SUPPLEMENTARY DATA

Supplementary Materials and Methods

Plasma Cholesterol and Triglyceride Levels

Plasma was collected from *ApoE*$^{-/-}$ and *Aif1*$^{-/-}$*;ApoE*$^{-/-}$ mice after 18 weeks of high fat diet feeding and overnight fasting. Total cholesterol and triglycerides were measured by commercially available colorimetric kits (FUJIFILM Wako Diagnostics; 999-02601 and Thermo Scientific; TR22421).

BrdU Incorporation

BMDMs were incubated with BrdU (10 μM) for 15 hours at specified days after bone marrow retrieval. Cellular DNA was denatured with HCl and stained with anti-BrdU antibody (Abcam; ab8152; 1:200 dilution). BrdU incorporation was assessed by FACS analysis using a FACSCalibur flow cytometer (BD Biosciences).

Propidium Iodide Staining

For cell cycle analysis, BMDMs at days 3, 6, 8 and 10 days of culture were washed with FACS buffer (10% FBS in PBS) fixed with 95% ethanol and stained with propidium iodide (2 ug/ml) in PBS containing 0.1 mg/ml RNase.

OxLDL Overload

To evaluate the effect of oxidized (ox)-LDL overload on the viability of wt and *Aif1*$^{-/-}$ BMDMs, 16x10^3 cells/well were plated in 96-well plates with six independent wells per treatment and genotype. BMDMs were incubated with 300 μg/ml of oxLDL (Kalen Biomedical; 7702026) for 24 or 48 hours. Evaluation of cell viability in culture was performed with the AlamarBlue assay (Bio-Rad; BUF012A). Absorbances at 570 nm and 600 nm were measured on a Synergy 2 microplate reader (BioTek). AlamarBlue reduction correlates with the number of viable cells, and was calculated according to the manufacturer's instructions and expressed relative to baseline.

OxLDL Uptake

BMDMs were cultured on glass bottom 6-well culture dishes (Corning; 3516). After overnight incubation with 0, 25, or 50 µg/ml of oxLDL (Kalen Biomedical; 7702026), cells were washed, fixed with 10% formalin and stained with Oil Red O for 18 minutes. After washing and drying, cells were observed under the microscope. Cells with at least 5 lipid granules were counted as positive. The number of positive BMDMs per total BMDMs in a given field were counted from least 15 random fields.

Expression of Lipid Uptake and Cholesterol Efflux Genes

Wt and *Aif1*$^{-/-}$ BMDMs were stimulated overnight with 50 µg/ml of oxLDL (Kalen Biomedical; 7702026). Total RNA was isolated as described in the main manuscript, cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen; 18080051) and the expression levels of *Sr-a1* (forward, 5'-GGAGTGTAGGCGGATC
-3'; reverse, 5'-GTCAATGGAGGCCCCA-3'), *CD36* (forward, 5'-TCCTCTGACATTTGCAGGTCTATC
-3'; reverse, 5'-AAAGGCATTGGCTGGAAGAA-3'), *Abca1* (forward, 5'-CCGAGGAAGACGTGGACACCTTC
-3'; reverse, 5'-CCTCAGCCATGACCTGCCTTGTAG
-3') and *Abcg1* (forward, 5'-CCTTCCTCAGCATCATGCG
-3'; reverse, 5'-CCGATCCCAATGTGCGA-3'), relative to the housekeeping *18S* (forward, 5'-AGTCCCTGCCCTTTGTACACA-3'; reverse, 5'-CGATCCGAGGGCCTCACTA-3') and normalized to unstimulated cells, were measured by real-time PCR using the PowerUp SYBR Green Master Mix (ThermoFisher; A25741) following manufacturer recommendations. Gene expression changes were calculated using the ΔΔCT method as described in the main manuscript.

