SUPPLEMENTAL MATERIAL

Deletion or inhibition of NOD1 favours plaque stability and attenuates atherothrombosis in advanced atherogenesis

Silvia González-Ramos[1,2,*,$], PhD; Victoria Fernández-García[1,2,$], MSc; Miriam Recalde[1], MSc; Cristina Rodríguez[2,3], PhD; José Martínez-González[2,4], PhD; Vicente Andrés[2,5], PhD; Paloma Martín-Sanz[1,2], PhD; Lisardo Boscá[1,2,*], PhD

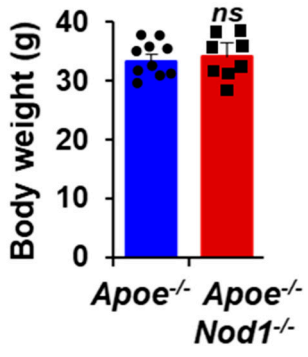
[1]*Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Madrid, Spain;*
[2]*Centro de Investigación Biomédica en Red en Enfermedades Cardiovasculares (CIBERCV), Madrid, Spain;*
[3]*Institut de Recerca del Hospital de la Santa Creu i Sant Pau-Programa ICCC, IIB Sant Pau, Barcelona, Spain;*
[4]*Instituto de Investigaciones Biomédicas de Barcelona (CSIC-IIBB), IIB Sant Pau, Barcelona, Spain;*
[5]*Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain.*

Running title: NOD1 deficiency promotes atheroma stability

Address correspondence to:
Lisardo Boscá *or* Silvia González-Ramos
Instituto de Investigaciones Biomédicas Alberto Sols
C/Arturo Duperier, 4
28029 Madrid
Phone +34-(0)91-497-2747 *or* -5345
Email lbsoca@iib.uam.es or sgramos@iib.uam.es

Supplementary Figures

a



b

mg/dL	*Apoe*$^{-/-}$	*Apoe*$^{-/-}$*Nod1*$^{-/-}$
Triglycerides	118.1 ± 2.3	112 ± 4.2
LDL	237.1 ± 4.6	244.6 ± 6.4
HDL	101.1 ± 4.5	103.6 ± 4.4
Total Cho.	372.1 ± 3.4	382.6 ± 8.1
Free Cho.	267.6 ± 6.7	279.9 ± 8.8

Figure S1. HFD-induced body weight increase and lipid profile changes in *Apoe*$^{-/-}$ mice are not mediated by *Nod1*. (a) Body weight in *Apoe*$^{-/-}$ (*n*=10) and *Apoe*$^{-/-}$*Nod1*$^{-/-}$ (*n*=8) mice after 16 weeks on a HFD. **(b)** Serum concentration of triglyceride (TGA), high-density lipoproteins (HDL), low-density lipoproteins (LDL), total cholesterol (TCHO) and free cholesterol (FCHO) in the same cohort of mice. Data is represented as mean ± s.e.m. of the indicated number (*n*) of repeats. ***ns***; Non-significant by Student's *t* test.

