Online-only Supplement
Complete Material and methods

Human Samples

Carotid endarterectomy samples were selected from the Maastricht Pathology Tissue Collection (MPTC). Collection and storage in the MPTC and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (http://www.fmwv.nl). Each atherosclerotic plaque sample was obtained by carotid endarterectomy, collected at the time of surgery and immediately processed. The endarterectomy specimen was cut into parallel, transverse segments of 2-3 mm thickness. Each alternating segment was snap frozen in liquid nitrogen. The flanking segments were fixed in formalin and processed for histological evaluation. Hematoxylin-Eosin (HE) stained slides from these flanking segments were evaluated for plaque stage using the Virmani classification criteria [1] by a cardiovascular pathologist (MJD) and an experienced researcher in cardiovascular pathology (MVH). Segments designated as stable featured either a fibrous cap atheroma or pathological intimal thickening. Segments designated as ruptured included a thrombus and/or presented intraplaque hemorrhage. For a frozen segment to be selected in the study, it had to be flanked by two segments of identical classification: stable or ruptured. The same classification was applied to the central segment. In addition, plaques from individual patients were only included when both a ruptured and a stable segment could be identified in the same endarterectomy specimen. This approach allowed us to include 24 stable-ruptured pairs of 24 patients/plaques.

Micro-array analysis

RNA was isolated using Guanidium Thiocyanate lysis followed by Cesium Chloride gradient centrifugation. After the extracting procedure, the RNA was further purified using the Nucleospin RNAII kit (Macherey-Nagel GmbH & Co. KG). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A). The RNA quality and integrity was determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). Samples that had an RNA Integrity Number (RIN) lower than 5.6 were excluded from the study. The average RIN was 7.23 ± 0.48.

Transcripts were measured by Illumina Human Sentrix-8 V2.0 BeadChip®. A total of 24,495 human transcripts and variants, as defined by RefSeqs (NCBI) sequences, were analyzed. The RNA labeling, hybridisation and data extraction were performed at ServiceXS (Leiden, The Netherlands). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's specifications starting with 100 ng total RNA. Per sample 750 ng of cRNA was used for hybridization. Hybridization and washing were performed according to the Illumina standard assay procedure. Scanning was performed on the Illumina BeadStation 500 (Illumina, Inc., San Diego, CA, U.S.A.). Image analysis and extraction of raw expression data was performed with Illumina Beadstudio v3 Gene Expression software with default settings (no background substraction) and no normalization.

Mice

In all experiments, female low-density lipoprotein receptor (LDLR-/-) mice (age: starting experiment ~10 weeks), on a C57BL/6J background, were used (Jackson Laboratories; Bar Harbor, Maine, USA). Male CatC-/- or CatC+/+ mice (> 10 backcrossed to C57BL/6) were obtained from Dr C. Pham and used as donor mice for the bone marrow transplantation studies. All mice were fed a standard fat diet (cat. V1535; ssniff Spezialdia¨ten GmbH, Soest, Germany) unless stated otherwise, had ad libitum access to food and water, and were housed under a 12-h light-dark cycle. Mice were kept according to Maastricht University animal facility regulations and all experiments were approved by the local Animal Ethical Committee in compliance with the Dutch government guidelines.

Bone Marrow Transplantation and *in vivo* Experimental Schemes

Eight week-old female littermate LDLR-/- mice were housed in filter-top cages and received acidified water supplemented with neomycin (100 mg/L) and polymyxin B sulfate (60 000 U/L) starting 1 week before until 5 weeks after bone marrow transplantation. Mice were lethally irradiated (10-Gy, Philips MUI5F/225kV; Hamburg, Germany) and received either bone marrow cells from CatC-/- or CatC+/+ donor mice 1 day after irradiation. Bone marrow cells were harvested by flushing the tibias and femurs, single cell suspensions were made and injected (~$5x10^6$ cells) into the recipients via the tail vein. A portion of the cells was also used for in vitro assays. After recovery for 8 weeks, mice put on a high fat diet (HFD, 1.25 % cholesterol, D12108C, Research Diets Inc, NJ, USA) for 13 weeks, unless stated otherwise.

Carotid atherosclerotic lesions were induced by bilateral placement of semiconstrictive perivascular collars as described by von der Thusen et al [2], at 6 weeks HFD. Chimerism was assessed to determine the efficiency of repopulation by donor bone marrow cells. Briefly, genomic DNA was extracted from blood drawn from the transplanted mice and copy numbers of LDLR and p50 subunit of NF-κB were quantified by qPCR (Supplement Table 1). The quotient of genomic LDLR relative to p50 served as measure of chimerism.

