Supplemental Materials

A Maresin 1/RORα/12-lipoxygenase autoregulatory circuit prevents inflammation and progression of nonalcoholic steatohepatitis

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Supplemental Methods

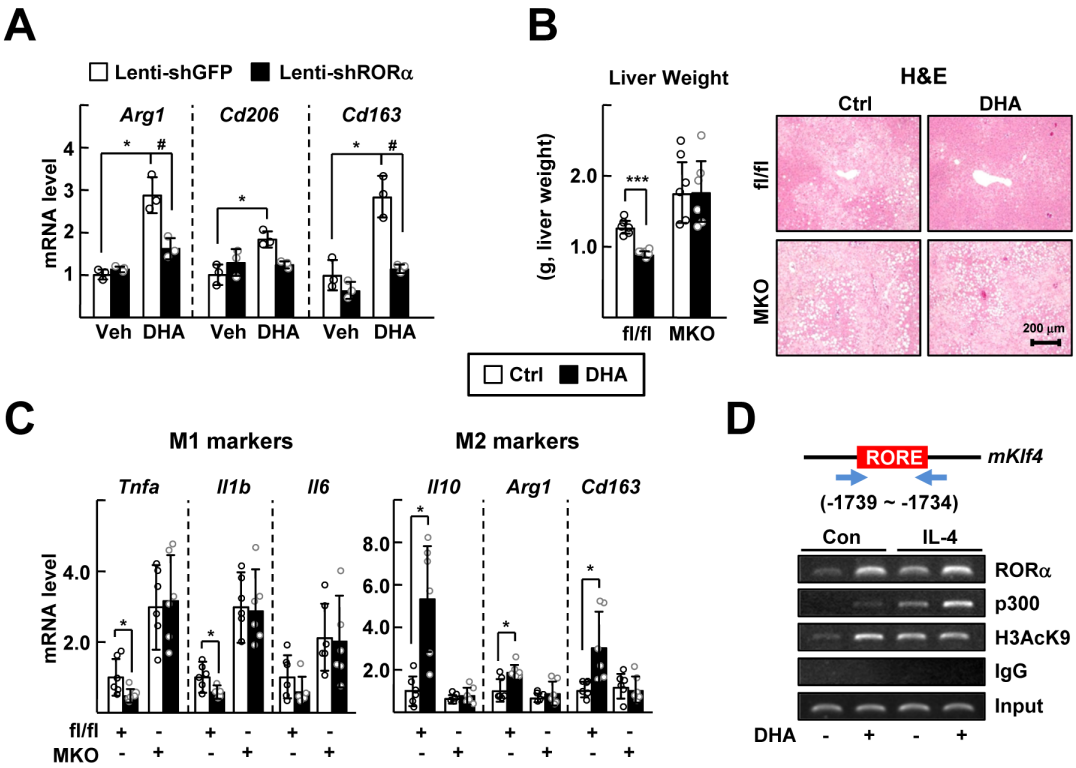
Gene expression microarray

RNA was extracted using the RNeasy Micro kit (Qiagen) and analyzed using SurePrint G3 Mouse Gene Expression v2 8x60K chip (Agilent). Differentially up-regulated genes that expressed higher than 1.5-fold versus vehicle were extracted ($P < 0.05$). The potential over-represented transcription factor enrichment analysis was performed by oPOSSUM software (http://opossum.cisreg.ca/). The microarray data have been deposited in the Gene Expression Omnibus (GEO) database with the accession number GSE122430.