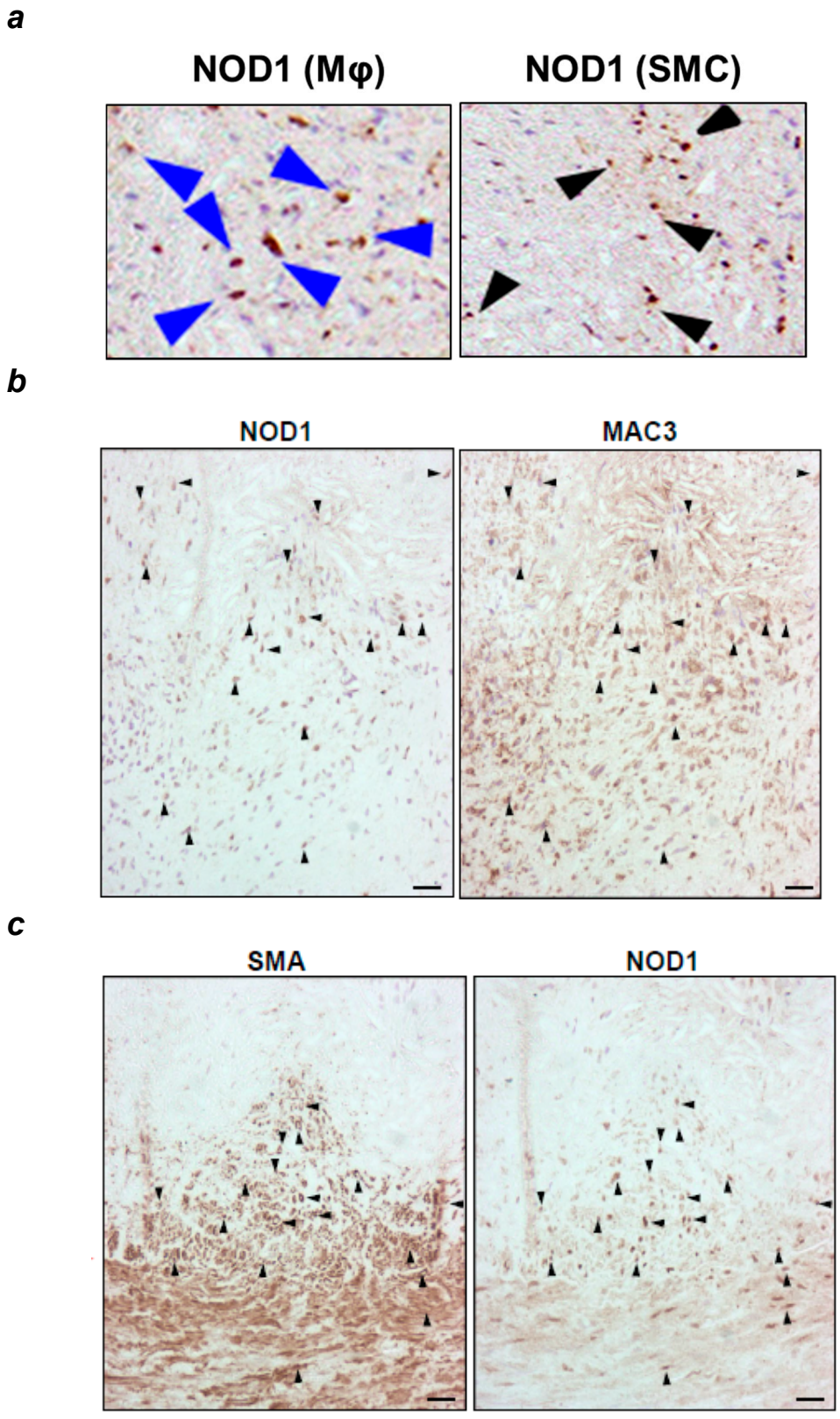


Figure S2. Inset of NOD1 from atherogenic human coronary arteries (from Fig. 1c) and NOD1 abundance in consecutive sections of human atherosclerotic coronary arteries presenting cholesterol crystals. (a) Amplification of atherogenic sections from Fig. 1c. **(b)** NOD1 co-localises with macrophage-rich areas (Mac3^{+}-cells) in the media and intima of atherosclerotic human arteries. **(c)** Representative images showing the immunohistochemical analysis of SMA and NOD1.Blue arrows point to NOD1^{+} cells in macrophages; black arrows point out NOD1^{+} cells in the smooth muscle cells.

