

DATA SUPPLEMENT

The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting macrophage emigration from plaques

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SUPPLEMENTARY TABLES

Supplementary Table 1. Metabolic parameters of *WT* → *Ldlr*^{-/-} and *Ntn1*^{-/-} → *Ldlr*^{-/-} chimeric mice fed a Western diet for 12 weeks.

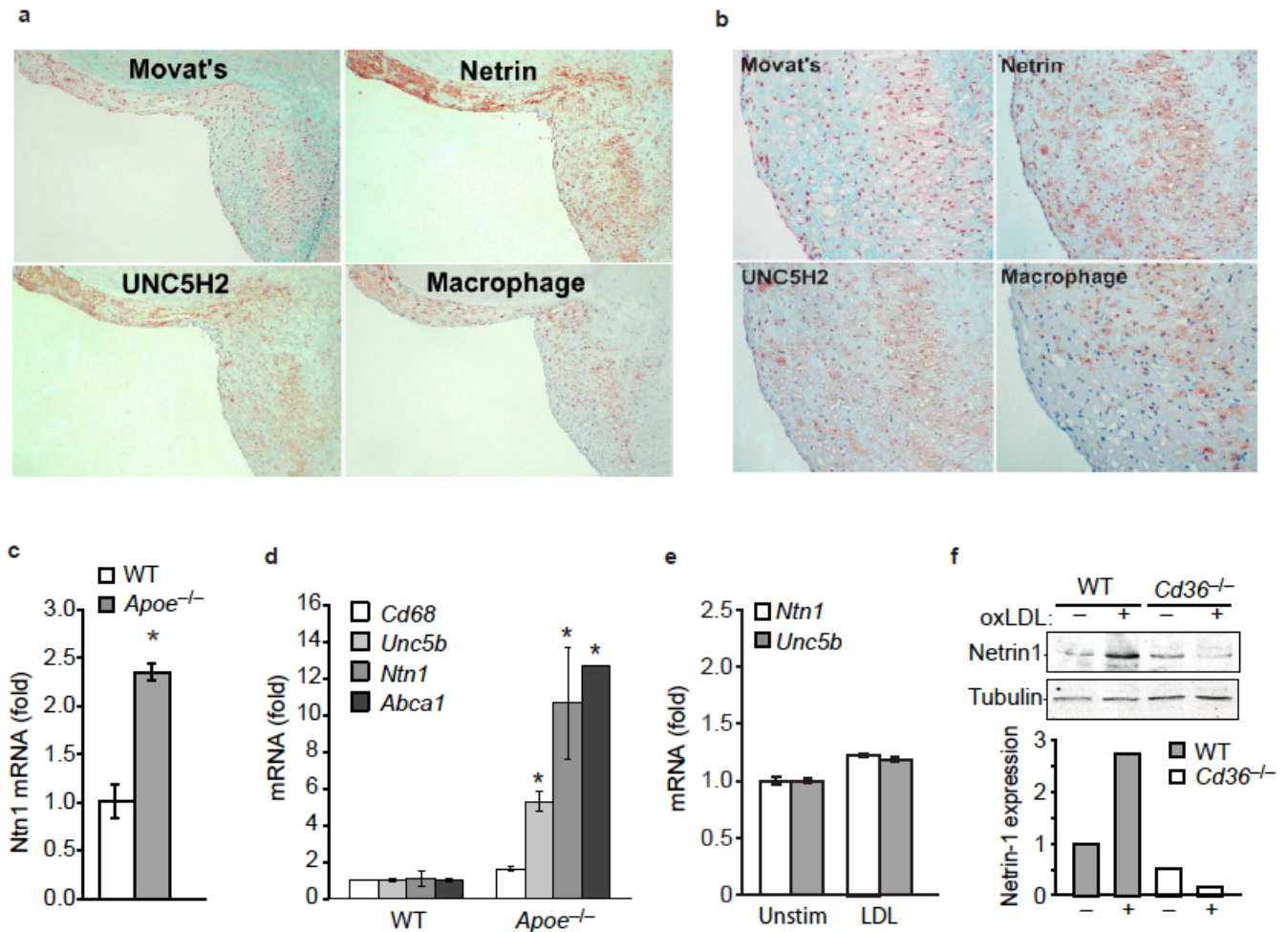
	<i>WT</i> → <i>Ldlr</i> ^{-/-}		<i>Ntn1</i> ^{-/-} → <i>Ldlr</i> ^{-/-}	
Weight (g)	20.7 (±0.90)	n=9	20.2 (±1.50)	n=14
Total cholesterol (mg/dl)	716 (±195)	n=10	738 (±138)	n=14
Triglycerides (mg/dl)	869 (±365)	n=9	936 (±588)	n=12
Reconstitution (%)*	99.7 (±0.20)	n=10	97.9 (±3.50)	n=10

* Analyzed by *Ldlr* WT and KO gene expression in blood leukocytes.