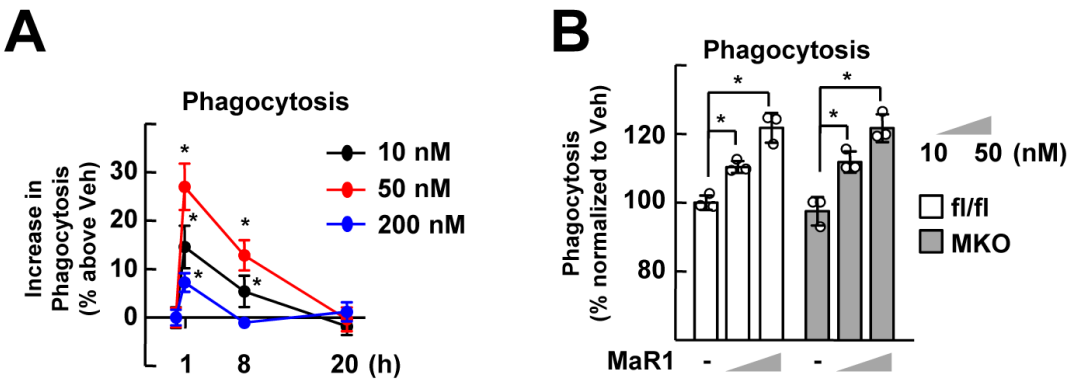
Measurement of cellular phagocytosis

Liver macrophages (50,000 cells/well) were seeded onto 96-well plates. Then 100 μl of fluorescein-labeled E. coli particles obtained from Vybrant^{TM} phagocytosis assay kit (Molecular Probes) was added and incubated at 37°C for 1 h. To remove unengulfed fluorescence of bioparticles, trypan blue was added and immediately washed. The intensity of engulfed fluorescein-labeled bioparticles was measured by SpectraMAX M5 microplate multi-reader (Molecular Devices).