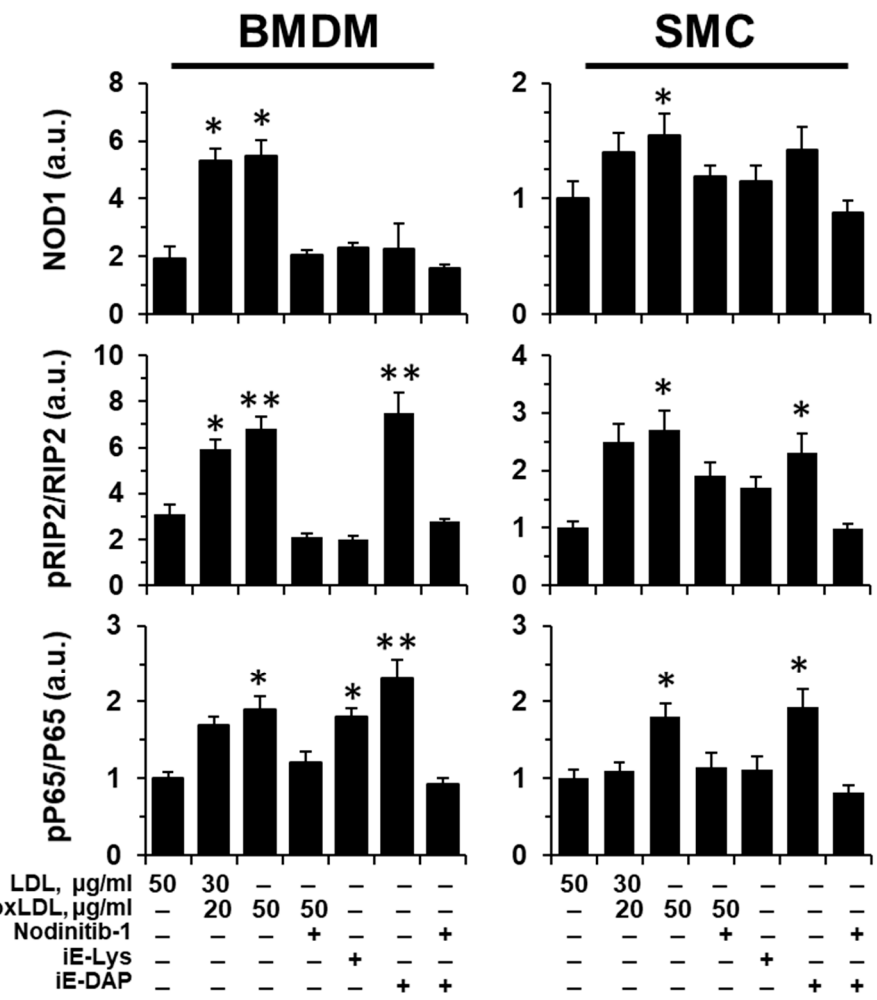


Figure S3. Quantification of the immunoblots shown in Fig. 1d. Analysis of NOD1, *p*RIP2/RIP2, pP65/P65 in *Wt* BMDM (n=6) and SMC (n=4) pre-treated with the NOD1 inhibitor Nodinitib-1 and/or stimulated with native LDL (as control for lipid load in the medium), oxLDL, c12-iE-DAP (an agonist for NOD1) and iE-Lys (an iE-DAP analogue that fails to activate NOD1) and c12-iE-DAP (an agonist for NOD1) for 24h or 48h, respectively. Protein levels were normalized to tubulin. Data are represented as mean ± s.e.m. of the indicated number (*n*) of independent experiments. **P* < 0.05, ***P* < 0.01 *vs.* the LDL condition by Student's *t* test. a.u., arbitrary units.

