Leukocyte Cathepsin C Deficiency Attenuates Atherosclerotic Lesion Progression by Selective Tuning of Innate and Adaptive Immune Responses

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Objective—The protein degrading activity of cathepsin C (CatC), combined with its role in leukocyte granule activation, suggests a contribution of this cystein protease in atherosclerosis. However, no experimental data are available to validate this concept.

Approach and Results—CatC gene and protein expression were increased in ruptured versus advanced stable human carotid artery lesions. To assess causal involvement of CatC in plaque progression and stability, we generated L.DLr−/−/CatC−/− chimeras by bone marrow transplantation. CatC−/− chimeras presented attenuated plaque burden in carotids, descending aorta, aortic arch and root, at both the early and advanced plaque stage. CatC was abundantly expressed by plaque macrophages and foam cells. CatC expression and activity were dramatically downregulated in plaques of CatC−/− chimeras, supporting a hematopoietic origin of plaque CatC. Our studies unveiled an unexpected feedback of CatC deficiency on macrophage activation programs and T helper cell differentiation in as much as that CatC expression was upregulated in M1 macrophages, whereas its deficiency led to combined M2 (in vitro) and Th2 polarization (in vivo).

Conclusions—Our data implicate CatC has a role in the selective tuning of innate and adaptive immune responses, relevant to a chronic immune disease, such as atherosclerosis. (Arterioscler Thromb Vasc Biol. 2015;35:79-86. DOI: 10.1161/ATVBHA.114.304292.)

Key Words: atherosclerosis ■ human ■ inflammation ■ mice ■ protease

Cathepsin C (CatC), also known as dipeptidyl peptidase I, is a lysosomal cystein protease that belongs to the papain super family.1 Unlike cathepsin S (CatS) and cathepsin K (CatK), it is expressed in many tissues, but highest in lymphoid organs, such as spleen,2 and homologues have been identified in a variety of species, suggesting an important and widespread role.2,4 In mice, CatC is most abundantly expressed in lung, liver, spleen, and small and large intestines; interestingly expressed in bone marrow, thymus, and stomach; and low expression in kidney, heart, and brain.7 CatC has a unique aminodipeptidyl peptidase activity2 and can progressively remove N-terminal dipeptides from various protein substrates and as such participates in post-translational processing. Indeed, studies in CatC knockout mice have revealed a central function in the activation of granule serine proteases in cytotoxic T lymphocytes and natural killer cells (granzymes A and B), mast cells (chymase and tryptase), and neutrophils (cathepsin G, proteinase 3, and elastase).2–11 Furthermore, alveolar macrophage and mast cell–derived CatC were seen to cleave extracellular matrix proteins, such as fibronectin and collagen types I, III, and IV, suggestive of a role of CatC in airway remodeling of chronic airway diseases, such as asthma.12 Finally, a contribution of CatC in coagulation as plasminogen13 and thrombin regulator14 and in angiogenesis have been documented.15

CatC-deficient mice seem healthy but have defects in serine protease activities in multiple hematopoietic lineages8 and show unexpected resistance to sepsis as compared with their wildtype (WT) littermates, possibly by attenuated tryptase-dependent IL-6 cleavage.16 Likewise, CatC−/− mice are protected against acute arthritis by reducing neutrophil recruitment to the joints, as well as by modulating the neutrophil production of cytokines and possibly chemokines.8,17

Its immunomodulatory effects on mast cells, macrophages, and neutrophils next to its intrinsic proteolytic capacity points to a role of CatC in inflammatory vascular remodeling processes. Indeed, CatC was seen to regulate neutrophil recruitment and CXCL12 production in...
Although several cathepsins, such as CatS, CatK, and cathepsin L, have already been implicated in atherosclerosis, the effect of CatC in its pathophysiology remains elusive, apart from its identification as a sensitive vascular injury marker in rabbits with experimental hypertension and cholesterol-fed mini pigs.