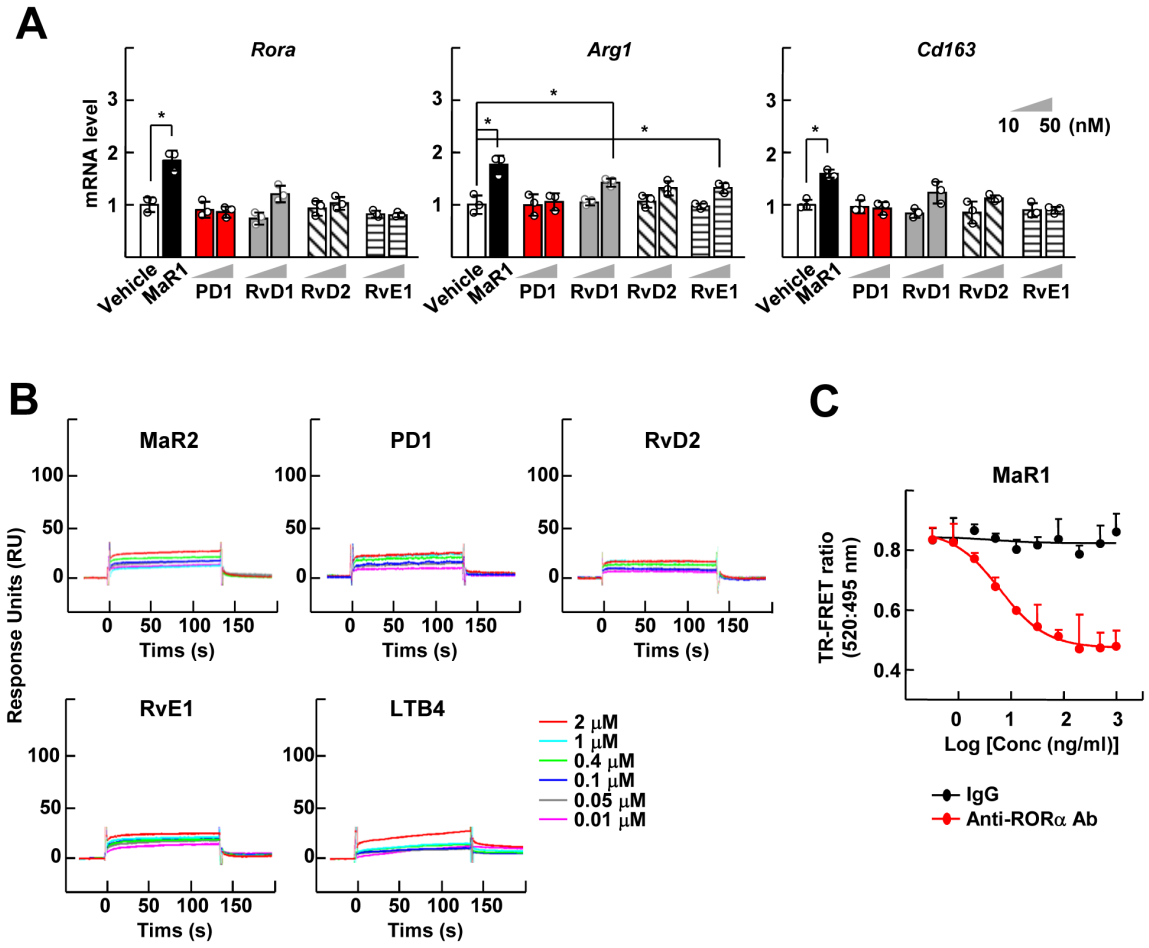
Supplemental Figures



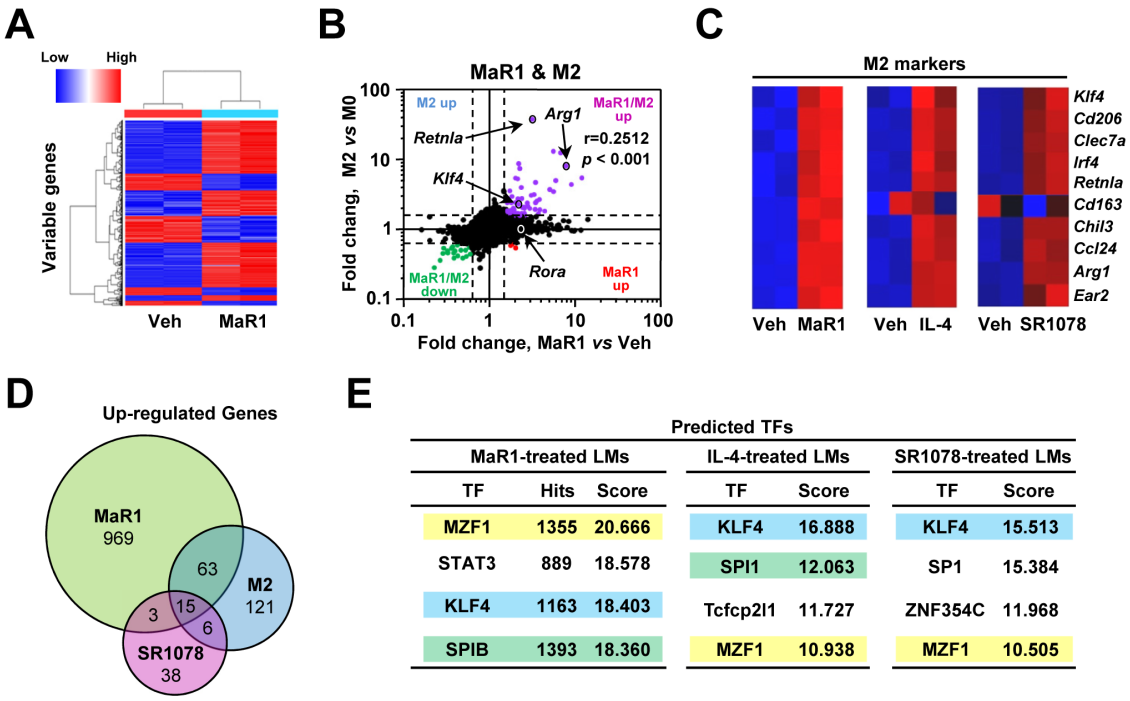
Supplemental Figure 1. RORα-dependent protective function of DHA against NASH.
(**A**) The liver macrophages were infected by lenti-shGFP or lenti-shRORα for 24 h and then treated with or without 50 μM DHA for 24 h. The mRNA levels of M2 markers were measured by qRT-PCR. The data represent mean ± SD. $^{*}P < 0.05$; $^{\#}P < 0.05$ (n=3).
(**B** and **C**) Seven week-old fl/fl and the RORα-MKO mice were fed with HFD for 12 weeks. After 10 weeks of diet feeding, DHA was administered daily at dose of 5 mg/kg BW/day for 2 weeks. (**B**) The liver weights of experimental mice at the end of experiments and H&E staining of liver sections. (**C**) The mRNA levels of M1 markers and M2 markers in liver tissues were analyzed by qRT-PCR. The data represent mean ± SD. $^{*}P < 0.05$, and $^{***}P < 0.001$ (n=5-6).
(**D**) Raw 264.7 cells were treated with 50 μM DHA in the presence or absence of 20 ng/ml IL-4 for 24 h. DNA fragments that contain flanking region of the ROREs on the *Klf4* promoter were immunoprecipitated with anti-RORα, anti-p300, or anti-histone (H3AcK9) antibodies and then amplified by PCR using specific primers.
Data were analyzed by Mann–Whitney U test for simple comparisons or Kruskal-Wallis test for multiple groups.



