Materials and Methods

Animals
C57BL/6J mice, *Ldlr*$^{-/-}$ mice, *Hif1a*$^{flox/flox}$ mice, *Cd11c*-cre mice, and OT-II mice (all C57BL/6J background) were obtained from the Jackson laboratory or Charles River. *Cd11c*-cre mice, *Hif1a*$^{flox/flox}$ mice and *Ldlr*$^{-/-}$ mice were crossed to generate littermate *Cd11c*-cre^{+}*Hif1a*$^{flox/flox}$ (*Hif1a*-CKO) and *Cd11c*-cre^{+}*Hif1a*$^{+/+}$ (*Hif1a*-WT) mice, as well as *Cd11c*-cre^{+}*Hif1a*$^{flox/flox}$ *Ldlr*$^{-/-}$ (*Hif1a*-CKO *Ldlr*$^{-/-}$) and *Cd11c*-cre^{+}*Hif1a*$^{+/+}$ *Ldlr*$^{-/-}$ (*Hif1a*-WT *Ldlr*$^{-/-}$) mice. 8 week old male *Hif1a*-WT *Ldlr*$^{-/-}$ and *Hif1a*-CKO *Ldlr*$^{-/-}$ mice or *Ldlr*$^{-/-}$ mice were placed on a high fat diet (15% fat, 1.25% cholesterol, Altromin) for 8 weeks. *LysM*-cre^{+}*Hif1a*$^{flox/flox}$ mice (C57BL/6J background)[1] were kindly provided by Randall Johnson. All experiments were approved by local authorities (Regierung von Unterfranken, or Maastricht University) and performed in compliance with directive 2010/63/EU of the European Parliament.

Immunohistochemistry and atherosclerotic lesion quantification
Hypoxia was detected using the Hypoxyprobe Plus kit (Hypoxyprobe Inc) according to the manufacturer's instructions. Briefly, mice were injected with 60 mg/kg Pimonidazole hydrochloride and the hearts were harvested after 90 minutes to generate fixed frozen cryosections (5 µm). Hypoxia was detected using the provided FITC-MAb1 primary antibody and anti-FITC-HRP secondary reagent and visualized by the DAB peroxidase substrate kit (Vector laboratories). CD11c^{+} cells and HIF1α were stained in cryosections of the aortic root using biotin-labeled anti-CD11c (Biolegend) and anti-HIF1α (Novus Biologics) antibodies, detected by Alexa-Fluor-555-Streptavidin (Molecular probes, Life Technologies) and Alexa-Fluor-488 anti-rabbit IgG (Molecular probes, Life Technologies), respectively. Nuclei were counter-stained by 4',6-Diamidino-2-phenylindole (DAPI, Vector laboratories). The extent of atherosclerosis was assessed in serial sections of paraffin-embedded aortic roots stained with aldehyde fuchsin (basic fuchsin and acid aldehyde, Sigma Aldrich) or hematoxylin-eosin (Sigma) and in *en face* prepared aortas by staining for lipid depositions with Oil-Red-O[2]. Macrophages, T cells and SMCs were immunostained using specific antibodies to Mac-2 (Cedarlane labs) or Mac-3 (Becton Dickinson), CD3 (AbD Serotec) and α-actin (Sigma Aldrich), respectively, using standard immunohistochemistry techniques[2]. The co-localization of different DC markers with markers for hypoxia in human plaques was measured by multispectral imaging of immunohistochemical staining. Paraffin embedded and frozen human plaque sections were stained for the DC marker S100 (Dako) or CD11c (BD Biosciences) and the hypoxia marker pimonidazole (Hypoxyprobe store) or HIF-1α (Novus Biologicals), respectively. In addition, APCs were visualized by staining for the DC marker S100 in combination with STAT3 (Cell Signaling Technology) or IL12 (abcam), on paraffin embedded human plaque sections. From double staining, spectral imaging data sets from maximal three random regions of interest were taken between 420-720 nm (10 nm interval) at a 20x (plan apo.) magnification using a Nuance spectral imaging system (Perkin Elmer/Caliper Life Sciences, Hopkinton, MA, USA) mounted on a Zeiss Axiophot microscope. Slides stained for a single chromogen only (Vector Red and Vector Blue, both Vector Laboratories) were used to create a spectral library. The spectral library was used for computational segregation of the individual image components using the Nuance^{TM} 3.0.2 software as described[3]. After spectral unmixing, pseudo-colors were assigned to unmixed images, and composite images showing co-localization were generated with the Nuance 3.0.2 software. Co-localization was quantified on a pixel-based measurement of the individual markers per microscopic field using the same software.