Here, we show increased CatC gene and protein expression in advanced stable compared with ruptured carotid human atherosclerotic plaques, mainly localizing in macrophages. Furthermore, we provide evidence for an attenuated atherogenic response in low-density lipoprotein receptor-deficient (LDLr−/−) mice with hematopoietic deficiency of CatC, via a selective tuning of innate and adaptive immune responses.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

CatC Is Differentially Expressed in Ruptured Human Atherosclerotic Plaques

In a candidate approach using microarray analysis, the cathepsin family was identified as differentially expressed between stable and ruptured segments of the same plaque (all P<0.001; Figure 1A). Giving its immunomodulatory effects, proteolytic capacity, and unknown role in atherosclerosis, we focused our follow-up research on CatC. Protein expression was validated on a series of early, stable, and ruptured human carotid plaques (Figure 1B). CatC expression localized to the same areas with abundant CD68+ macrophages presence (Figure 1B, panel iii and iv). CatC was expressed significantly higher in ruptured plaques compared with both stable (P<0.05) and early plaques (P<0.05; Figure I in the online-only Data Supplement).

LDLr−/− Mice With Hematopoietic CatC Deficiency Display Reduced Atherosclerosis

To address the causal involvement of CatC in atherogenesis, we generated LDLr−/− chimeras with hematopoietic CatC deficiency by a bone marrow transplantation strategy. After recovery, mice were put on a high fat diet for 13 weeks and equipped with semiconstrictive carotid artery collars after 6 weeks of high fat diet to induce lesion formation.

Figure 1. A, Cathepsin (Cat) family members were differently expressed in human ruptured carotid endarterectomies, as determined by microarray analysis. Fold changes stable versus ruptured lesion, all P<0.0001. B, Immunohistochemical staining of human carotid atherosclerotic lesions using CatC and CD68 antibodies. Panel (i) represents CatC expression in an early lesion, panel (ii) represents CatC expression in a stable lesion, panel (iii) represents CatC expression in a ruptured lesion, and panel (iv) represents parallel section of panel (iii), showing that CatC expression localizes to the same areas of intense CD68-positive staining cells.
Body weight and plasma total cholesterol levels did not differ between groups throughout the experiment (Figure II in the online-only Data Supplement). At point of killing, lesion formation was analyzed at 4 sites: the common carotid artery, the aortic arch, the aortic root (all cross-sectional), and the descending aorta (en face). Carotid artery plaques were 55% smaller in CatC−/− chimeras compared with WT controls (P<0.05; Figure 2B) and had smaller lipid core areas (Figure IIIB in the online-only Data Supplement). A representative picture of the carotid lesions is shown in Figure 2A.

The aortic arch showed a similar pattern with reduced plaque size in CatC−/− chimeras. Plaque area of the early lesions was decreased in the CatC−/− chimeras (P=0.009; Figure IVA in the online-only Data Supplement), whereas advanced lesion size did not differ between groups (Figure IVB in the online-only Data Supplement). Consequently, the early and total plaque burden (mean number of aortic arch lesions per mouse) was reduced in CatC−/− chimeras, but advanced lesion burden was unaltered (Figure IVC in the online-only Data Supplement). Given the observations in the advanced plaques of the aortic arch, an additional location was investigated: the lesions of the aortic root. In the advanced lesions of the aortic root, both lesion size (P<0.05; Figure 2C) and necrotic core content (P<0.05; Figure 2D) were smaller in the CatC−/− chimeras. En face analysis of the thoracic and abdominal aorta showed the scanty presence of lipid deposits that had not progressed beyond the early stage. Consistent with the carotid and aortic arch data, the WT group presented a larger area of initial fatty streak lesions compared with the CatC−/− chimeras (P=0.009; Figure IVD in the online-only Data Supplement).

