Materials and Methods

Reagents

OxLDL was prepared by as we described by first dializing LDL (0.25 mg/ml) in PBS for 24 hours at 4ºC and then incubating with 5μM $CuSO_4$ for 6 hours at 37ºC[1]. To stop the reaction, 0.2 μM EDTA and 50 μM BHT were added. The extent of oxidation of oxLDL was determined by measuring thiobarbituric acid-reactive substances (TBARS) according to the manufacturer's instructions (CellBiolabs) and was between 15-20 nmol/mg. Cobalt chloride ($CoCl_2$) and dimethyloxalylglycine (DMOG) were from Sigma Aldrich (15862 and D3695, respectively). The NFκB inhibitor (Bay11-7082) and HIF-1α inhibitor (400083) were from Calbiochem.

Cell culture

L929 conditioned medium was used as a source of macrophage colony stimulating factor for the differentiation of primary bone marrow derived macrophages (BMDM). Briefly, L929 cells were grown to confluence in DMEM supplemented with 10% FBS, media was changed to DMEM supplemented with 2% FBS and the conditioned media was harvested 3 days later and filtered through a 0.2 μm filter. Bone marrow was flushed from the tibias and femurs of 6-8 week old C57BL/6 mice as we described[2], and cultured in DMEM supplemented with 15% L929-conditioned media, 10% FBS and 1% penicillin/streptomycin for 7 days. BMDM or human THP-1 monocytes were treated with 50 μg/ml oxLDL or subjected to hypoxic conditions in a Billups-Rothenberg modular incubator chamber at 1% O_2, 5% CO_2 and 94% N_2 for 24 hours at 37°C. J774 control (J774con) and HIF-1a overexpressing (J774Hif) macrophages[2] were cultured in 5.5 mmol/L glucose containing DMEM supplemented with 15% FBS and 1% penicillin/streptomycin.

Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) and RNA (0.5–1 μg) was reverse transcribed with an iScript cDNA Synthesis kit according to the manufacturer's instructions (Bio-Rad). RT-PCR analysis was performed using iQ SYBR green Supermix (Biorad) and a Mastercycler Realplex (Eppendorf) as we described[3]. The change in mRNA expression was calculated by the comparative change-in-cycle-method (ΔΔCT) relative to GAPDH mRNA levels. The following primer sets were used;
Mouse *Ntn1* 5'CAGCCTGATCCTTGCTCGG3',3'GCGGGTTATTGAGGTCGGTG5',
Mouse *Unc5b* 5'CTGGGGACCGGGAAAGAAC3', 3'CTGATGGGTAGGAGTCTGGG5',
Mouse *Vegf* 5'CTCCGCTCTGAACAAGGCT3', 3'GCACATAGAGAGAATGAGCTTCC5'
GAPDH 5'AGGTCGGTGTGAACGGATTTG3',
3'TGTAGACCATGTAGTTGAGGTCA5'
Human *NTN1* 5'CTCACACTGTCCCTCGGCAAGAAGT3',
3'CTCCGAGTCGTCCTCGTTCTCGTC5', *UNC5B*
5'CAGCCTTAAGGTCAAGGTCTACAGCTC3', 3'GTGACTGGATCTTTCAGCTCAAGACC5'.
RT2 Custom Profiler PCR Arrays (Qiagen) were obtained for neuronal guidance molecules and their receptors (Supplemental Table 1). 1 μg of total RNA was reverse transcribed and quantitative RT-PCR analysis was performed according to manufacturer's protocol. Data analysis was performed using the manufacturer's integrated web-based software package of the PCR Array System using ΔΔCt based fold-change calculations.

Western Blotting: Protein was extracted in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40) and 60 μg of protein were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, blocked in TBST-5% milk and blotted for Netrin-1 (R&D Systems Inc) (1:500 dilution), Unc5b (Millipore) (1:1000 dilution), GAPDH (1:10000 dilution). Horseradish peroxidase conjugated secondary antibodies followed by SuperSignal West Pico Chemiluminescent Substrate (Pierce 34080) were used and the signals were detected by using LI-COR Odyssey Fc Imaging system.