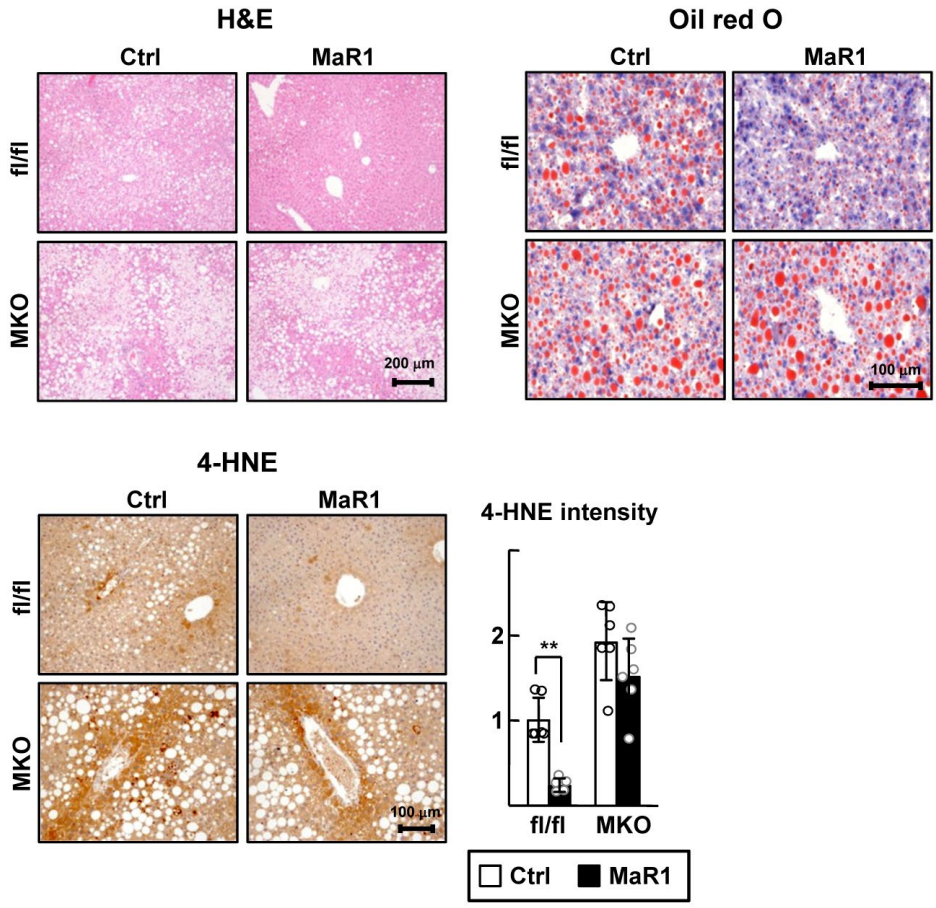
Supplemental Figure 2. RORα does not influence on the phagocytic function of the liver macrophages
(**A**) The primary culture of the liver macrophages was treated with 10, 50, or 200 nM MaR1 for 1, 8, or 20 h prior to incubation with fluorescence-labled Escherichia coli particles for 1 h. The fluorescence intensity was measured by fluorescence microplate reader. The data represent mean ± SD. **P* < 0.05 versus 0 h.
(**B**) The liver macrophages obtained from the livers of fl/fl and RORα-MKO mice were treated with or without 10, or 50 nM MaR1 for 1 h prior to incubation with fluorescence-labled Escherichia coli particles for 1 h. The fluorescence intensity was measured by fluorescence microplate reader. The data represent mean ± SD. **P* < 0.05 (n=3).
Data were analyzed by Mann–Whitney U test for simple comparisons.