Further morphometric analysis of the carotid lesions did not show any effects of hematopoietic CatC deficiency on lumen area, media area, and total vessel area (data not shown). Zooming in on plaque composition, we first quantified the contribution of hematopoietic CatC to total plaque CatC. Clearly, almost all plaque CatC was derived from leukocytes, as CatC expression was almost abrogated in CatC−/− chimeric mouse, whereas it was predominant in foam cells and macrophages of WT plaques (Figure 3A; Figure IIIA in the online-only Data Supplement). In fact, only 18% of the lesions of the CatC−/− chimera mice showed CatC staining, while 93% of the WT lesions showed robust CatC staining. Of interest, we did observe scanty CatC expression in the carotid adventitia, colocalizing with granular cells, possibly mast cells, and resident macrophages (see arrows; Figure 3A). In concert with the low plaque CatC expression in CatC−/− chimeras, CatC activity was extremely low in aortic arch tissue homogenates of CatC−/− chimeras, whereas elastolytic activity was comparable to WT (Figure 3B and 3C).

Compatible with the reduced plaque progression, CatC−/− chimera carotid lesions were almost devoid of lipid cores that were prominent in plaques of WT controls. In fact, 10/14 WT mice had lipid cores compared with only 1/11 CatC−/− chimeras (P=0.002; Figure IIIB in the online-only Data Supplement). Lesions from CatC−/− chimeras tended to have lower collagen content (P=0.165; Figure IIIC in the online-only Data Supplement), but did not differ in CD45+ leukocyte content (not shown), Mac3+ macrophage (Figure IIIID in the online-only Data Supplement), and CD3+ T cell content (Figure IIIE in the online-only Data Supplement). Mast cells were present in the plaque adventitia, at numbers that did not...
differ between the groups. Plaque granulocytes tended to be increased in WT mice (8/14 plaques) versus CatC−/− chimeras (3/11 plaques), but this effect did not reach statistical significance (P=0.22). Essentially similar findings were obtained for aortic arch plaques with borderline statistical significance in plaque granulocyte content (number of granulocytes in WT aortic arch lesions 0.53±0.22, in CatC−/− aortic arch lesions 0.00±0.00, NS [P=0.053]).

Effects of CatC Deficiency on Monocyte/Macrophage Function

The profound reduction in plaque size, the rather subtle effects on plaque composition and the abundant expression of CatC by plaque macrophages, foam cells, and granulocytes, prompted further study of monocyte/macrophage function in CatC deficiency. We found that CD11b+Ly6G− blood monocyte levels tended to be increased in CatC−/− mice, albeit not significantly (Figure VA in the online-only Data Supplement).

As the number of macrophages in plaques did not differ significantly between WT and CatC−/− chimeras, we next examined whether CatC expression affects or is affected by macrophage polarization. As expected, CatC−/− bone marrow-derived macrophages (BMDM), whether at baseline (M0) or IL-4 (M2) or IFN-γ primed (M1), did not express CatC (Figure 4A). However, CatC expression is upregulated >20-fold in WT M1 macrophages (P<0.001), and, conversely,
downregulated by >70% in WT M2 (P<0.01; Figure 4A). Thus, CatC seems to be a selective M1 marker.

CatK and CatS were next measured to verify whether any of these proteases are overexpressed to compensate for CatC deficiency and potentially contribute to the CatC phenotype. The results obtained show that CatS but not CatK expression is slightly but significantly increased in CatC−−/− chimeras (P<0.001; Figure VB and VC in the online-only Data Supplement). CatK and CatS gene expression was also assessed in WT- versus CatC−−/− macrophages, polarized to M0, M1, and M2. CatS expression was not affected by M1 or M2 polarization, whereas CatK was downregulated in M1 macrophages (Figure 4B). However, CatC deficiency did not affect macrophage CatK expression at activity level because macrophage CatK activity was similar in M1 and M2 macrophages of both WT and CatC−−/− BMDM (Figure VD in the online-only Data Supplement). This suggests that a differential regulation at the posttranscriptional level resulted in comparable CatK activity levels in M1 and M2 macrophages in vitro.