Quantitative real-time RT-PCR
Total RNA was isolated from APCs and BMMs using the RNeasy Mini kit (Qiagen), and from aortas using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. cDNA was reverse transcribed from total RNA using random primers and first strand cDNA synthesis kit (Fermentas). Genomic DNA (gDNA) was prepared from isolated CD11c^{+} and CD4^{+} T cells. qPCR analyses were performed from 20 ng cDNA or gDNA using SYBR green mix (Fermentas) and gene specific primer sets (*Hprt*: 5'-TCCTCCTCAGACCGCTTTT-3', 5'-

CCTGGTTCATCATCGCTAATC-3'; *Hif1a*: 5'-CAAGATCTCGGCGAAGCAA-3', 5'-GGTGAGCCTCATAACAGAAGCTTT-3'; *Cd80*: 5'-TCGTCTTTCACAAGTGTCTTCAG-3', 5'-TTGCCAGTAGATTCGGTCTTC-3'; *Cd86*: 5'-GAAGCCGAATCAGCCTAGC-3', 5'-CAGCGTTACTATCCCGCTCT-3'; *Cd74*: 5'-CACCGAGGCTCCACCTAA-3', 5'-GCAGGGATGTGGCTGACT-3'; *Il4*: 5'-CAACGAAGAACACCACAAGAG-3', 5'-ATGAATCCAGGCATCGAAAAGC-3'; *Il6*: 5'-GTGGCTAAGGACCAAGACCA-3', 5'-ACCACAGTGAGGAATGTCCA-3'; *Il10*: 5'-TGCACTACCAAAGCCACAAGG-3', 5'-TGGGAAGTGGGTGCAGTTATTG-3'; *Il12a*: Quantitect primer assay QT01048334 (Qiagen); *Tgfb*: 5'-GACGTCACTGGAGTTGTACGG-3', 5'-GGTTCATGTCATGGATGGTGC-3'; *Tnfa*: 5'-CTGTAGCCCACGTCGTAGC-3', 5'-GGTTGTCTTTGAGATCCATGC-3'; *Nfkb1/p105*: 5'-GAACTTCTCGGACAGCTTCG-3', 5'-CGTAGTTCGAGTAGCCATACCC-3'; *Rela/p65*: 5'-CATGCGATTCCGCTATAAATG-3', 5'-TCCTGTGTAGCCATTGATCTTG-3'; *Stat3*: 5'-CTACCTCTACCCCGACATTCC-3', 5'-GATGAACTTGGTCTTCAGGTACG-3'; *Nos2*: 5'-GTTCTCAGCCCAACAATACAAGA-3', 5'-GTGGACGGGTCGATGTCAC-3'; *Igf1*: 5'-TCGGCCTCATAGTACCCACT-3', 5'-ACGACATGATGTGTATCTTTATTGC-3'; *Mrc1*: 5'-CACTCATCCATTACAACCAAAGC-3', 5'-CAGGAGGACCACGGTGAC-3'; *Ccr7*: 5'-CTCCTTGTCATTTTCCAGGTG-3', 5'-TGGTATTCTCGCCGATGTAGT-3') in triplicates (7900HT Thermal cycler, Applied Biosystems). For analysis of the expression of genes in human atherosclerotic lesions, RNA was isolated from paraffin-embedded tissue sections adjacent to slides used for histological characterisation by the High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. In all cases 2 sections of 10 µm thickness were used, yielding 10-20 ng/µl of RNA per tissue sample. Isolated RNA was reverse transcribed using the cDNA Synthesis Kit RevertAid and oligo-dT primers supplied by the manufacturer (Fermentas, St. Leon-Rot, Germany). Quantitative Real-Time PCR was performed with cDNA using SYBRgreen fluorescence dye (peqLab, Erlangen, Germany) and specific primer pairs from Qiagen, QuantiTect Primer Assays using the ABI Prism 7700 Sequence Detector (Applied Biosystems).