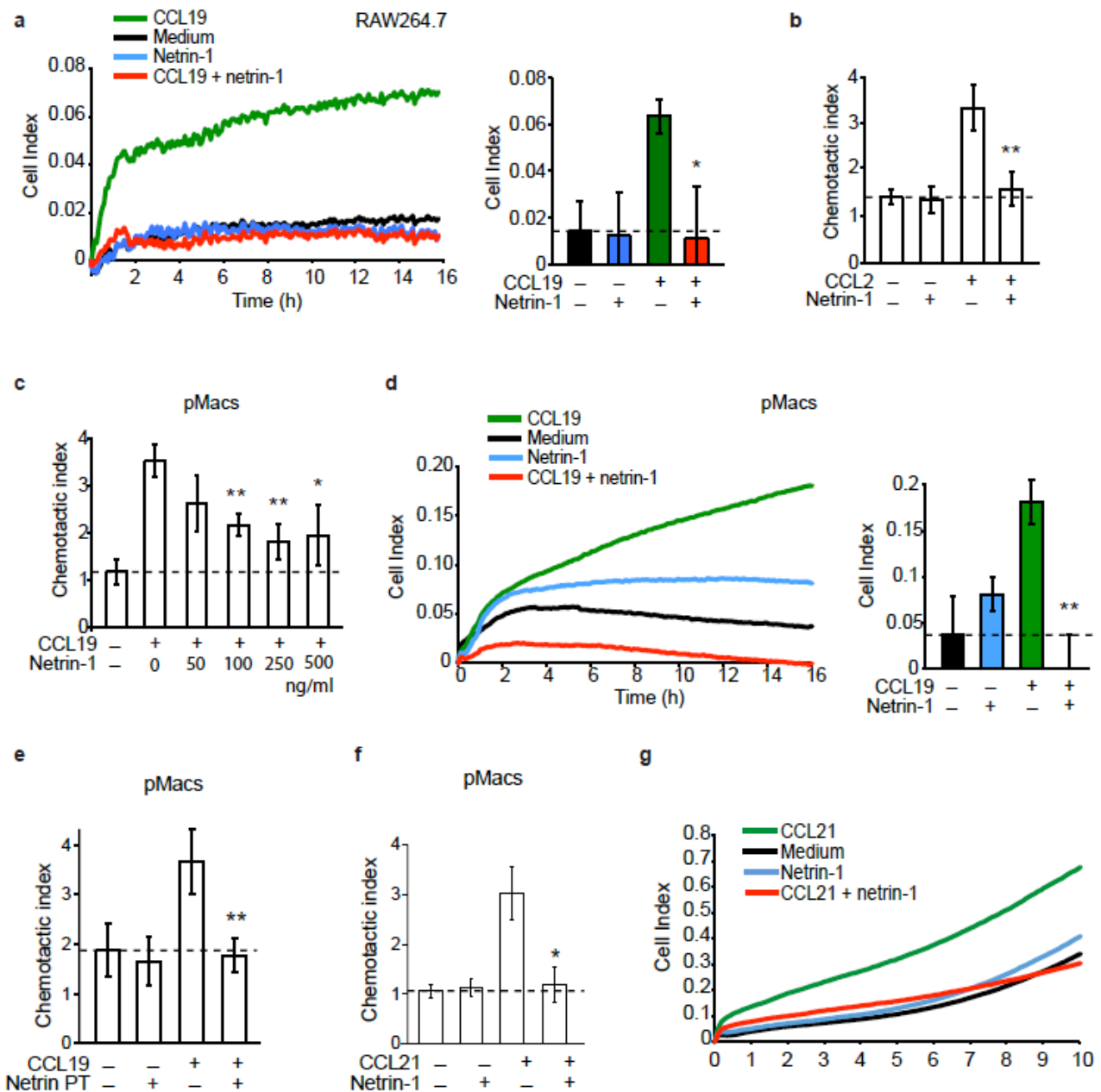
Supplementary Table 2. PCR Primer sequences.