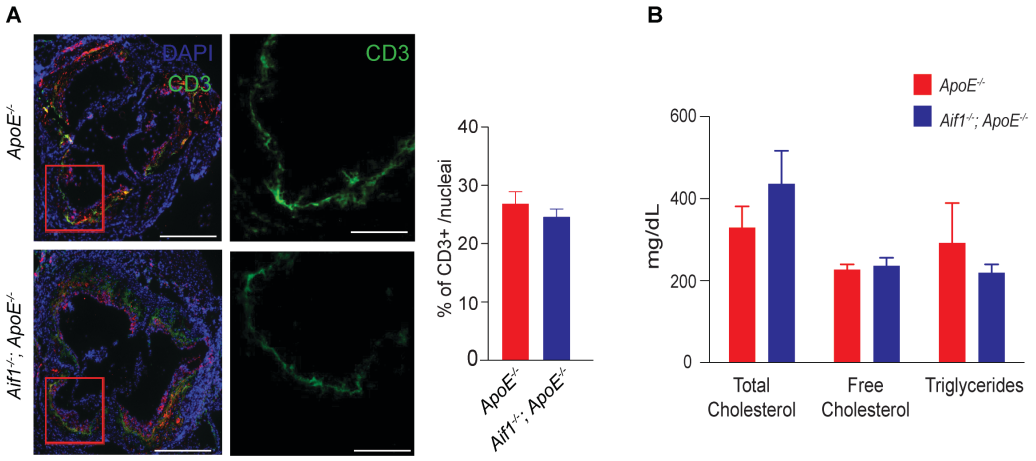
Cytokine Levels

Wt and *Aif1*$^{-/-}$ BMDMs were stimulated overnight with 50 µg/ml of oxLDL (Kalen Biomedical; 7702026). Cell culture supernatants were collected and IL-6 and TNF-α pro-inflammatory cytokine levels were determined using the ELISA MAX™ Deluxe Sets for mouse IL-6 (Biolegend; 431304) and mouse TNF-α

(Biolegend; 430904) following manufacturer´s recommendations. Secreted IL-6 and TNF-α levels were calculated after oxLDL stimulation, relative to baseline.

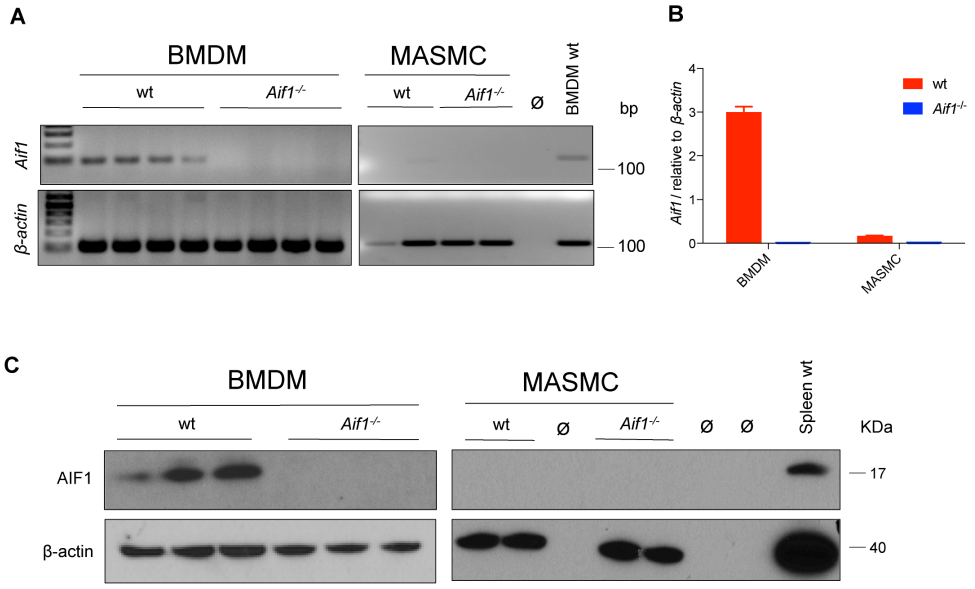
Supplementary Figures and Tables

Egaña-Gorroño L. et al., Supplementary Figure S1.