Western blotting

Whole cellular lysates were separated by 8% or 10% SDS-PAGE, followed by semi-dry Western blotting onto a Nitrocellulose or PVDF-membrane (Whatman, GE Healthcare). Protein detection was conducted using antibodies from Cell Signaling Technology to NFκB p65 (C22B4), phosphor-NFκB p65-Ser536 (93H1), IκBα (polyclonal), GAPDH (14C10), from Sigma Aldrich to tubulin (B-5-1-2), Santa Cruz biotechnology to β-Actin (J1509) and from Abcam to HIF1α (GR30496-1), NFκB p105/p50 (E381), and enhanced chemiluminescence kit (Thermo Fisher Scientific Inc.) according to the manufacturers' instructions. Quantification of the chemiluminescence signal was carried out on the FluorChemQ using the AlphaView® software (ProteinSimple). Equal loading of the gel was verified by stripping the membrane in 62.5 mM Tris HCl (pH 6.7) containing 2% SDS and 100 mM β-mercaptoethanol at 70°C for 20 minutes and redetection with antibodies recognizing the protein irrespective of its phosphorylation status or by detection of β-Actin, tubulin or GAPDH.

Serum cholesterol and triglyceride assays

Serum cholesterol and triglyceride levels were quantified using the Amplex Red cholesterol assay kit (Life technologies) or standard enzymatic techniques automated on the Cobas Fara centrifugal analyzer (Roche) and Triglyceride assay kit (Biotrend), according to the manufacturer's instructions.

Flow cytometric analysis

Spleens and lymph nodes from mice were dissociated into single-cell suspensions. Blood and spleen were subjected to erythrocyte lysis. Aortic tissue was enzymatically dissociated using Liberase Blendzyme TL solution (Roche). Staining was performed using combinations of specific antibodies from BD Biosciences to CD19 (1D3), CD3 (500A2 or 17A2), CD45 (30-F11), CD8a (53-6.7), Gr1 (RB6-8C5), IFNγ (XMG1.2), MHC II (2G9); from eBioscience to CD115 (AFS98), CD11b (M1/70), CD11c (N418), CD4 (RM4-5 or GK1.5), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL1), F4/80

(BM8), Foxp3 (FJK-16s), IL-12 (C15.6), IL-17a (eBio17B7), CCR7 (4B12); from Cell Signaling Technology to STAT3 (124H6); R&D Systems to STAT3 (232209). Intracellular staining was performed using the Cytofix/Cytoperm solution (BD Biosciences) on cells treated with 2.5 µg/ml brefeldin, 50 ng/ml PMA and 750 ng/ml ionomycin for IFNγ and IL-17a, and 2.5 µg/ml brefeldin and 1 µg/ml LPS for IL-12. Intracellular staining for Foxp3 and STAT3 was performed using the Transcription factor staining buffer set (eBioscience). Data were acquired using a FACSCanto II (BD Bioscience) or a FACS Calibur (BD Biosciences), and results analyzed by FlowJo 7.6 software (Tree Star).

Immunization assay
Mice were subcutaneously injected with 100 µg OVA protein (Sigma Aldrich) and complete Freund's adjuvant (Sigma Aldrich). After 10 days, spleens and inguinal lymph nodes were harvested, and single cell suspensions re-stimulated with 5 µg/ml brefeldin, 10 ng/ml PMA and 1 µg/ml ionomycin overnight. Thereafter, cells were stained for IFNγ as described above and analyzed by flow cytometry.
Bone marrow transplantation (BMT)
Recipient 12 week old female *Ldlr*$^{-/-}$ mice were given acidified water (pH=2) containing neomycin (100 mg/L, Gibco) and polymyxin B sulphate ($6x10^4$U/L, Gibco) from 1 week before until 4 weeks after BMT. One day after lethal irradiation (10 Gy), *Ldlr*$^{-/-}$ mice were injected intravenously with bone marrow cells (10^7 cells) isolated from femurs and tibias of male donor *LysM*-cre^{+}*Hif1a*$^{flox/flox}$ and *LysM*-cre^{+}*Hif1a*$^{+/+}$ mice. Mice were placed on a high fat diet (20% fat, 1.25% cholesterol, Research Diets) starting 4 weeks after BMT for a total of 6 weeks.