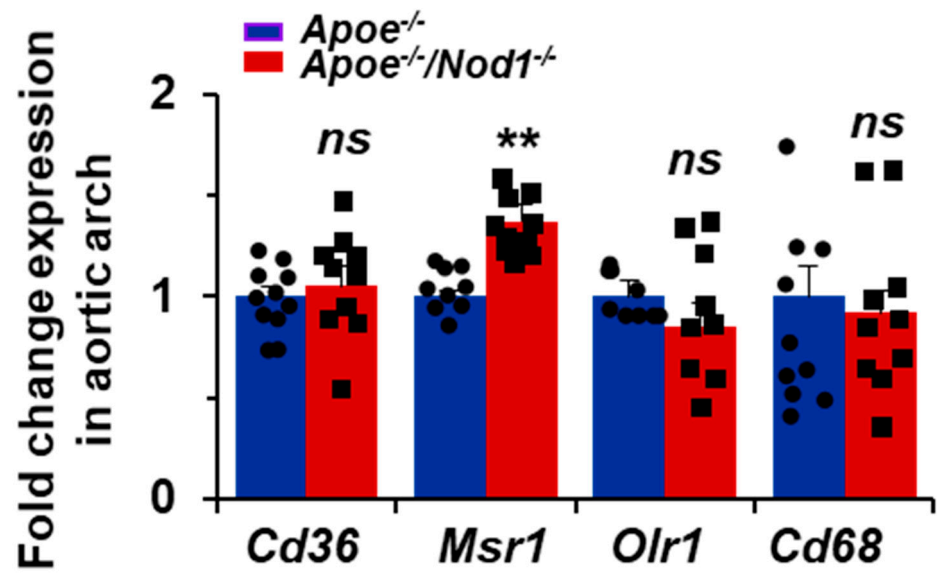


Figure S4. mRNA levels of scavenger receptors in athero-prone *Apoe*$^{-/-}$ mice in the presence and absence of *Nod1*. Quantitative PCR analysis of the scavenger receptors *Cd36,* mouse scavenger receptor 1 (*Msr1*), oxidised low-density receptor 1 (*Olr1)* and the low-density lipoprotein receptor *Cd68* expression in the aortic arch of *Apoe*$^{-/-}$ (*n*=10) and *Apoe*$^{-/-}$*Nod1*$^{-/-}$ (*n*=10) mice after 16 weeks on HFD. *m*RNA levels were normalised to *mRplp0* expression. Data are represented as mean ± s.e.m. of the indicated number (*n*) of repeats. ***P* < 0.01 *vs. Apoe*$^{-/-}$ by Student's *t* test. ***ns***; Non-significant by Student's *t* test.

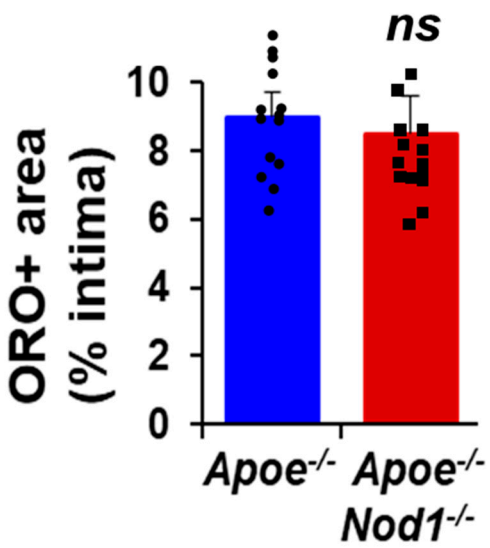


Figure S5. Nod1 does not affect lipid atherosclerotic area in *Apoe*$^{-/-}$ mice. Quantification of positive Oil Red O (ORO) lesion area in the semilunar valve cups of *Apoe*$^{-/-}$ (*n*=13) and *Apoe*$^{-/-}$ *Nod1*$^{-/-}$ (*n*=13) mice fed HFD for 16 weeks. Data are represented as mean ± s.e.m. of the indicated number (*n*) of repeats. Non-significant by Mann-Whitney U test. ***ns***; Non-significant by Student's *t* test.