Tissue preparation and histological analysis

Mice were euthanized by pentobarbital injection (115 mg/kg), blood was collected by heart puncture, organs were excised and processed for further analysis. The arterial tree was perfused with phosphate-buffered saline (PBS) containing 0.1 mg/mL sodium nitroprusside (Sigma-Aldrich, St Louis, MO) via the left cardiac ventricle, subsequently with 1% paraformaldehyde and carotids, aortic arch and heart excised and processed for paraffin embedding. The descending (thoracic and abdominal) aorta was stained for lipid accumulation with Sudan IV, and lesions were visualized “en face” and analyzed for size by ImageJ (http://rsb.info.nih.gov/ij). Carotids, aortic arch and aortic roots were analyzed histologically and morphometrically from cross-sections (4um) after staining with HE. Atherosclerotic lesions were classified as either initial (fatty streaks, containing macrophage-derived foam cells) or advanced (containing extracellular lipid, a lipid core and/or a fibrous cap). Lawson solution (elastin; lumen, intima, and media and total vessel area), anti-CD45 (1:50; Pharmingen, San Diego, CA) (leukocyte infiltration), anti-CD3 (1:50; Lab Vision, Fremont, CA) (T cells), anti-Mac3 (1:50; Pharmingen) (macrophages), picrosirius red (collagen) and Movat’s modified pentachrome stain (matrix components). Modified congo red-acidified toluidine blue was used for detection of mast cells [3] and naphtol AS-D chloroacetate specific esterase kit (Sigma) for granulocyte detection. Aortic arches (including the brachiocephalic, left and right carotid, and subclavian artery)

were cut longitudinally and analyzed at 4 different levels (HE). Quantitative analyses were performed blindly (intra-observer variability <10%) using the Leica QWin software.

CatC protein expression in human and mouse plaques was visualized by polyclonal goat antibody (1:10, AF1071-R&D systems), with simultaneous co-staining with anti-CD68 antibody (human plaques; 1:100 KP1 clone, DAKO) to visualize macrophages. CatC expression in human carotid plaques (early lesions n=8, advanced n=9, ruptured n=11, derived from MPTC) was evaluated qualitatively on the basis of staining intensity (no staining =0; scanty (±) = 1 Clear positive (+) = 2; strong positive (++) = 3; stronger positive (+++) = 4), as described in Miserus et al. [4]

A staining with rabbit-anti-mouse iNOS (1:20, Abcam, UK) and rabbit-anti-mouse arginase-1 (1:1250, kindly provided by Paul van Dijk, department of Anatomy and Embryology, Maastricht University, The Netherlands) was performed to semi-quantitatively assess the presence of respectively M1 and M2 (see Figure Question 7A-D for representative examples). A score of 0 (negative), 1 (few positive cells), 2 (small positive areas) or 3 (intense staining in several areas of the lesion) was assigned to the (single) lesion in the carotid artery or the 3 lesions in the aortic root area (using the sum of scores in these 3 lesions).

Elastolytic and CatC activity assays

Aortic arches of wt (n=7) and CatC chimeras (n=7) were homogenized in a modified RIPA buffer (50 nM Tris-HCl, 0.5% Sodium Deoxycholate, 150 nM NaCl, 1% NP40 and 1 mM EDTA). Protein concentration was determined by Pierce BCA Protein Assay kit (Thermo Scientific). Elastolytic activity was determined using an EnzChek Elastase Kit (Molecular Probes) according the manufactures instructions. CatC activity was determined using a Gly-Arg-AMC substrate, which is degraded by recombinant mouse CatC (R&D systems, standard curve) or CatC present in the homogenate. All measurements were done in duplo.

Cholesterol analysis

Total serum cholesterol content was measured at 3 time points during the study (1 day before HFD feeding, at 6 weeks of HFD and at sacrifice (week 13) by a colorimetric assay (Roche diagnostics).