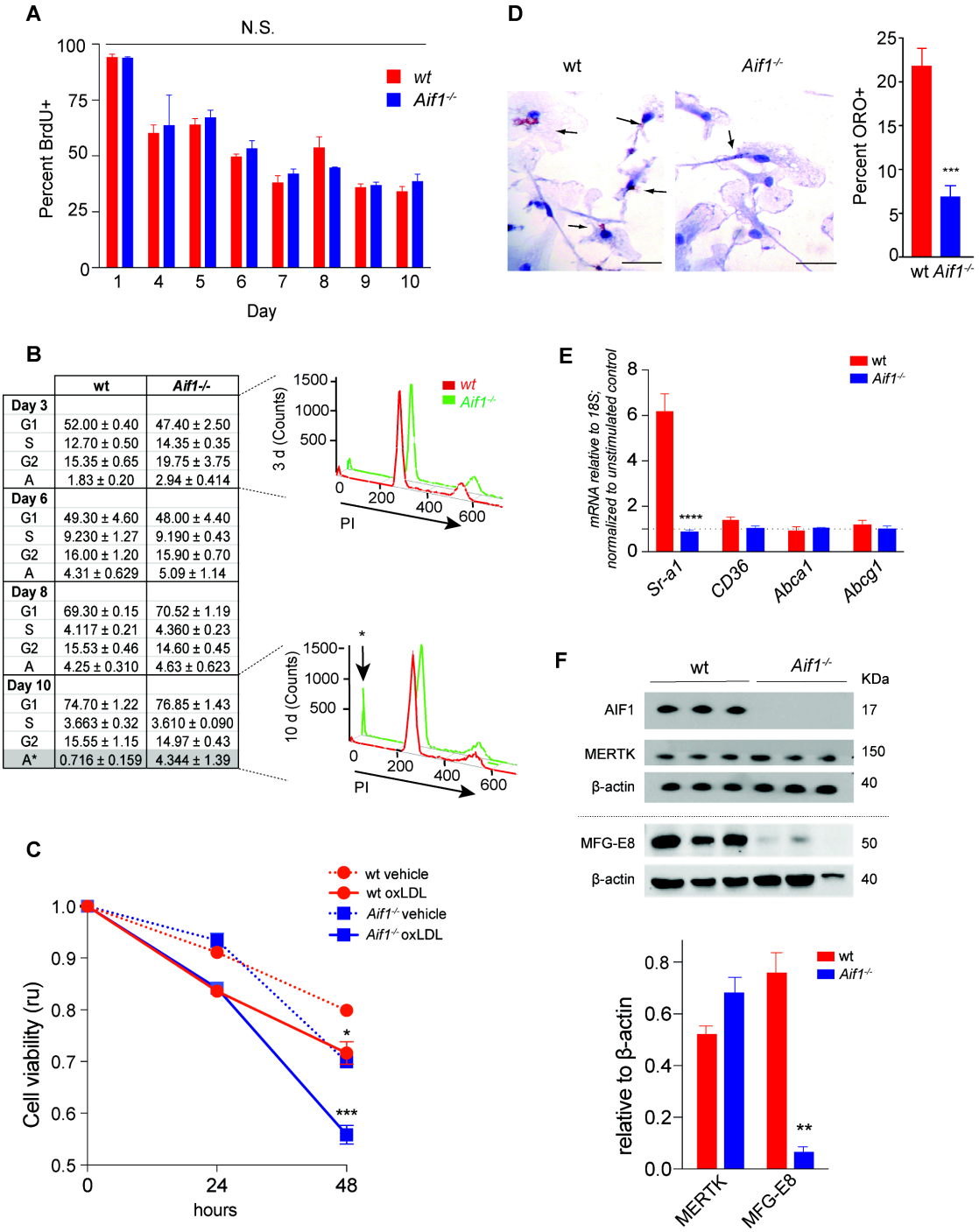


Supplementary Figure S1. A) No differences in $CD3^+$ T-cell content between plaques from *ApoE*$^{-/-}$ (n=3) and *Aif1*$^{-/-}$;*ApoE*$^{-/-}$ (n=3) mice; quantification on the left. B) Cholesterol and triglyceride levels are similar between *ApoE*$^{-/-}$ (n=11) and *Aif1*$^{-/-}$;*ApoE*$^{-/-}$ (n=11) mice placed on high fat diet for 18 weeks. Error bars indicate ± SEM.