Immunoflurescent staining

J774con or J774Hif were plated on coverslips and fixed in 4% paraformaldehyde and permeabilised with 0.1% TritonX100 in PBS. Non-specific binding was blocked with 5% BSA for 30 minutes and then coverslips were incubated with anti-HIF-1alpha (Novus Biologicla, NB100-479) and anti-netrin-1 chicken polyclonal (Abcam, ab39370), or mouse monoclonal anti-Unc5b antibody (Abcam, ab54430). Secondary fluorescent antibodies used were AlexaFluor 568-anti-rabbit, Alexafluor 488-anti-chicken antibody, or Alexafluor 488-anti-mouse antibodies (Molecular Probes, A11011, A11039, A11008, respectively). Experimental negative controls were incubated in PBS with isotype matched control antibodies (Santa Cruz Biotechnology). Cells were visualized under x40 using a Nikon Eclipse microscope

Mouse Atherosclerosis

C57BL/6 and *Ldlr*$^{-/-}$ mice were from Charles River Laboratories and maintained in a pathogen-free facility. Experimental procedures were done in accordance with the USDA Animal Welfare Act and the PHS Policy for the Human Care and Use of Laboratory Animals and New York University School of Medicine's Subcommittees on Research Animal Care and Use. Eight-week old *Ldlr*$^{-/-}$ mice from Jackson Laboratories were fed a Western diet (WD; 21% [wt/wt] fat, 0.3% cholesterol; Research Dyet) for 12 weeks. Mice were injected prior to sacrifice with a hypoxia probe (hpi, Hypoxiaprobe, Inc) at a dose of 60mg/kg *i.p.* Mice were anesthetized with tribomoethanol (0.4 mg/g *i.p.*) and ex-sanguinated by cardiac puncture. Aortas were flushed with PBS and perfused with 10% sucrose. Aortic roots were embedded in OCT medium, snap frozen and cross-sections (7 μm thick) were taken for analysis. For immunofluorescent staining, sections were fixed with ice cold acetone for 10 minutes and non-specific binding sites were blocked with 5% BSA for 30 minutes at room temperature. Thereafter, either anti-pimonidazole antibody (clone 4.3.11.3, MAb1) or anti-netrin-1 antibody (Calbiochem, ab-2), or anti-Unc5b antibody (Abcam, ab54430) or anti-CD68 (Lifespan biosciences, LS-C33253) were applied to the sections. After PBS washes, sections were incubated with anti-mouse biotin followed by FITC-conjugated streptavidin to detect the hypoxia probe. The segments were stained with DAPI to detect nuclei and mounted with Dako fluorescent mounting medium (Dako, S3023). The slides were visualized under Nikon Eclipse microscope and images captured under x20 objective. For mice with conditional deletion of HIF1a, lethally irradiated 8 week old *Ldlr*$^{-/-}$ mice were reconstituted with bone marrow (3×10^6 cells) from *Hif1a*$^{flox/flox}$*LysMcre/-* (Mac-*Hif1a*$^{-/-}$) and *Hif1a*$^{flox/flox}$ (WT) donor mice. After four weeks recovery, mice were fed a Western diet for 20 weeks and injected with a hypoxiaprobe-1 (pimonidazole HCl, hpi-Hypoxiaprobe, Inc; 1.5 mg/25g mouse) *i.v.* prior to sacrifice. Laser capture microdissection (Arcturus Bioscience, Mountain View, CA) was carried out on 6-μm frozen sections that were dehydrated in ethanol and xylene and air-dried. At 100-μm intervals, sections were immunostained with a FITC-Hypoxyprobe-1 antibody (Hypoxyprobe-1 plus kit, hpi-Hypoxiaprobe, Inc) and CD68 primary antibody (ABD Serotec, Raleigh, NC) to identify hypoxic macrophages and normoxic macrophages. These guide sections were used as templates for the isolation of hypoxic and nomoxic macrophages for the next five serial sections. RNA was isolated using the PicoPure RNA Isolation Kit (Life technologies, Grand Island, NY) and the concentration and quality of RNA was determined by Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA). RNA was converted to cDNA and amplified using the WT-Ovation Pico RNA Amplification kit (NuGEN, San Carlos, CA) and qRT-PCR was carried out as described above.