Spleen cell proliferation assay

Spleen tissue from HFD fed chimeric mice were aseptically dissociated into single cell suspension and seeded at a density of 1 x 10^6 cells/ml in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) and cultured at 37℃ (5% CO_2; 95% humidity) in complete RPMI-1640. Cells were incubated with concanavalin A (conA, 2.5 µg/ml; Sigma), LPS (1µg/ml; Sigma) or oxLDL (9 µg/ml) for 48 h (ConA and LPS) and 24 h (OxLDL). Optimal culture and mitogen concentrations were previously determined for each agent. During the final 8-18 h of culture, 1 µCi [^{3}H]-thymidine (Amersham, GE Healthcare UK) was added to each well. The cultures were harvested into glass fiber filters, processed and cell associated radioactivity was counted in a ß-counter. Cultures were set up in triplicates and expressed as mean DPC/min.

Fluorescence-activated cell sorting (FACS) analysis

Blood, spleen and peripheral LN were removed before perfussion and used for flow cytometry analysis of B/T-cell subsets CD3e FITC (eBioscience 11-0031-82); CD4

PerCp, (Beckton & Dickinson, 553052); CD8a eFLUO450 (eBioscience 48-0081-82); CD25 APC (eBioscience 17-0251-82); B220 PE-Cy7 (eBioscience, clone RA3-6B2); CD44 PE (BD, clone IM7), CD62L PE-Cy7 (eBioscience, clone MEL14, 25-0621-82); FoxP3 PE (eBioscience, 12-5775-82), of DC subsets (CD11cPE-Cy7, Beckton & Dickinson (BD) 558079; CD4 APC-H7 (Beckton & Dickinson); CD8α eFluo450 (eBioscience 48-0081-82); MHCII FITC (eBioscience 11-5322-82); B220 PE (BD 553089); nonCD3/19PerCp-Cy5.5 (CD3e Miltenyi 45-0031-82; CD19 Miltenyi 45-0193-82); CD11cPE-Cy7 (BD 558079); monocytic and granulocytic cells Ly6C FITC (Miltenyi 130-093-134); Ly6G PE (BD 551461); CD11b PE-Cy7 (BD, 552858).
Flow cytometry of CD4+CD25+Foxp3+ Tregs, CD4+CD25+IFNγ+ TH1 and CD4+CD25+IL17+ TH17 populations was done after intracellular staining of splenocytes that had been stimulated for 2h with PMA (50ng/ml, Sigma)/Ionomycin (1ug/ml, Sigma) and incubated for 4h with Golgistop (1 ul/ml; monensin, Beckton & Dickinson). Cell suspensions were stained with αCD3e-FITC (e-Bioscience 11-0031-82), αCD4-PerCp (Calbiochem, polyclonal, 219384) and αCD8-eFLUOR450 (eBioscience, clone 53-6.7, 48-0081-82), and after permeabilization stained for FoxP3-PE (eBioscience, 12-5775-82), for IFNγ PE-Cy7 (BD Biosciences, clone XMG1.2, 557649) or for IL17A-Alexa647 (BD, clone TC11-18H10, 560184). FACS analysis was performed with FACS Canto II (BD).

Cytokine Analysis
Plasma cytokine profiles were assessed on Luminex 100 via the Bio-Plex cytokine assay (Bio-Rad Laboratories, Inc; Hercules CA, USA), covering 15 cytokines (IL-1alpha, IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-10, IL12(P40), IL-12 (P70), IL-17, Eotaxin, Keratinocyte chemoattractant (KC), monocyte chemoattractant protein (MCP), monocyte inflammatory protein-1 alpha (MIP-1alpha), tumor necrosis factor alpha (TNF-alpha).

In vitro studies
Bone marrow derived macrophages: Bone marrow cells were isolated from the tibias and femurs of WT or CatC-/- mice as described and macrophages selected and cultured in standard RPMI media containing 1% L-glutamine, 10% fetal calf serum, 2.5% HEPES, 100IU/ml penicillin/streptomycin and 15% L929 cell-conditioned medium for at least 7 days before performing any experiment.
Macrophage differentiation : Bone marrow derived macrophages (BMDM) were either left unstimulated (M0 macrophages), or differentiated in M1 (18 h Interferon (IFN)-gamma stimulation (24 hrs INF-gamma 100 uU/ml, Peprotech via Bioconnect), or M2 (24 h interleukin (IL)-4 stimulation, 20 ng/ml, Peprotech via Bioconnect).
Phagocytosis: BMDM were seeded in triplicate at a density of 150,000 BMDM/well (48-well plate) and labeled with cell tracker red (Invitrogen). Calcein-AM green-labeled Jurkat cells, rendered apoptotic by UV-light exposure were added to the macrophages at a 3:1 numerical ratio; after 1h of incubation, cells were washed to remove non-bound Jurkat cells and fixed with 4% paraformaldehyde for 10 min. Jurkat cell phagocytosis was assessed by fluorescent microscopy after nuclear counterstain with DAPI and photo's analyzed using ImageJ.
Wound Healing: BMDM were seeded at 500,000 cells/well in gelatin (Bovine skin type B, Sigma) coated 24-well plates (n=4) and allow to grow to confluence overnight at 37^{o}C. Next day a transverse scratch was made with a sterile glass tip. Unbound cells were washed carefully, photos were made at t=0 (baseline), 2, 4, 8, 10 and 24