Egaña-Gorroño L. et al., Supplementary Figure S2.

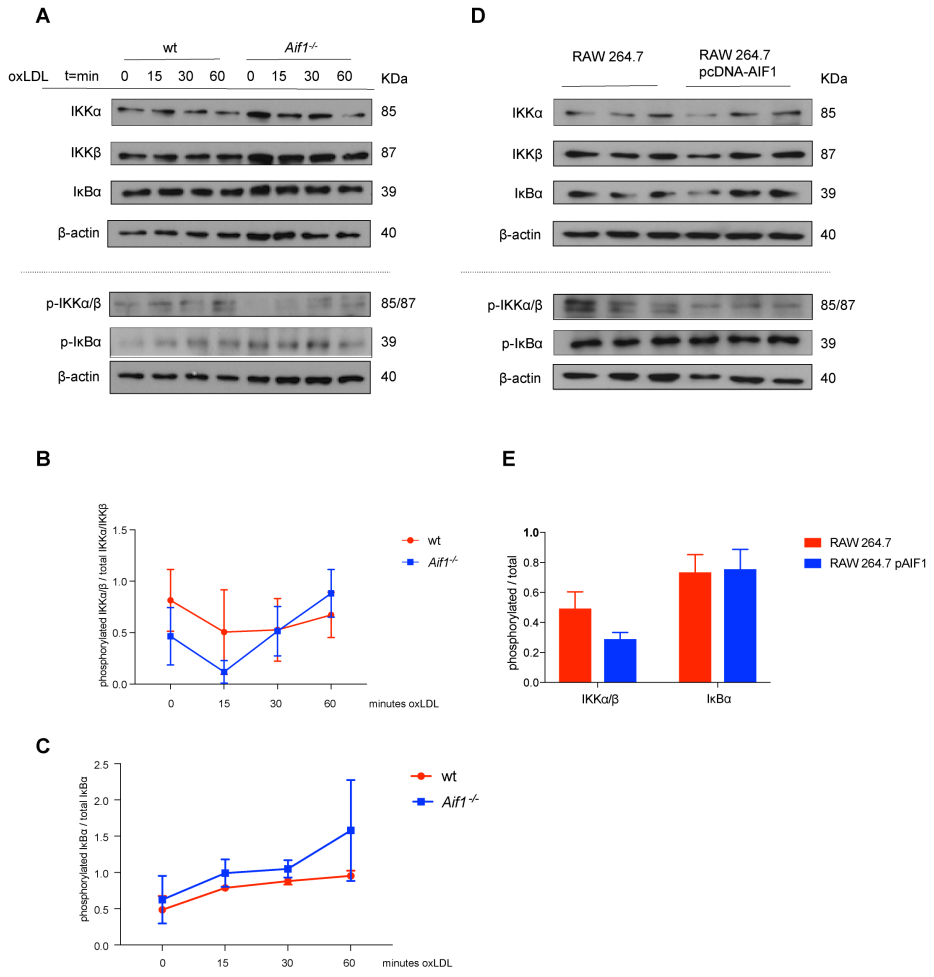


Supplementary Figure S2. Physiologic AIF1 levels were detected in BMDMs but not in MASMCs. AIF1 levels were measured in BMDMs and MASMCs using A) end-point PCR, B) real-time PCR and C) western blot. Error bars indicate ± SEM.