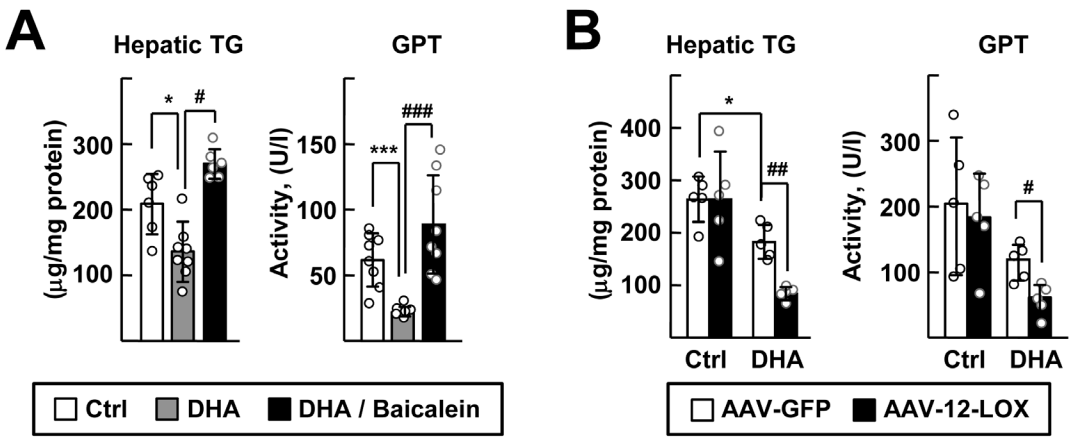
Supplemental Figure 3. Specificity of MaR1 on the RORα binding and M2 polarity switch.
(**A**) The primary culture of liver macrophages was treated with 50 nM MaR1, 10 or 50 nM protectin D1 (PD1), resolvin D1 (RvD1), resolvin D2 (RvD2), or resolvin E1 (RvE1) for 24 h. The mRNA levels of *Rora*, and M2 marker genes were measured by qRT-PCR. The data represent mean ± SD. **P* < 0.05 (n=3).
(**B**) BIAcore analysis for binding of MaR2, PD1, RvD2, RvE1, or leukotriene B4 (LTB4) to RORα. The increasing concentrations of ligands were injected over immobilized GST-RORα-His proteins on the sensor chip.
(**C**) TR-FRET assay was performed using Lanthascreen RORα co-activator assay kit. Blocking RORα antibody and corresponding IgG was pre-incubated with GST-RORα recombinant proteins and reaction with 10 μM MaR1 was conducted. Y-axis represents ratio of fluorescence intensity at 520 nm (signal) and at 495 nm (background). X-axis represents log scale of RORα antibody concentration.
Data were analyzed by Mann–Whitney U test for simple comparisons.