hrs of incubation and “wound” ingrowth was analyzed by LeicaQWin using a tailored-made algorithm.
Di-I labeling: LDL (3 ml; 150 ug) was labeled overnight with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; 150 ul, 3 mg/ml in DMSO). Unbound DiI was removed by gel filtration (PD10; Amersham Biosciences). BMDM were incubated with 25 µg/ml DiI-LDL for 3 h. Cells were lifted from the culture dish, washed 3 times with PBS resuspended in 400 µl PBS and analyzed by flow cytometry on a FACS Calibur (BD Biosciences).
CatK activity assay: BMDM were differentiated in M1 and M2 macrophages. CatK activity was measured by Cathepsin K Activity Assay Kit (Fluorometric, Abcam, ab65303), according to manufacturer’s instructions.

Quantitative RT-PCR:
Total RNA was extracted from cell or tissue lysates using the Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany). cDNA was generated using iScript cDNA synthesis kit (Bio-Rad). Real time PCR was made using Taqman IQ SYBR Green Super Mix (Bio-Rad). mRNA expression of T cell differentiation markers (TBX21, GATA3, Ror-gamma-T), CCR3, FoxP3, CatK, CatS, eotaxin, IL-10, and TGF-beta were performed in RNA preps isolated from spleens of HFD fed WT and CatC-/- chimeric mice.

M1/M2 polarization marker expression (iNOS, IL-18, IL-12B, TNF-alpha, IL-1beta, IL-6, INF-gamma, Arg-1, IL-10, mannose receptor (MR), YM1, FIZZ-1, TGF-beta) was measured in BMDM (500,000 cells/well; n=4) exposed 24 hrs to INF-gamma 100 uU/ml), IL-4 (20 ng/ml, both from Peprotech), LPS (10 ng/ml Sigma), OxLDL (50 ug/ml) or non-stimulated cells, after which total RNA was isolated from cell lysates. For the primer sequences and characteristics, see Supplement Table 1.

Statistical Analyses
Microarray analysis: A paired univariate model to detect individual genes that were differentially expressed between stable and ruptured plaques was used. The analyses were performed using the R bioconductor Lumi and Limma packages [5], applying the linear models and empirical Bayes methods included in the package. We applied the variance stabilizing transformation that is incorporated in the Lumi package [6, 7]. The data were normalized using the Robust Spline Normalization (RSN) algorithm, which combines the features of quantile and loess normalization. The probe intensities that are from potentially differentially expressed genes are heuristically determined as follows: First, a quantile normalization is performed. Next, the fold-change of a gene measured by a probe is estimated based on the quantile-normalized data. The weighting factor for a probe is calculated based on a Gaussian window function. This results in normalized data that is corrected for technical variations introduced by the microarray analysis.
Subsequently, we used a paired univariate model to detect individual genes that are differentially expressed between stable and ruptured segments. Samples from the same patient were considered a pair. The analyses were performed using the R bioconductor Limma package, applying the linear models and empirical Bayes methods included in the package.
In vivo and in vitro experiments: Statistical analyses were performed using the non-parametric Mann-Whitney U test (corrected for multiple testing), except for the phagocytosis assay (pooled cells in triplicate), and FACS CD4CD25FoxP3 data (WT

(n=4), vs CatC-/- mice (n=3)), where T-test was used. Fisher's exact test was used to test ratio's. In all instances, differences were considered significant when $P<0.05$.

References

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Supplement Table 1. Gene name, characterization and primer sequences used for QPCR analyses.