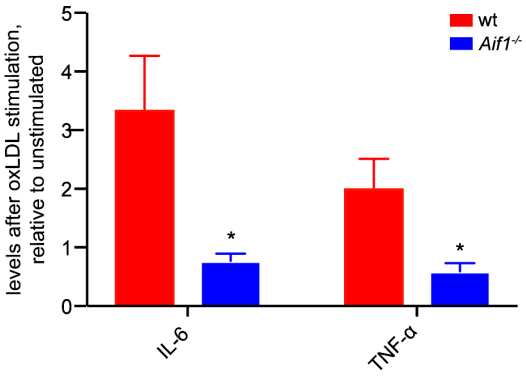


Supplementary Figure S3. AIF1 limits apoptosis, promotes survival and supports oxLDL uptake and efferocytosis in macrophages. (A) BrdU incorporation was assessed by FACS analysis in Wt and *Aif1*$^{-/-}$ BMDMs. (B) Propidium Iodide (PI) analysis of the cell cycle of BMDMs at days 3, 6, 8 and 10. Cells in the G1, S, G2 and A phases of the cell cycle are shown as a

percentage of total cells in each gate (±SD). PI analysis of cell cycle at days 3 and 10 is zoomed; arrowhead indicates apoptotic cells at day 10 (with <2N DNA). (C) Wt and *Aif1*$^{-/-}$ BMDMs were stimulated with oxLDL (300 μg/ml) for 24 and 48 hours and cell viability was assessed using AlamarBlue assay. (D) Left; bright field image showing BMDMs cultured with oxLDL (50 μg/ml) overnight and stained with ORO (bar, 20 μM). Right; BMDMs with at least five intracellular lipid granules were counted as ORO positive. Percentage of ORO positive BMDMs per total macrophages per field were quantified from at least 15 random fields. (E) Wt and *Aif1*$^{-/-}$ BMDMs were stimulated with oxLDL (50 μg/ml) for 12 hours and expression changes of lipid uptake (*Sr-a1*, *CD36*) and lipid efflux (*Abca1* and *Abcg1*) genes were measured by real time qPCR, relative to *18S*. Dashed grey line represents no changes after stimulation. (F) Lysates from wt and *Aif1*$^{-/-}$ BMDMs were immunoblotted for MERTK and MFG-E8 markers of efferocytosis and quantified relative to β-actin levels. Dashed grey line represents distinct gels. Error bars indicate ± SEM (N.S. non-significant, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$).



Supplementary Figure S4. AIF1 does not affect the levels of phosphorylated IKKα/β, and phosphorylated IκBα. (A) Wt and *Aif1*$^{-/-}$ BMDMs were stimulated with oxLDL (50 μg/ml) for 15, 30 and 60 minutes. Lysates were immunoblotted for total and phosphorylated IKKα, IKKβ and IκBα. (B) Quantification of phosphorylated IKKα/β protein levels, relative to total IKKα/IKKβ levels. (C) Quantification of phosphorylated IκBα protein levels, relative to total IκBα levels. (D) RAW 264.7 cells were transfected with 2 μg/ml of pcDNA3.1-AIF1 or control pcDNA3.1. 48 hours after transfection lysates were immunoblotted for total and phosphorylated IKKα, IKKβ and IκBα. (E) Quantification of phosphorylated IKKα/β and IκBα protein levels, relative to total IKKα/IKKβ and IκBα levels, respectively. Dashed grey line represents distinct gels. Error bars indicate ± SEM.