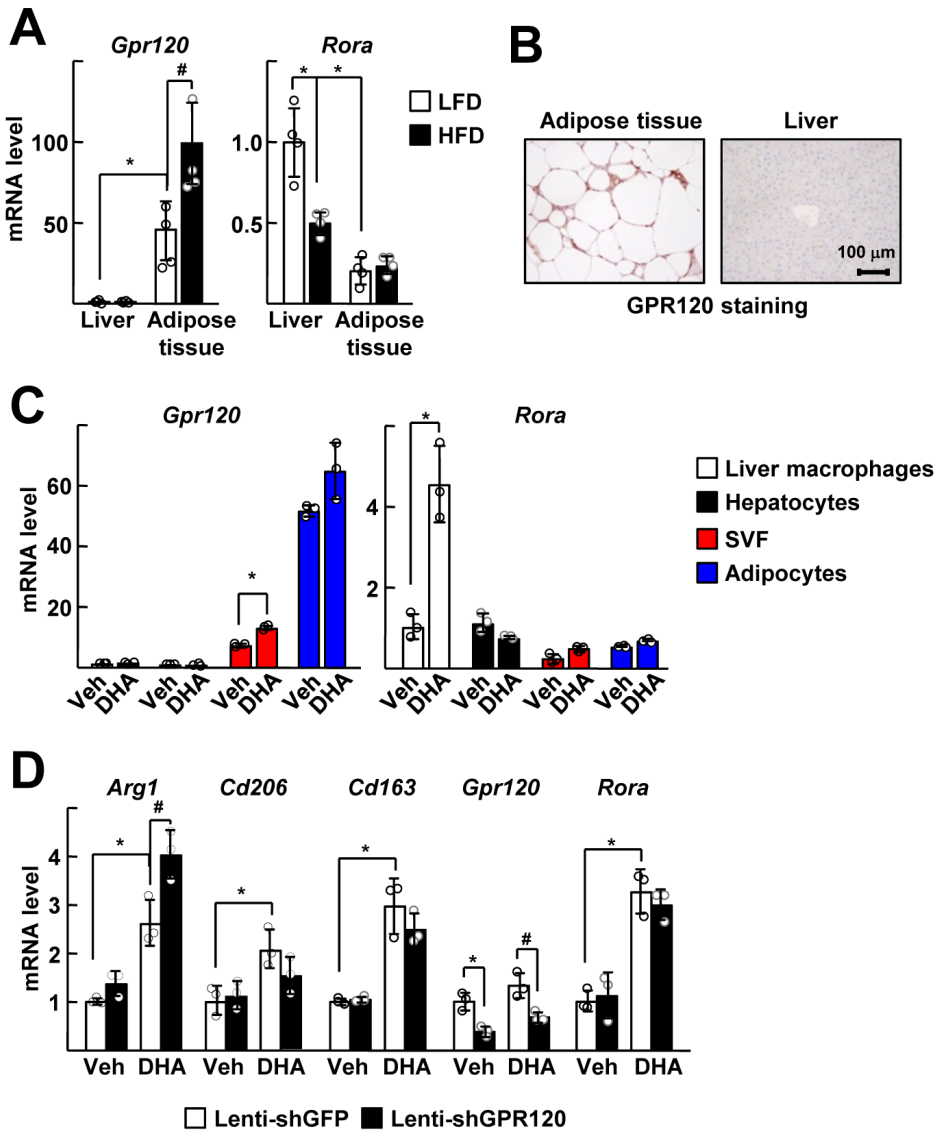
Supplemental Figure 4. Gene expression profiling of the MaR1-treated liver macrophages.
(**A**) Hierarchical Clustering global gene expression map of the MaR1-treated liver macrophages.
(**B**) Distribution of gene expression in IL4 (M2)- and MaR1-treated liver macrophages were compared by their expression level. Black dotted lines represent cut-off (±1.5 fold-change).
(**C**) Gene expression heatmap of the MaR1-treated liver macrophages was compared with those of the IL-4 (M2)- and the SR1078-treated macrophages (3).
(**D**) Venn-diagram of up-regulated genes (fold change > 1.5) in the MaR1-, IL-4 (M2)-, or SR1078-treated liver macrophages.
(**E**) Enriched transcription factors (TFs) at promoter regions of the MaR1-, IL-4 (M2)-, or SR1078-treated liver macrophages responsive genes. Top four overrepresented conserved transcription factor binding sites were ranked by Z score, which reflects the occurrence of the transcription factor binding sites in the promoters of the target gene set compared to background gene set.