APC isolation, bone marrow derived dendritic cells (BMDCs) and macrophages (BMMs)
Splenic APCs were isolated using CD11c-microbeads (Miltenyi Biotec). For bone marrow-derived cell cultures, femurs were excised and cells flushed to prepare single cell suspension. For the generation of BMDCs, cells were cultured in RPMI-1640 (with 2 mM L-Glutamine, Gibco) supplemented with 10% FCS (PAA), 100 U/ml penicillin-streptomycin (Gibco), 50 µM β-mercaptoethanol (Gibco) and 50 ng/ml murine GM-CSF (Peprotech) for 7 days[2] and matured with 100 ng/ml murine TNFα (Peprotech) for 24 h to induce *Hif1a* mRNA expression[4] (2.4±0.3 fold over unstimulated controls, n=5, $p<0.001$). Alternatively, cells were cultured in RPMI-1640 (with 2 mM L-Glutamine) supplemented with 10% FCS, 100 U/ml Penicillin-Streptomycin, 50 µM β-mercaptoethanol and 15% L929-conditioned medium for 7 days to generate BMMs[5]. Some BMMs were treated with 100 ng/ml murine TNFα (Peprotech) for 24 h.

BMDC transmigration assay
Serum starved BMDCs were allowed to migrate towards 100 ng/ml CCL19 (Peprotech) through 8 µm transmigration inserts (ThinCert, Greiner Bio-one) for 2 hours at 37°C. Transmigrated cells collected from the bottom chamber were enumerated using a Neubauer chamber.

ELISA
IL-12 protein levels were quantified in cell culture supernatants using the murine IL-12 mini ELISA development kit (Peprotech) according to the manufacturer's instructions.

Transfection of STAT3 vectors in BMDCs
BMDCs were transfected with pCAGGS-STAT3 wild type vector, pCAGGS-STAT3D dominant negative mutant vector (kindly provided by K. Nakajima and T. Hirano)[6], or empty vector using the Amaxa P4 Primary Cell 4D-Nucleofector kit (Lonza) following manufacturer's instructions. Transfected cells were cultured in RPMI-1640 (with 2 mM L-Glutamine, Gibco) supplemented with 10% FCS (PAA), 100 U/ml penicillin-streptomycin (Gibco), 50 µM β-mercaptoethanol (Gibco) and 100 ng/ml murine TNFα (Peprotech) for 16 hours and then harvested for RNA isolation. Vector overexpression was confirmed by observing dramatically increased *Stat3* mRNA expression in *Hif1a*-WT and *Hif1a*-CKO BMDCs transfected with wild

type (2,982 ± 756, and 1,186 ± 490 fold increase, respectively, n=3 each) and mutant vector (3,221 ± 1,045, and 3,530 ± 2,425 fold increase, respectively, n=3 each).

Chromatin Immunoprecipitation (ChIP) assay

Predicted HIF1α binding sites were determined within the *Stat3* promoter using the MatInspector software (Genomatix)[7] (GATTCCCA**CGTG**GTAAG at anchor position 117bp and CGCCCCCA**CGTG**GTGCC at anchor position 147bp from start of *Stat3* gene; bases in bold show core HRE sequence). ChIP assays were performed using the ExactaChIP Human/Mouse HIF1α chromatin immunoprecipitation kit (R&D) according to the manufacturer's instructions. In brief, BMDCs generated from C57BL/6J mice and treated with $CoCl_2$ (Sigma Aldrich) in vitro for 16 hours were cross-linked using 1% formaldehyde (Sigma Aldrich). After formaldehyde quenching with 125 mM glycine (Sigma Aldrich), cells were lysed, the lysates sonicated to shear chromatin and lysate supernatants prepared (serving as input DNA sample). The remaining supernatants were incubated with biotin anti-HIF1α or biotin normal goat IgG antibody overnight at 4°C. Antibodies were precipitated using Streptavidin agarose beads (Sigma Aldrich) and washed using kit buffers. Reverse cross-linking was done by boiling the samples with a Chelating Resin solution. DNA fragments were purified using the QIAquick DNA purification kit (Qiagen) and equal volumes from the eluate were added to a PCR reaction with primers against HIF1α binding site in the *Stat3* gene promoter region (5'-GCCCTGATACGGCTCGCTTCTGC-3', 5'-TGGGGACCGCCTAAGTGGCTG-3'). Quantitative PCR was carried out at an annealing temperature of 60°C and results were analyzed and presented as percent input. Amplified products were also separated by gel electrophoresis (1% agarose).