The observation that CatC is selectively expressed in M1 macrophages raised the question whether CatC may have a regulatory role in macrophage polarization and, in this way, could affect atherosclerosis. CatC−−/− BMDM showed greatly reduced induction of the M1 markers iNOS (P<0.05; Figure 4C), IL-18 (Figure VE in the online-only Data Supplement, in IL4 and IFN-γ-stimulated BMDM; P<0.05), and TNF-α (Figure VF in the online-only Data Supplement; P<0.05). In contrast, we observed increased expression of the M2 marker YM1 (baseline) in CatC−−/− BMDM (Figure 4D; P<0.05), supporting the hypothesis that M2 polarization in vitro is favored in the absence of CatC. To further investigate this phenomenon, immunohistochemical stainings for a M1 marker (iNOS) and a M2 marker (Arg-1) were performed on the initial collar-induced carotid lesions and advanced aortic root lesions. M1 macrophages were more dominant both in early and advanced lesions, this in contrast to a report of Khallou-Laschet et al, that showed a predominance of M2 macrophages in early lesions. An increased expression of the M2 marker was evident in the advanced aortic root lesions (Figure VI in the online-only Data Supplement). There was no difference in M1 and M2 presence in the carotid and aortic root lesions of the WT and CatC−−/− chimeras. Thus, despite the clear in vitro M2 phenotype of CatC−−/− BMDM, this did not lead to a higher presence of M2 macrophages in the lesions of the CatC−−/− chimeras, albeit that we used a limited panel of M1/M2 markers and assessed only 1 (end) timepoint.

Congruent with the M2 skewed macrophage polarization observed in CatC deficiency, of the 7 cytokines detectable in plasma of WT and CatC−−/− chimeras, on chow and high fat diet, only eotaxin levels differed between both groups. High fat diet upregulated plasma eotaxin in CatC−−/− (P<0.01), but not WT chimeras (Figure 5A). Moreover, TGF-β as well as eotaxin expression by spleen was increased in CatC−−/− chimeras (P<0.01; Figure 5B and 5C).

Macrophage polarization is a major determinant of the cell’s phagocytotic capacity. However, the CatC-dependent shift in macrophage polarity was not accompanied by an altered ability to ingest or accumulate Dil-labeled LDL or oxLDL (Figure VIIA in the online-only Data Supplement), excluding that CatC deficiency affects macrophage lipid uptake. In contrast, CatC−−/− BMDM showed a marginal decreased uptake of apoptotic Jurkat cells at baseline compared with WT mice (P=0.053; Figure VIIB in the online-only Data Supplement); intriguingly phagocytosis by CatC−−/− BMDM was largely unresponsive to lipopolysaccharide (LPS) challenge: although LPS increased efferocytosis of WT BMDM by 30.4% (P<0.003), it had little effect on CatC−−/− BMDM (6.8%, P=0.38; Figure VII in the online-only Data Supplement). Consequently, a significantly higher phagocytosis index was observed in WT compared with CatC−−/− BMDM (P<0.05; Figure VII in the online-only Data Supplement).
In addition, when testing cell migration/tissue repair in BMDM, we found no difference between the groups in any of the conditions tested (no stimulation, LPS stimulation, and data not shown).

Altogether, the above data point to autocrine-positive feedback of CatC in macrophage polarization toward M1.

**Peripheral Effects on T-Cell Function**

The increased eotaxin and TGF-β expression in CatC-deficient mice might also be reflective of or affect Th2-skewed T-cell responses. We did observe increased CD4 and lower CD8 numbers in lymph node and blood with CatC deficiency. Hence, CD4/CD8 ratios were elevated in both tissues of CatC−/− chimeras (P<0.001 and P<0.05, respectively; Figure VIII A–VIII C in the online-only Data Supplement). Moreover, relative expression of GATA3 (Th2 marker) was 2-fold increased (P<0.01), whereas that of TBX21 (Th1 marker) was unchanged (Figure IX A and IX B in the online-only Data Supplement). Of note, Foxp3 expression was also increased, albeit weakly (Figure IX B in the online-only Data Supplement), which is in accordance with the flow cytometry data showing enrichment in spleen of CatC−/− chimeras of CD4+CD25+Foxp3+ cells (P<0.05), as well as CD4+CD25+Foxp3–activated T-cells (Figure VIIIA–VIIIC in the online-only Data Supplement).