Supplemental Figure 5. MaR1 attenuates lipid accumulation in a RORα-dependent manner.
Seven week-old fl/fl and RORα-MKO mice were fed with HFD for 12 weeks. After 10 weeks of diet feeding, MaR1 was administered daily at dose of 5 μg/kg BW for 2 weeks. Representative H&E staining and Oil red O staining of liver sections were shown (top). Immunohistochemistry staining of 4-hydroxynonenal (4-HNE) in liver sections were shown. The intensity of stained 4-HNE in the liver sections was analyzed by Image J (bottom). The data represent mean ± SD (n=5-6). $^{**}P < 0.01$.
Data were analyzed by Mann–Whitney U test for simple comparisons or Kruskal-Wallis test for multiple groups.



Supplemental Figure 6. Modulation of 12-LOX activity affects the DHA-mediated improvement of NASH. (**A**) Seven-week-old wild-type male C57BL/6 mice were fed with HFD for 12 weeks. After 10 weeks of diet feeding, DHA was i.p. injected daily at dose of 5 mg/kg BW with vehicle or 5 mg/kg BW baicalein for 2 weeks. Hepatic TG levels and serum GPT activities were analyzed at the end of experiments. The data represent mean ± SD. *$P < 0.05$, and ***$P < 0.001$; $^{\#}P < 0.05$, and $^{\#\#\#}P < 0.001$ (n=6-8).
(**B**) Seven-week-old wild-type male C57BL/6 mice were fed with HFD for 16 weeks. Seven-week-old wild-type male C57BL/6 mice were fed with HFD for 16 weeks. After 13 weeks of diet feeding, an intravenous injection of either AAV-GFP or AAV-12-LOX at 5 X 10^9 virus genomes was conducted. DHA was i.p. injected daily at doses 1 mg/kg BW for 3 weeks after virus injection. Hepatic TG levels and serum GPT activities were analyzed at the end of experiments. The data represent mean ± SD. *$P < 0.05$; $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ (n=5).
Data were analyzed by Mann–Whitney U test for simple comparisons or Kruskal-Wallis test for multiple groups.



Supplemental Figure 7. GPR120 does not affect polarity of the liver macrophages.
(**A**) Seven-week-old wild-type male C57BL/6 mice were fed with LFD or HFD for 12 weeks. The mRNA levels of *Gpr120* and *Rora* were analyzed by qRT-PCR. The data represent mean ± SD. **P* < 0.05; $^{\#}P$ < 0.05 (n=4).
(**B**) Immunohistochemistry staining of GPR120 in the liver and adipose tissue sections.
(**C**) mRNA levels of *Gpr120* and *Rora* in the liver macrophages, hepatocytes, stromal vascular fraction (SVF), and adipocytes were measured by qRT-PCR. The data represent mean ± SD. **P* < 0.05 (n=3).
(**D**) The liver macrophages were infected by lenti-shGFP or lenti-shGPR120 for 24 h and then treated with or without 50 μM DHA for 24 h. mRNA levels of M2 markers, *Gpr120,* and *Rora* were measured by qRT-PCR. The data represent mean ± SD. **P* < 0.05; $^{\#}P$ < 0.05 (n=3).
Data were analyzed by Mann–Whitney U test for simple comparisons or Kruskal-Wallis test for multiple groups.

Supplemental Table

Supplemental Table 1. Oligonucleotide sequences used in the present investigation.