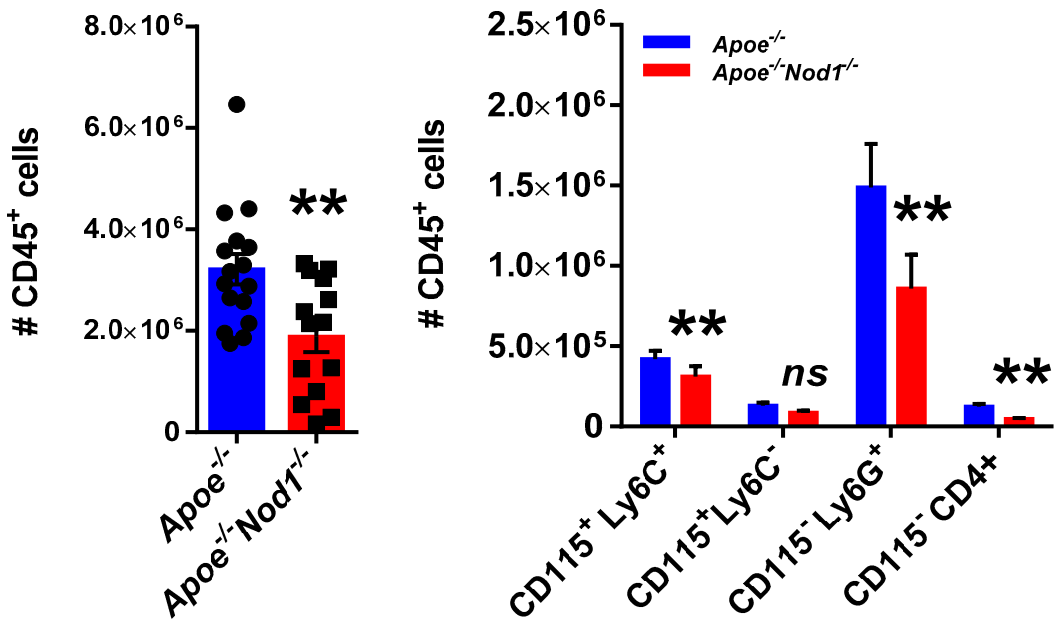


Figure S6. *Apoe*$^{-/-}$*Nod1*$^{-/-}$ mice present less leukocyte cell content in blood comparing to their *Apoe*$^{-/-}$ counterparts. Quantification of leukocyte, monocyte/macrophage, neutrophil and CD4+ lymphocyte cells in blood collected from *Apoe*$^{-/-}$ (*n*=16) and *Apoe*$^{-/-}$*Nod1*$^{-/-}$ (*n*=16) mice (16 weeks HFD-fed), performed by flow cytometry. Data are represented as mean ± s.e.m. of the indicated number (*n*) of repeats. *$P < 0.05$, **$P < 0.01$ *vs. Apoe*$^{-/-}$ by Student's *t* test. ***ns***; Non-significant by Student's *t* test.

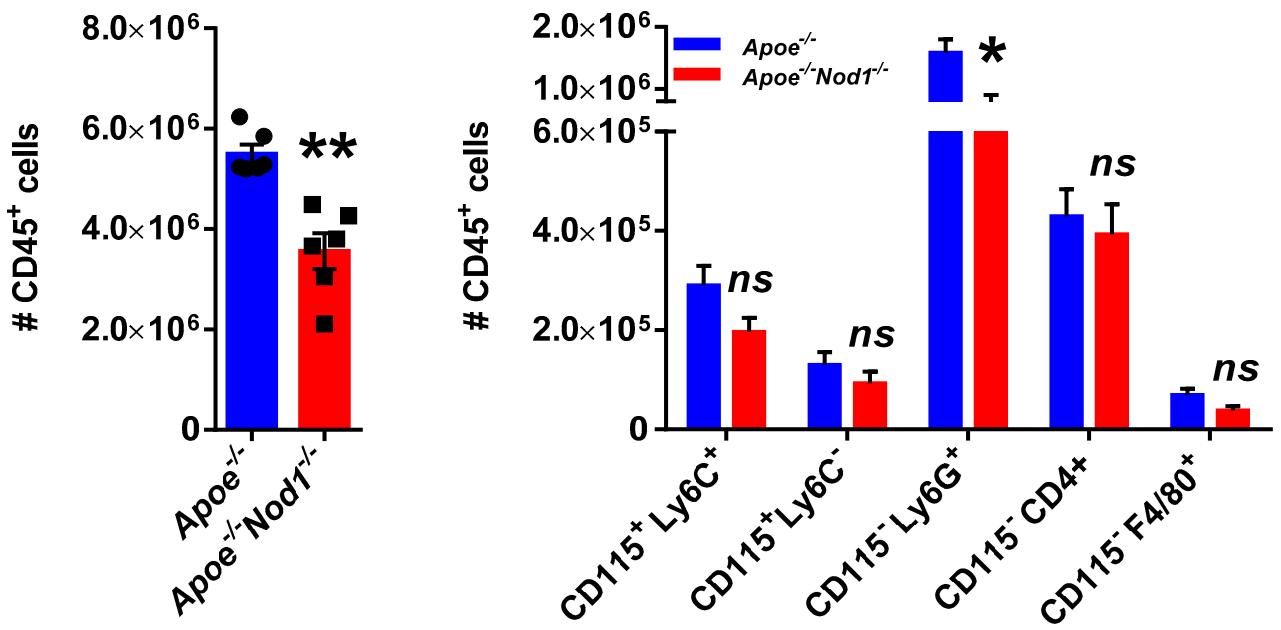


Figure S7. *Nod1* inactivation in mice results in a lower number of total CD45^{+} cells and neutrophils in spleen, while there are no significant changes in other white cells subsets. Leukocyte, monocyte/macrophage, neutrophil and CD4^{+} lymphocyte number of cells was assessed in the spleen obtained from *Apoe*$^{-/-}$ (*n*=6) and *Apoe*$^{-/-}$*Nod1*$^{-/-}$ (*n*=6) mice (16 weeks HFD-fed), using flow cytometry techniques. Data are represented as mean ± s.e.m. of the indicated number (*n*) of repeats. *$P < 0.05$, **$P < 0.01$ *vs. Apoe*$^{-/-}$ by Student's *t* test. ***ns***; Non-significant by Student's *t* test.