**Discussion**

CatC is reported to exert immunomodulatory effects on mast cells, macrophages, and neutrophils, which next to its intrinsic proteolytic capacity points to a potential role of CatC in inflammatory vascular remodeling processes, such as atherosclerosis. We show here that CatC is not only upregulated with lesion progression in human atherosclerotic lesions, but may also be causally involved in the pathogenesis of this disease. Hematopoietic CatC deficiency led to attenuated lesion formation in the carotid artery, the aortic arch, and the descending aorta in our mouse model. Although CatC expression appeared to be reflective of macrophage M1 polarization, it surprisingly fostered an autocrine-positive feedback loop for M1 polarization in vitro. Combined with the CatC deficiency–associated Th2 skewing, this effect may well be responsible for the observed phenotype.

In our study, the results pointed to a T cell–dependent effect of leukocyte CatC deficiency in lesion development. This is in contrast to the effect of CatC deficiency in a model of collagen-induced arthritis.17 Here, CatC deficiency reduced the development of collagen-induced arthritis, independently of T cell and B cell function. Specific measures of humoral immunity (IgG1 and IgG2a anticollagen antibodies) were comparable in WT and CatC−/− mice as was ConA-induced T cell proliferation. These are specific parameters of T and B cell function, which do not exclude difference in the parameters that were assessed in our atherosclerosis study. In addition, in our model, T cell proliferation was comparable in the WT and CatC−/− chimeric mice (data not shown). Thus, the mechanism via which CatC modulate disease outcome may differ in distinctive pathologies.

The majority of cathepsin family members showed upregulation in an advanced yet stable segment compared with a ruptured segment of a carotid lesion of the same patient. CatC was among the top 3 of upregulated cathepsins (Figure 1A). CatC was also significantly upregulated on the protein level in ruptured lesions and was largely confined to inflammatory foci within the plaque. To study the role of CatC, a mouse model of atherogenesis was used. In the collar-induced atherosclerotic lesion model, a semiconstrictive but nonstenotic collar is placed around the carotid artery in a hyperlipidemic background. The lesions are the result of perturbed hemodynamics, and not of restenosis. Several papers have shown that the processes and mechanisms that are involved in collar-induced atherosclerosis are also involved in longitudinal, age-induced models of atherosclerosis, that is, inflammation, lipid metabolism, and intraplaque hemorrhage.25–29 Mouse models model human atherogenesis, but show inherent differences with the human pathophysiology,30 the most striking of which being the virtual absence of plaque rupture; therefore, this study focused on the role of CatC in early and more advanced stages of disease progression. Our experimental mouse study suggested that CatC deficiency (or therapeutic intervention in its activity) during atherogenesis may be beneficial. Given limitations intrinsic to the animal model, extrapolating these findings to the human situation should be done cautiously, especially as CatC proteolysis could contribute to processes that are relevant to vulnerable plaque formation and rupture (eg, hemorrhage, coagulation, cap erosion, fibrinolysis). Also, human-ruptured plaques likely have entered an active wound-healing phase, and the overexpressed CatC might well be involved in the healing process after plaque rupture. Importantly, in the mouse model, the effect of CatC deficiency on disease progression was not only based on collar-induced atherosclerotic lesions, but also on other locations that were investigated (aortic arch and descending aorta, aortic root). Collectively, these data suggested that CatC deficiency affected lesion progression, with some site specificity, because the effect in the advanced lesions of the aortic arch was less evident. CatC was abundantly expressed in mouse plaques, where it colocalized mainly with foam cells and plaque macrophages. Because intimal CatC expression and CatC activity were almost absent in chimeric mice with hematopoietic CatC deficiency, we may infer that plaque CatC is of leukocyte origin.

The reduced plaque formation during CatC deficiency was similar to murine intervention studies with other cathepsin family members, such as CatS and CatK.19,20 Similar to leukocyte CatS deficiency, CatC deficiency appeared to prevent plaque progression beyond the early macrophage-rich stage. CatC deficiency did not affect CatK expression but slightly induced CatS expression. As the chromosomal location of both genes (chr.3, 3 F2.1) differs from that of CatC (chr.7), CatS upregulation may be compensatory, rather than a transgenesis artifact.