	Forward (5' – 3')	Reverse (3' – 5')
Human ACTB	TGCTATCCCTGTACGCCTCT	CCATCTCTTGCTCGAAGTCC
Human DCC	CGACCGAGGAGTTCCAGTGATCAAG	GGTTCTTCTGCCAGTGGATTGTTGG
Human NEOGENIN	ACCCAGCCTGTGATTAGTG	TGTGATGGTTCAGAGCTTGC
Human UNC5b	CAGCCTTAAGGTCAAGGTCTACAGCTC	GTGACTGGATCTTTCAGCTCAAGACC
Mouse <i>Abca1</i>	GGTTTGGAGATGGTTATACAATAGTTGT	CCCGGAAACGCAAGTCC
Mouse <i>Cd3</i>	ATCGCCTGGAACACTTTCTGG	GCACGTCAACTCTACACTGGT
Mouse <i>Cd11c</i>	GCAGGAGTGTCCAAAGCAAGA	CGTGTGCTAGGTCTCTGAAGC
Mouse <i>Cd68</i>	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
Mouse <i>Gapdh</i>	TGTGAGGGAGATGCTCAGTG	TGTTCTACCCCAATGTGT
Mouse <i>Ldlr</i> (KO)	CCATATGCATCCCCAGTCTT	AATCCATCTTGTTCAATGGCCGATC
Mouse <i>Ldlr</i> (WT)	CCATATGCATCCCCAGTCTT	GCGATGGATACACTCACTGC
Mouse <i>Ntn1</i>	GCGGGTTATTGAGGTCCGGTG	CAGCCTGATCCTTGCTCGG
Mouse <i>Elastase</i>	TTGCCAGGAATTCGTCATGT	TGTGAGGGAGATGCTCAGTG
Mouse <i>Unc5b</i>	TGGATCTTTCAGCTCAAGACCCAG	AAGATGGCCAGCTGGAGCCG
Mouse <i>Ntn1</i> (Taqman)	GCAAGCCCTTCCACTACGAC	CGCGAGCTCCATGTTGAATCTGC
Mouse <i>Ntn1</i> Taqman probe	GAGGCCAACGAGTGCG	