Gene name	Sequence	Characterization
TH profile		
TBX21	5' GGGAACCGCTTATATGTCCA 3' 5' GGGCTGGTACTTGTGGAGAG 3'	TH1 lineage
GATA3	5' CAGCTGCCAGATAGCATGAA 3' 5' GCAGGCATTGCAAAGGTAGT 3'	TH2 lineage
Ror-gamma -T	5' CGACTGGAGGACCTTCTACG 3' 5' TTGGCAAACTCCACCACATA 3'	TH17
M1/M2 profile		
iNOS	5' CCTGGTACGGGCATTGCT 3' 5' GCTCATGCGGCCTCCTTT 3'	M1
IL-18	5' CAGGCCTGACATCTTCTGCAA 3' 5' TCT GAC ATG GCA GCC ATT GT 3'	M1
IL-12B	5 'CGCAGCAAAGCAAGATGTGT 3' 5' TGGAGACACCAGCAAAACGA 3'	M1
TNF-alpha	5' CATCTTCTCAAAATTCGAGTGACAA3' 5' TGGGAGTAGACAAGGTACAACCC 3'	M1
IL-1beta	5' CAACCAACAAGTGATATTCTCCATG 3' 5'-GAT CCA CAC TCT CCA GCT GCA-3'	M1
IL-6	5' CTGCAAGAGACT TCC ATC CAG TT 3' 5' GCTCATGCGGCCTCCTTT 3'	M1
INF-gamma	5' CAGGCCTGACATCTTCTGCAA 3' 5' TCT GAC ATG GCA GCC ATT GT 3'	M1
Arg-1	5' ATGGAAGAGACCTTCAGCTAC 3' 5' GCTGTCTTCCCAAGA GTTGGG 3'	M2
IL-10	5' TGCTCCTAGAGCTGCGGACT 3' 5' CTTGATTTCTGGGCCATGCT 3'	M2/ anti-inflammatory
Mannose Receptor	5' GCAAATGGAGCCGTCTGTGC 3' 5' CTCGTGGAT CTC CGTACAC 3'	M2
YM1	5' TGGCCCACCAGGAAAGTACA 3' 5' CAGTGGCTCCTTCATTCAGAAA 3'	M2
FIZZ-1	5' CTGCCCTGCTGGGAT GAC 3' 5' TCCACTCTGGATCTCCCAAGA 3'	M2
TGF-beta	5' GCCCTTCCTGCTCCTCATG 3' 5' CCGCACACAGCAGTTCTTCTC 3'	M2/anti-inflammatory
Other		
CatC	5' CAACTGCAC CTACCCTGATC 3' 5' CTCGTCGTAGGCAGTATCCA 3'	CatC mouse
CatK	5' GGG CCA GGA TGA AAG TTG TA 3' 5' CAC TGC TCT CTT CAG GGC TT 3'	CatK mouse
CatS	5' AGA GAA GGG CTG CGT CAC T 3' 5' GAT ATC AGC TTC CCC GTT TTC AG 3'	CatS mouse
Eotaxin	5' CAACTTCCTGCTGCTTTATC 3' 5' CCTGGACCCACTTCTTCTTG 3'	
CCR3	5' TGGCATTCAACACAGATGAAA 3' 5' TGACCCCAGCTCTTTGATTC 3'	Pro-inflammatory

FoxP3	5' CCCAGGAAAGACAGCAACCTT 3' 5' TTCTCACAACCAGGCCACTTG 3'	Immunoregulatory T cells
CD3	5' AACACGTACTTGTACCTGAAAGCTC 3' 5' GATGATTATGGCTACTGCTGTCA 3'	CD3 mouse
CD4	5' ACACACCTGTGCAAGAAGCA 3' 5' GCTCTTGTTGGTTGGGAATC 3'	CD4 mouse
CD8	5' GGCTCTGGCTGGTCTTCA 3' 5' GACGAAGGGGTCTGAATGAG 3'	CD8 mouse
18SRNA	5' GTAACCGCTTGAACCCCATT 3' 5' CCATCCAATCGGTAGTAGCG 3'	Housekeeping gene
NF-kappaB p50 subunit	5'-AACCTGGGAATACTTCATGTGACTAA-3' 5' -GCACCAGAAGTCCAGGATTATAGC -3'	Chimerism
LDR-R	5'- GCTGCAACTCATCCATATGCA -3' 5'- GGAGTTGTTGACCTCGACTCTAGAG-3'	Chimerism
Cyclophilin	5' CAA ATG CTG GAC CAA ACA CAA 3' 5' TTC ACC TTC CCA AAG ACC ACA T 3'	Housekeeping gene