 35mm-dishes and grown to 70% confluence. Cells were co-transfected with 0.01 µg pGl4.74[*hRluc*/TK] vector (Invitrogen, Karlsruhe, Germany) for normalization and 1 µg of expression vectors using the diethylaminoethyl(DEAE)-dextran method:[4] Cells were incubated for 24 h at 37°C, 5% CO_2 and harvested in 500 µL Passive Lysis Buffer (Promega, Mannheim, Germany). Firefly and renilla luciferase activities were measured sequentially using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany) in a Sirius Luminometer (Berthold Detection Systems, Pforzheim, Germany).

Statistical Analysis

All data are given as mean ± standard deviation unless otherwise indicated. Normality of distribution was assessed using the Kolmogorov–Smirnov test implemented in PRISM statistical software (GraphPad, San Diego, CA). Comparison of multiple groups was done using ANOVA and Tukey was performed as post-test. Comparison of two groups of normally distributed samples was done using the t-test. Linkage analysis for single QTLs was done using MAPMANAGER QTX B20, freely available at www.mapmanager.org.[5] Levels of significance were determined empirically by permutation testing in 1 cM steps.

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