SUPPLEMENTARY FIGURES

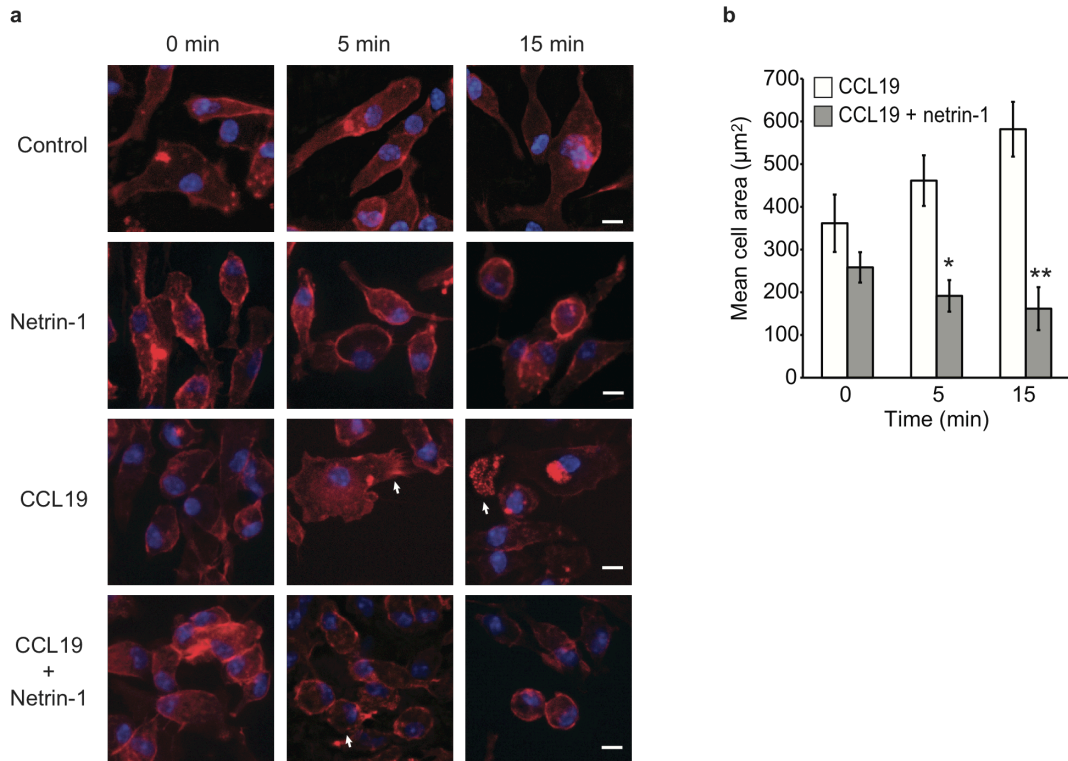


Supplementary Figure 1. Netrin-1 and its receptor UNC5b are expressed in human and *Apoe*^{-/-} mouse atherosclerotic plaques. (a-b) Immunohistochemical staining for netrin-1, UNC5H2 (UNC5b) and macrophages (HAM56) in serial sections of a human atherosclerotic plaque from the left anterior descending coronary artery, also stained with Movat's pentachrome stain to show histological features of the plaque. Staining is representative of plaques from 3 individuals (original Mag (a) x20, (b) x 200). (c) qPCR analysis of *Ntn1* mRNA isolated from the aortic arch of C57BL/6 or *Apoe*^{-/-} mice fed a chow diet for 6 months. (d) qPCR analysis of *Ntn1* and *Unc5b* mRNA in peritoneal macrophages isolated from C57BL/6 or *Apoe*^{-/-} mice fed chow for 6 months. Expression of the macrophage marker *Cd68* and the cholesterol responsive gene *Abca1* were measured as controls. (e) qPCR analysis of *Ntn1* and *Unc5b* mRNA in peritoneal macrophages treated with 50 µg/ml LDL (6 h). (f) Immunoblot of netrin-1 protein in WT or *Cd36*^{-/-} macrophages stimulated with oxLDL (50 µg/ml, 48 h). Data are mean ± s.d. of triplicate samples in a single experiment and are representative of 3 independent experiments. *P<0.05.