Antigen-specific T cell proliferation and polarization

Naïve $CD4^+$ T cells were isolated from spleens and lymph nodes of OT-II mice using the $CD4^+CD62L^+$ T cell isolation kit (Miltenyi Biotec) and labeled with 5μM CFSE (Sigma Aldrich) for 10 minutes at 37°C. APCs were loaded with 1 μg/ml $OVA_{323-339}$ peptide (AnaSpec Inc.) for 45 minutes at 37°C. Naïve $CD4^+$ T cells (1.5×10^5 cells) were co-cultured with OVA peptide-loaded $CD11c^+$ APCs (0.5×10^5 cells) in RPMI-1640 medium (supplemented with 2mM L-glutamine, 10% FCS, 100 U/ml Penicillin/Streptomycin and 50 μM β-mercaptoethanol) for 3 days. For IL-12 blockade, neutralizing *α*IL-12 p35 antibody (eBioscience) was added. Flow cytometric analysis was performed to quantify T cell proliferation and polarization by CFSE dye-dilution and intracellular cytokine staining respectively.

Transplantation of lentivirus transduced bone marrow cells

A pLB2-Ubi-FLIP-STAT3 vector was prepared by cloning *Stat3* cDNA (Open Biosystems library, clone ID 4923137) in the reverse orientation and flanked by inverted repeats of loxP sequences behind the Ubiquitin promoter[8]. To test the specificity of the vector, HEK293F cells were co-transfected with the pLB2-Ubi-FLIP-STAT3 vector and a Cre recombinase producing pIC-cre vector (kindly provided by Dr. Bernhard Nieswandt, Würzburg, Germany). Lentivirus particles incorporating the pLB2-Ubi-FLIP-STAT3 or empty control vector were generated as described[9]. BM cells from *Cd11c*-cre^+ mice or lineage-depleted BM cells from *Hif1a*-WT and *Hif1a*-CKO mice (Lineage Cell Depletion Kit, Miltenyi Biotec) were transduced with lentivirus particles (50 MOI) in the presence of DEAE-dextran[10] (10 μg/ml, Sigma Aldrich). After 24 hours, cells were washed to remove free virus, and differentiated into BMDCs, or injected retro-orbital into lethally irradiated $Ldlr^{-/-}$ recipients ($1x10^6$ cells) treated with neomycin sulphate (2g/L, Bela pharm) for 2 weeks before switching to a high fat diet for 4 weeks.

Human atherosclerotic plaques

For immunohistochemistry, a total of 40 atherosclerotic carotid arteries with advanced atherosclerotic lesions were obtained from patients undergoing vascular surgery or at autopsy to analyze expression in cryosections or paraffin-embedded sections, respectively. In addition, in 7 symptomatic patients the hypoxia marker pimonidazole (Hypoxyprobe-1, 0.5 g/m^2, hypoxyprobe store) was used to detect hypoxia in human carotid atherosclerosis. The

investigation was approved by an external ethical committee and written informed consent was obtained. All patient data has previously been described[11].

For real-time PCR analyses, atherosclerotic plaques were obtained from patients with high-grade carotid artery stenosis (>70%) after carotid endarterectomy. The study was performed according to the Guidelines of the World Medical Association Declaration of Helsinki. The local ethics committee (Klinikum rechts der Isar der Technischen Universität München) approved the study and written informed consent for being included in the Biobank was given by all patients. Carotid plaques were segmented in blocks of 3 to 4 mm, fixed in formalin and embedded in paraffin. Haematoxylin-eosin and Elastica van Gieson staining were performed in order to assess the stage/type of atherosclerosis. Histological classification of carotid atherosclerotic lesions was performed as described by Stary *et al*.[12, 13] by two independent investigators blinded for the study in close collaboration with an experienced pathologist. Study specimens were divided into groups of early (stage I-III, n=10) and advanced atherosclerosis (stage V-VII, n=10).

Statistics

Data are presented as mean ± SEM. Data were analyzed by unpaired Student t tests, and if more than 2 groups were compared by ANOVA 1-way analysis of variance followed by Tukey's or Dunnett's multiple comparison post-hoc tests. Differences with $p<0.05$ were considered to be statistically significant.

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