Human atherosclerosis

The use of human endarterectomy carotid tissue was approved by the New York University School of Medicine IRB. For immunofluorescent staining, sections from 5 subjects were fixed with ice-cold acetone for 10 minutes and permeabilized for 15 minutes with 0.2% Triton X-100 in PBS. Non-specific binding sites were blocked with 5% BSA for 30 minutes at room temperature. Thereafter, either goat anti-netrin-1 antibody (R&D Systems, AF 1109), or mouse monoclonal anti-Unc5b antibody (Abcam, ab54430) or rabbit polyclonal anti-HIF1-a (Novus Biologicals, NB100-479) were applied to the sections. After PBS washes, Alexafluor 568 or 488-coupled secondary antibodies (Molecular Probes, A11057, A11034, A11004), were applied for one hour

each at room temperature. The segments were stained with DAPI to detect nuclei and mounted with Dako fluorescent mounting medium (Dako, S3023). The slides were visualized under Nikon Eclipse microscope and images captured under x20 objective.

Promoter luciferase assays
Human NTN1 or UNC5B promoter-luciferase reporter plasmids (Switchgear Genomics) were transfected into HEK293T using lipofectamineTM 2000 (11668-019, Invitrogen). After transfection, media was changed and cells were treated with oxLDL (50 μg/ml) or $CoCl_2$ (0.1 mM), or DMOG (1 mmol/l) for 24 hours, in the presence or absence (PBS control) of inhibitors of NFκB (10 μmol/L Bay11-7082) or HIF1a (100 μmol/L HIF-1α inhibitor). Luciferase activity was measured using the DUAL-Glo Luciferase Assay system (E1910, Promega) and normalized to a constitutively expressed renilla reporter or to protein concentration.

Migration assays
Macrophage chemotaxis was measured using the Real-Time Cell Invasion and Migration xCelligence Assay System with monitoring every 5 min (Roche Applied Science). BMDM migration towards MCP-1 (100 ng/ml; R&D Systems) was measured in the presence or absence of oxLDL (50μg/ml) and/or recombinant rat UNC5b-Fc chimera (1006-UN; R&D Systems) to block the effects of netrin-1. In a subset of experiments, cells were pre-treated with 0.1 mmol/L $CoCl_2$ for 1 h prior to migration. J774 macrophage migration was carried out in the presence or absence of UNC5b-Fc chimera. Results of chemotaxis assays are representative of at least three independent experiments performed on triplicate samples.

Apoptotic assays
To induce apoptosis, BMDMs were grown in the absence of L929 conditioned media (CM) for 3 days under normoxic or hypoxic conditions. In some assays, cells were treated with 100 μmol/L HIF-1α inhibitor or Unc5b-FC fragment or control IgG. After treatment, apoptotic cells were labeled with PE-active caspase-3 (557091, BD pharmingen) or by the TUNEL method using an *in situ* cell detection kit (Roche Diagnostics). Active Caspase-3, TUNEL and DAPI staining were analyzed with a Nikon Eclipse fluorescent microscope. Only TUNEL positive cells that colocalized with DAPI stained nuclei were considered apoptotic.

Statistical analysis
The difference between two groups was analyzed by two-tailed Student's *t*-test or for multiple comparisons, by one-way analysis of variance. A P value of <0.05 was considered significant.

References:

1. Kunjathoor, V.V.*, et al.* Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem* **277**, 49982-49988 (2002).
2. Parathath, S.*, et al.* Hypoxia is present in murine atherosclerotic plaques and has multiple adverse effects on macrophage lipid metabolism. *Circ Res* **109**, 1141-1152 (2011).
3. van Gils, J.M.*, et al.* The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques. *Nat Immunol* **13**, 136-143 (2012).