The lack of effect of CatC deficiency on plaque collagen content led us to investigate peripheral effects of CatC on leukocyte function. Because no overt differences were
seen in granulocyte and monocyte counts, we focused on effects of CatC on monocyte/macrophage differentiation. CatC expression was downregulated in IL-4–primed macrophages and upregulated in IFN-γ–primed macrophages. In this regard, the CatC expression profile mimics that of established M1 markers, such as IL-19 and iNOS, and the proinflammatory proteases MMP9 and MMP12. CatC expression was previously reported to be responsive to IRF8 and PU.1, suggesting a link to macrophage differentiation and interferon signaling.31 The most intriguing finding, however, was that CatC deficiency dampened M1 but not M2 polarization in vitro, the net effect being macrophage skewing toward M2. Although macrophage motility on a gelatin matrix was not affected during CatC inhibition, effecytosis of apoptotic cells by CatC-deficient macrophages was impaired during proinflammatory conditions. The CatC deficiency–associated reduction in necrotic core expansion seems to confirm that apoptotic cell handling is not notably defective in CatC−/− plaque macrophages. The blunted LPS response on effecytosis in CatC deficiency is interesting and may suggest interference with Toll-like receptor signaling, in analogy to the previously reported inhibition of TLR9 signaling by CatK32 for protein kinases.33 Direct evidence for CatC for such interaction is, however, lacking. Despite the clear in vitro polarization phenotype, a concomitant shift in local plaque macrophage polarization was not observed, although this was assessed using a single M1 and M2 marker and only at 1 (end) timepoint. This is possibly related to the overt dominance of M1 macrophages in early and more advanced stages of plaque development, as also reported by Stöger et al.34 Of note, this does not exclude that CatC deficiency could be associated with a dampened M1 polarization stage in plaques.

In relation to the M2 shift in vitro, increased eotaxin plasma levels and a concomitant increase in spleen eotaxin and TGF-beta expression were noted. Eotaxin is generally viewed as a prototypical Th2 cytokine,35 and compatible with this notion, CatC deficiency led to reduced CD4+/CD8+ ratios in blood and spleen and elevated GATA3, but not TBX21, expression in spleen T cells. Whether Th2-favored immune responses ameliorate or worsen atherosclerosis is still subject to controversy36; thus, consequently, whether the CatC deficiency–associated Th2 shift affords atheroprotection remains to be determined.

Of note, as shown by Devadas et al, a critical step in Th2/Th1 cell differentiation involves selective granzyme B–mediated apoptosis of Th2 cells.37 Hence, leukocyte deficiency in CatC, a key enzyme in granzyme activation, could well favor survival of Th2 cells. Although conceivable, further studies will be required to establish this pathway. The CD4+ T cell pool was enriched in CD25+Foxp3+ Tregs numbers, and in agreement, Foxp3 expression by spleen T cells was elevated as well. As demonstrated in numerous studies, Tregs display atheroprotective activity (for a review, see Ref. 38). Whether this effect is directly attributable to CatC deficiency remains to be elucidated.

In conclusion, we are the first to show that CatC gene and protein expression is upregulated in atherosclerotic lesions of both mice and man. Its expression seems confined to inflammatory foci within the plaque and, in particular, involves intimal foam cells and macrophages. CatC deficiency led to an impaired atherogenic response. Although effects of CatC deficiency on plaque matrix composition seemed subtle, our studies unveiled an unexpected feedback effect on macrophage activation programs in vitro, as well as on T helper cell differentiation. In that regard, our findings could open the way for selective tuning of innate and adaptive immune responses, relevant to chronic immune diseases, such as atherosclerosis.

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Disclosures
None.