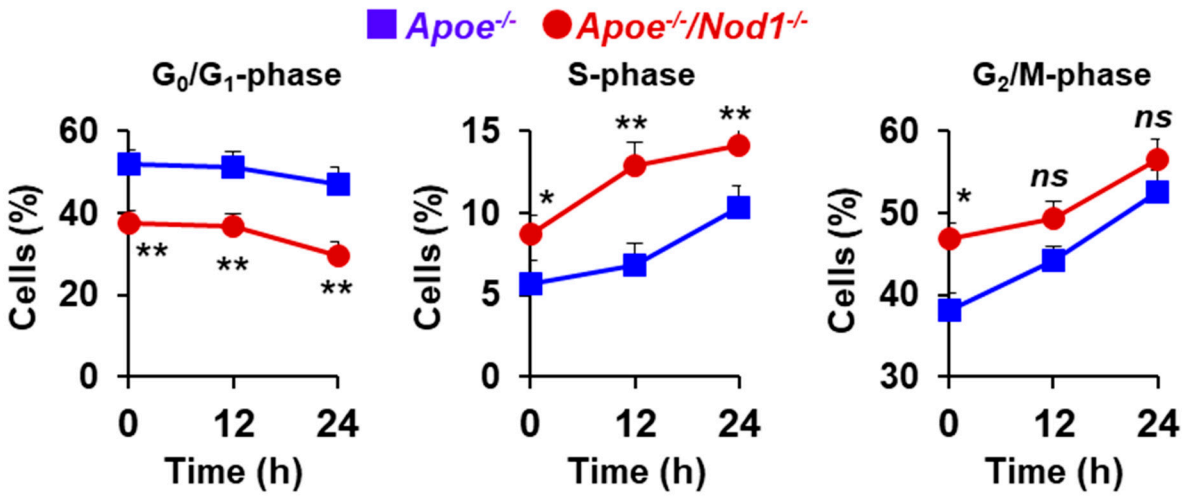


Figure S8. *Nod1* inactivation in cultured SMCs promotes faster cell cycle entry into S-phase. Cell cycle profile of primary vascular SMCs from *Apoe*$^{-/-}$ (*n*=10) and *Apoe*$^{-/-}$*Nod1*$^{-/-}$ (*n*=10) mice was assessed in a flow cytometer by propidium iodide staining. Cells were synchronised in G_0/G_1 phase by 72-h serum deprivation and restimulated for the indicated times. Results are average representative of three independent experiments. Data are represented as mean ± s.e.m. of the indicated number (*n*) of repeats. **P* < 0.05, ***P* < 0.01 *vs. Apoe*$^{-/-}$ by Student's *t* test. ***ns***; Non-significant by Student's *t* test.

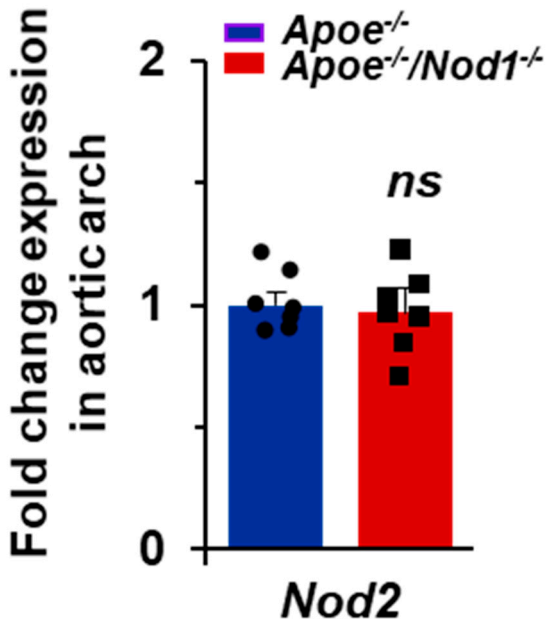


Figure S9. mRNA levels of Nod2 in the aortic arch of athero-prone *Apoe*$^{-/-}$ mice in the presence and absence of *Nod1*. Quantitative PCR analysis of *Nod2* expression in the aortic arch of *Apoe*$^{-/-}$ (*n*=7) and *Apoe*$^{-/-}$*Nod1*$^{-/-}$ (*n*=7) mice after 16 weeks on HFD. *m*RNA levels were normalised to *mRplp0* expression. Data are represented as mean ± s.e.m. of the indicated number (*n*) of repeats. ***ns***; Non-significant by Student's *t* test.