Supplementary Figure S5. AIF1 promotes pro-inflammatory cytokine production after oxLDL stimulation. Wt and *Aif1*$^{-/-}$ BMDMs were stimulated overnight with 50 µg/ml of oxLDL. IL-6 and TNF-α levels were determined after oxLDL stimulation in cell culture supernatants, relative to baseline. Error bars indicate ± SEM (* $p < 0.05$).

Supplementary Table S1

Position	Gene	Up/Down Regulation (comparing to unstimulated) wt BMMP 6 hours oxLDL vs. wt BMMP		Up/Down Regulation (comparing to unstimulated) Aif-/- BMMP 6 hours oxLDL vs. Aif1-/- BMMP	
		Fold Change	*p*-value	Fold Change	*p*-value
A01	Adm	1,2787	0,665825	-1,1045	0,60447
A02	Agt	-1,0154	0,956727	-1,2002	0,295387
A03	Akt1	1,4287	0,054574	-1,2483	0,364301
A04	Aldh3a2	1,2014	0,119041	-1,2311	0,282142
A05	Bcl2a1a	-1,161	0,038915	-1,1147	0,26693
A06	Bcl2l1	1,3177	0,017852	**-1,5192**	**0,025318**
A07	Birc2	1,1903	0,129071	-1,2397	0,275587
A08	Birc3	1,0314	0,490646	-1,0892	0,495549
A09	C3	1,2097	0,246245	-1,3724	0,217192
A10	C4a	**-2,0167**	0,300802	1,2426	0,998305
A11	Ccl12	**-1,597**	0,155011	**-2,114**	**0,017154**
A12	Ccl22	**2,0392**	0,412561	-1,4914	0,310159
B01	Ccl5	-1,0757	0,377704	-1,0377	0,42768
B02	Ccnd1	-1,3213	0,000835	**-3,4581**	**0,001979**
B03	Ccr5	1,2409	0,741537	**-1,9408**	0,138803
B04	Cd40	-1,2912	0,092076	-1,4241	0,251636
B05	Cd74	1,0314	0,679001	-1,0644	0,650905
B06	Cd80	1,2611	0,092006	1,0377	0,744013
B07	Cd83	1,0009	0,952161	**-1,5911**	**0,001391**
B08	Cdkn1a	1,4621	0,031154	1,1303	0,530253
B09	Cfb	1,2266	0,511959	-1,2628	0,550072
B10	Csf1	-1,0415	0,825125	-1,2628	0,398404
B11	Csf2	**2,1856**	0,070538	-1,1019	0,831685
B12	Csf2rb	1,2817	0,064332	-1,257	0,266444
C01	Csf3	**-1,8176**	0,255882	**2,1189**	0,170956
C02	Cxcl1	-1,0154	0,956727	-1,0163	0,973367
C03	Cxcl10	**-1,7475**	**0,030865**	**-3,249**	**0,008424**
C04	Cxcl3	1,0629	0,572313	1,4273	0,409928
C05	Cxcl9	-1,0154	0,956727	-1,0093	0,803167
C06	Egfr	1,1287	0,361254	1,3915	0,391606
C07	Egr2	1,0314	0,829691	-1,181	0,502372
C08	F3	1,058	0,815974	1,0425	0,860726
C09	F8	1,4621	0,316969	-1,3074	0,409326
C10	Fas	1,2669	0,045033	1,1892	0,349834
C11	Fasl	-1,3183	0,165145	**2,1238**	0,119904
C12	Gadd45b	**-1,5641**	**0,029601**	**-1,7092**	**0,033519**
D01	Icam1	1,2876	0,268238	**-1,5227**	0,133656
D02	Ifnb1	**-2,3598**	**0,020021**	-1,3195	0,755823
D03	Ifng	-1,0154	0,956727	-1,0093	0,803167
D04	Il12b	-1,0154	0,956727	-1,0093	0,803167
D05	Il15	1,0629	0,440404	-1,1567	0,303168
D06	Il1a	**5,3939**	**0,041189**	-1,0116	0,737412