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Significance

Cysteine cathepsins are a family of proteases involved in protein turnover and extracellular matrix degradation. Given their central role in extracellular matrix remodeling, cathepsins have been linked to several diseases, such as osteoporosis, cancer, and cardiovascular disease. Cathepsin (Cat) C has a unique aminodipeptidyl peptidase activity and has a role in leukocyte granule activity. Although there is considerable interest for the role of the Cathepsin family members, such as CatS, CatK, CatL in vascular diseases, there is no data yet on the role of CatC in experimental atherosclerosis. In this study, it was shown that CatC deficiency attenuated plaque progression in mice with atherosclerosis. CatC deficiency had unexpected effects on macrophage activation programs and T helper cell differentiation. These data suggest that CatC has a role in the selective tuning of innate and adaptive immune responses, relevant to a chronic immune disease, such as atherosclerosis.
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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 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.ª, 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 (4μm) 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°C (5% CO₂; 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 [³H]-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 β-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); CD8a eFluor450 (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°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:** 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
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. 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

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

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Legend Supplemental Fig. I: Semi-quantitative analysis of CatC expression in early, stable and ruptured human lesions as determined by immunohistochemistry. *= p<0.05.
Legend Supplement Fig. II: Serum cholesterol levels pre-, after 6 w diet and at sacrifice of WT (n=15) and CatC-/- chimeric mice (n=11)
Legend Supplemental Fig. III: Further analysis of the collar-induced atherosclerotic lesions in the carotid artery, A: Contribution of hematopoietic CatC to total plaque CatC expression in WT and CatC-/− mice. Further analysis of plaque components is shown in B: Lipid core area, C: Collagen content, D: Macrophage content and E: CD3+ cells.
Legend Supplement Fig. IV. A: plaque area of early and B: advanced lesions in the aortic arch, C: number of total, early and advanced lesions in the aortic arch, D: “En face” analysis of the percentage of lipid accumulation relative to abdominal and thoracic aorta surface area. * = p<0.05, ** p=<0.01.
Supplement Fig. V

A. Percentage of monocytes in the blood (Lys6high-positive cells). Relative mRNA expression of CatS (B) and CatK (C) in spleen of WT (n=15) and CatC-/- chimeric mice (n=11). D: CatK activity (relative to protein concentration, normalized to M0) in lysates of Wt and CatC-/- BMDM, in M0, M1 (stimulation with IFNγ) and M2 macrophages (stimulation with IL-4) in vitro (cells of n=4 mice). E-F: Relative mRNA expression of IL-18 (E) and TNF-alpha (F) in BMDM in basal conditions and after stimulation with IL-4 and IFNγ in vitro (in four wells from 3 pooled mice). * = p<0.05, *** p=<0.001.
Legend Supplemental Fig. VI. Immunohistochemical staining of aortic root atherosclerotic lesions of WT (panel A, C) and CatC-/- chimera mice (panel B, D) for iNOS (M1 marker, panel A-B) and Arg-1 (M2 marker, panel C-D)
Legend Supplemental Fig. VII. A.: Uptake of Dil labeled LDL and ox-LDL in BMDM of WT (n=4) and CatC--/- mice (n=3), B: Uptake of apoptotic cells by BMDM in basal and LPS-stimulated conditions (triplicate wells, from 3 pooled donor WT and CatC--/- mice, analysis of 4-6 photos/well).
Supplement Fig. VIII

A. Lymph nodes

B. Blood

C. CD4/CD8 Ratio

Supplemental Fig. VIII, continued next page, legend on next page
Legend Supplemental Fig. VIII. A-C: shows FACS analysis of lymph nodes (left panels) and blood (right panels) in WT (n=11) and CatC-/ chimeric mice (n=9), A: CD4+ T cells, B: CD8+ T cells, C: CD4/CD8 ratio. D-E: Analysis of splenic CD4 T cells subpopulations CD4CD25FoxP3neg (D) and CD4+CD25+FoxP3+ (E) in WT (n=4) and CatC/- mice (n=3). *= p<0.05, ** p=<0.01, ***= p<0.001.
Legend Supplemental Fig. IX: A-D. Relative mRNA levels corrected for CD4 expression in spleen of TBX21 (A), FoxP3 (B), RorγT (C) and GATA3 (D) in WT (n=15) and CatC/-/- chimeric mice (n=15)