Gene	Accession number		Nucleotide sequence	Species	Purpose
RORα	NM_013646 NM_001289916	Sense Antisense	5'- GATCGCTCGTGGCTTCAGGAA -3' 5'- TGGAGGAAAATGGAGTCGCACA -3'	Mouse	qRT-PCR
	NM_134261 NM_134262	Sense Antisense	5'- AAACAAGCAGCGGGAGGTGA -3' 5'- TGGCAAACTCCACCACATAC -3'	Human	
KLF4	NM_010637	Sense Antisense	5'- CCAGACCAGATGCAGTCACAA -3' 5'- ACGACCTTCTTCCCCTCTTTG -3'	Mouse	
IL-10	NM_010548	Sense Antisense	5'- GCTCTTACTGACTGGCATGAG -3' 5'- CGCAGCTCTAGGAGCATGTG -3'	Mouse	
Arg1	NM_007482	Sense Antisense	5'- CTCCAAGCCAAAGTCCTTAGAG -3' 5'- AGGAGCTGTCATTAGGGACATC -3'	Mouse	
CD206	NM_008625	Sense Antisense	5'- CAGGTGTGGGCTCAGGTAGT -3' 5'- TGTGGTGAGCTGAAAGGTGA -3'	Mouse	
CD163	NM_001170395 NM_053094	Sense Antisense	5'- TGGGTGGGGAAAGCATAACT -3' 5'- AAGTTGTCGTCACACACCGT -3'	Mouse	
TNFα	NM_013693	Sense Antisense	5'- AATGGCCTCCCTCTCATCAGTT -3' 5'- CCACTTGGTGGTTTGCTACGA -3'	Mouse	
IL-1β	NM_008361	Sense Antisense	5'- AGAGCCCATCCTCTGTGACTCA -3' 5'- TGCTTGGGATCCACACTCTCCA -3'	Mouse	
IL-6	NM_031168 NM_001314054	Sense Antisense	5'- GAACAACGATGATGCACTTGC -3' 5'- TCCAGGTAGCTATGGTACTCC -3'	Mouse	
PPARα	NM_011144 NM_001113418	Sense Antisense	5'- CGACCTGAAAGATTCGGAAA -3' 5'- CTTTCCCGCGAGTATGACC -3'	Mouse	
PPARβ	NM_011145	Sense Antisense	5'- GATGACAGTGACCTGGCGCT -3' 5'- AGGCCTGGCCGGTCTC -3'	Mouse	
PPARγ	NM_001127330 NM_001308352	Sense Antisense	5'- CTCCAAGAATACCAAAGTGCGA -3' 5'- GCCTGATGCTTTATCCCCACA -3'	Mouse	
RXRα	NM_011305	Sense Antisense	5'- CATGAGTTAGTCGCAGACATGGAC -3' 5'- ACCCGTTGGAGAGTTGAGGG -3'	Mouse	
Alox15	NM_009660	Sense Antisense	5'- CAGGGATCGGAGTACACGTT -3' 5'- GATTGTGCCATCCTTCCAGT -3'	Mouse	
	NM_001140	Sense Antisense	5'- CTTCAAGCTTATAATTCCCCAC -3' 5'- GATTCCTTCCACATACCGATAG -3'	Human	
Alox12	NM_007440 NM_001331118	Sense Antisense	5'- GATCACTGAAGTGGGGCTGT -3' 5'- CACACATGGTGAGGAAATGG -3'	Mouse	
	NM_000697	Sense Antisense	5'- AGTTCCTCAATGGTGCCAAC -3' 5'- GCAGCCAGGTATTGCTTCTC -3'	Human	
Alox5	NM_009662	Sense Antisense	5'- CTACGATGTCACCGTGGATG -3' 5'- GTGCTGCTTGAGGATGTGAA -3'	Mouse	
GPR120	NM_181748	Sense Antisense	5'- CGAGTGTCCCAACAAGACTAC -3' 5'- CAAGATGAGGAGGATGGTGATG -3'	Mouse	
18S rRNA	NR_003278	Sense Antisense	5'- GTAACCCGTTGAACCCCATT -3' 5'- CCATCCAATCGGTAGTAGCG -3'	Mouse	
β-actin	NM_007393	Sense Antisense	5'- CGTGGGCCGCCCTAGGCACCA -3' 5'- TGGCCTTAGGGTTCAGGGGGG -3'	Mouse	

KLF4 pro RORE	Sense 5'- CAGAGTTAAACTGGCCTAGTTCCA -3' Antisense 5'- CTTTCTCTTGGTTTTGGCAGAGGA -3'	Mouse	ChIP
Alox12 pro RORE	Sense 5'- GCCTGGAGAGTGTGCAATAG-3' Antisense 5'- GATGTTCTAGTTTGGTGGCTAGG-3'	Mouse	
shGFP	5'- GTTCAGCGTGTCCGGCGAG -3'		
shRORα	5'- GCAGAGAGACAGCTTGTACGC -3'	Mouse	Lentivirus
shGPR120	5'- GCACCCACTTCCCTTTCTTCT -3'	Mouse	