D07	Il1b	**2,5222**	0,06027	**1,5655**	**0,028327**
D08	Il1r2	**1,9159**	0,346039	**2,2501**	0,095035
D09	Il1rn	1,3299	0,013437	-1,0521	0,714705
D10	Il2	-1,0154	0,956727	-1,0093	0,803167
D11	Il2ra	-1,0154	0,956727	-1,0093	0,803167
D12	Il4	-1,2558	0,931504	**2,4396**	0,195641
E01	Il6	**3,4614**	0,067785	**-3,0596**	0,127271
E02	Ins2	-1,0154	0,956727	-1,0093	0,803167
E03	Irf1	1,0386	0,703487	-1,4811	0,09248
E04	Lta	1,2069	0,341604	-1,014	0,6944
E05	Ltb	**2,2212**	0,110478	**2,2553**	0,169958
E06	Map2k6	**-1,5859**	**0,020742**	**-2,2501**	**0,002008**
E07	Mitf	1,2995	0,022084	-1,0943	0,4794
E08	Mmp9	1,1903	0,492971	-1,3597	0,092715
E09	Myc	1,2817	0,176786	1,192	0,266034
E10	Myd88	1,4025	0,070328	-1,4273	0,195098
E11	Ncoa3	-1,0658	0,472457	-1,4811	0,085786
E12	Nfkb1	1,1055	0,214216	-1,3074	0,210545
F01	Nfkb2	1,3768	0,110465	-1,0792	0,692771
F02	Nfkbia	-1,1214	0,391476	-1,3472	0,054173
F03	Nqo1	**2,4083**	**0,000074**	**1,9816**	**0,019207**
F04	Nr4a2	**2,2679**	**0,02167**	1,1567	0,468858
F05	Pdgfb	**1,6714**	0,738347	**-2,5374**	**0,023506**
F06	Plau	1,0056	0,926428	**-2,9485**	**0,048977**
F07	Ptgs2	**2,3972**	**0,012825**	1,4983	0,01197
F08	Rel	-1,0958	0,048679	-1,3819	0,032325
F09	Rela	**1,5706**	0,099503	-1,3629	0,299764
F10	Relb	**1,8678**	0,089362	-1,2483	0,346681
F11	Sele	1,452	0,353984	-1,0093	0,803167
F12	Selp	1,4553	0,467806	-1,0093	0,803167
G01	Snap25	-1,3648	0,180549	1,398	0,380579
G02	Sod2	-1,0107	0,921709	1,0595	0,669863
G03	Stat1	-1,0807	0,35352	**-1,617**	**0,026262**
G04	Stat3	1,3864	0,023655	-1,1329	0,637861
G05	Stat5b	1,1157	0,597123	-1,3287	0,25978
G06	Tnf	-1,1009	0,806463	**-1,6586**	0,088411
G07	Tnfrsf1b	**1,6111**	**0,008289**	-1,0943	0,732519
G08	Tnfsf10	1,0654	0,743247	**-2,3134**	0,22424
G09	Traf2	1,3864	0,06299	-1,1947	0,438716
G10	Trp53	1,058	0,431247	-1,1225	0,306121
G11	Vcam1	-1,0154	0,956727	-1,0093	0,803167
G12	Xiap	1,1339	0,526546	-1,1434	0,395783

Supplementary Table S1. Wt and *Aif1*$^{-/-}$ BMDM fold change expression of NF-κB signaling target genes after oxLDL stimulation (relative to unstimulated cells). Cells were stimulated for 6 hours with oxLDL (50 µg/ml). Gene expression changes were assessed using the NF-κB - signaling target gene array and calculated using the ΔΔCT method. Upregulated gene expression changes (fold change > 1.5) are depicted in red and downregulated gene expression changes (fold change < -1.5) are depicted in green. Statistically significant expression changes ($p < 0.05$) are depicted in blue. Genes are organized alphabetically.