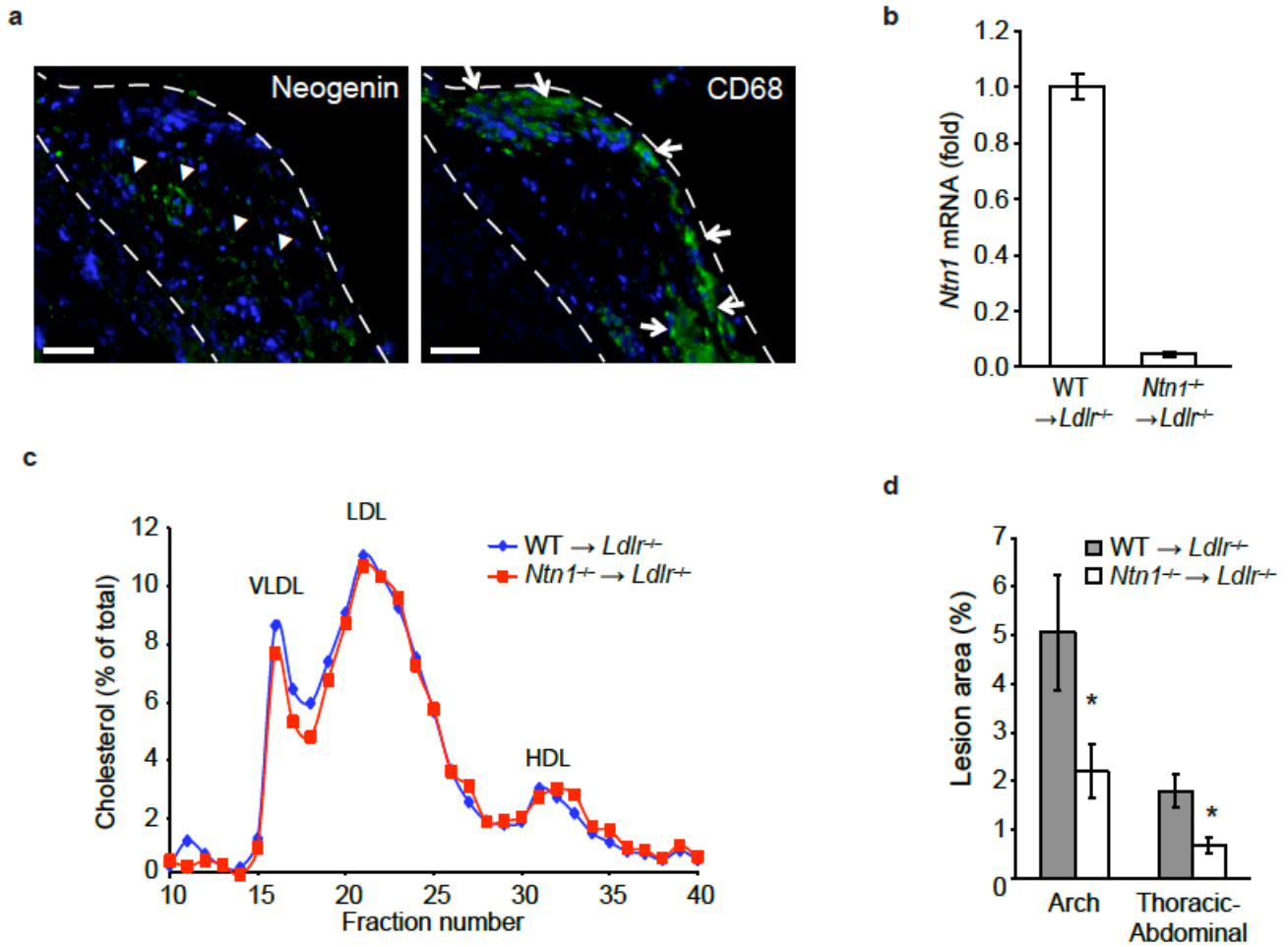


Supplementary Figure 2. Netrin-1 inhibits macrophage migration to CCL2 and CCL19.

(a) Measurement of RAW264.7 cell migration to 250 ng/ml netrin-1, 500 ng/ml CCL19, or both, using the Real-time Cell Invasion and Migration xCelligence system. Graph at right shows the mean cell index \pm s.d. of triplicate samples at 16 h. (b) Migration of RAW264.7 cells to 250 ng/ml netrin-1, 100 ng/ml CCL2, or both as measured by Boyden chamber assay. (c) Migration of pM ϕ to CCL19 (500 ng/ml) in the presence of increasing concentrations of recombinant netrin-1. (d) Real time measurement of pM ϕ migration to 250 ng/ml netrin-1, 500 ng/ml CCL19, or both. Graph at right shows the mean cell index \pm s.d. of triplicate samples at the 16 h endpoint. (e) Migration of pM ϕ pretreated (PT) with 250 ng/ml netrin-1 for 1 hour and washed 3x prior to exposure to CCL19 (500 ng/ml). (f) Migration of mouse peritoneal macrophages to 250 ng/ml netrin-1, 500 ng/ml CCL21, or both as measured by Boyden chamber assay. (g) Real time measurement of macrophage migration to 250 ng/ml netrin-1, 500 ng/ml CCL21, or both. (a-f) Data are mean \pm s.d. of triplicate samples in a single experiment and are representative of ≥ 3 independent experiments. *P<0.05, **P<0.01.



Supplementary Figure 3. Mouse peritoneal macrophages were incubated with 500 ng/ml CCL19 with/without 250 ng/ml netrin-1 pretreatment. After the indicated times the cells were fixed, permeabilized and stained with phalloidin to detect polymerized actin. **(a)** Cell morphology was analyzed with fluorescence microscopy and arrows indicate membrane ruffles. Scale bar, 10 µm. **(b)** mean surface area of cells in (a). Data are the mean of 5 high power fields ± s.d. *P<0.05.



Supplementary Figure 4. (a) Immunofluorescent staining of neogenin (left, green) or CD68 (right, green) and DAPI (blue) in serial sections of atherosclerotic plaques of *Ldlr*^{-/-} mice fed a Western diet. Arrowheads indicate neogenin staining in lesion areas distinct from CD68⁺ regions, indicated by arrows. Scale bar, 50 μ m. (b) qPCR analysis of *Ntn1* mRNA in M ϕ of *WT* \rightarrow *Ldlr*^{-/-} and *Ntn1*^{+/-} \rightarrow *Ldlr*^{-/-} chimeric mice. (c) FPLC fractionation of pooled plasma from *WT* \rightarrow *Ldlr*^{-/-} and *Ntn1*^{+/-} \rightarrow *Ldlr*^{-/-} chimeric mice (n=3/group) fed a Western diet for 12 weeks. (d) Regional analysis of lesion distribution in the en face aorta of *WT* \rightarrow *Ldlr*^{-/-} and *Ntn1*^{+/-} \rightarrow *Ldlr*^{-/-} chimeric mice fed a Western diet for 12 weeks (n=10/group). Data are expressed as mean % lesion area \pm s.e.m. *P<0.05.