Auto-Antigenic Protein-DNA Complexes Stimulate Plasmacytoid Dendritic Cells to Promote Atherosclerosis

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Background—Inflammation has been closely linked to auto-immunogenic processes in atherosclerosis. Plasmacytoid dendritic cells (pDCs) are specialized to produce type-I interferons in response to pathogenic single-stranded nucleic acids, but can also sense self-DNA released from dying cells or in neutrophil extracellular traps complexed to the antimicrobial peptide Cramp/LL37 in autoimmune disease. However, the exact role of pDCs in atherosclerosis remains elusive.

Methods and Results—Here we demonstrate that pDCs can be detected in murine and human atherosclerotic lesions. Exposure to oxidatively modified low-density lipoprotein enhanced the capacity of pDCs to phagocytose and prime antigen-specific T cell responses. Plasmacytoid DCs can be stimulated to produce interferon-α by Cramp/DNA complexes, and we further identified increased expression of Cramp and formation of neutrophil extracellular traps in atherosclerotic arteries. Whereas Cramp/DNA complexes aggravated atherosclerotic lesion formation in apolipoprotein E−deficient mice, pDC depletion and Cramp-deficiency in bone marrow reduced atherosclerosis and anti–double-stranded DNA antibody titers. Moreover, the specific activation of pDCs and interferon-α treatment promoted plaque growth, associated with enhanced anti–double-stranded–DNA antibody titers. Accordingly, anti–double-stranded DNA antibodies were elevated in patients with symptomatic versus asymptomatic carotid artery stenosis.

Conclusions—Self-DNA (eg, released from dying cells or in neutrophil extracellular traps) and an increased expression of the antimicrobial peptide Cramp/LL37 in atherosclerotic lesions may thus stimulate a pDC-driven pathway of autoimmune activation and the generation of anti–double-stranded-DNA antibodies, critically aggravating atherosclerosis lesion formation. These key factors may thus represent novel therapeutic targets. (Circulation. 2012;125:1673-1683.)

Key Words: atherosclerosis ■ dendritic cells ■ immune system ■ inflammation ■ leukocytes

Atherosclerosis is a chronic inflammatory disease of the arterial wall, modulated by innate and adaptive immune responses. Besides monocytes/macrophages, T cells and notably also plasmacytoid dendritic cells (pDCs) can be detected within human atherosclerotic lesions.1–4 Plasmacytoid DCs uniquely express high levels of Toll-like receptor (TLR) 7 and 9 specialized for sensing pathogenic single-stranded nucleic acids during viral and microbial infections, resulting in the production of large amounts of type-I interferons (IFN-α/β).5,6 Although pDCs usually do not respond to self-DNA,7 it became evident that cathelicidin antimicrobial peptide LL37 (known as Cramp in mice) can convert self-DNA into a trigger of pDC activation in human autoimmune diseases (eg, systemic lupus erythematosus or psoriasis).7 Additionally, neutrophil extracellular traps (NETs) and circulating immune complexes containing self-DNA and antimicrobial peptides were recently shown to trigger pDC activation via TLR9.8–11

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Previously, human plaque–residing pDCs were shown to respond to type-A oligonucleotides (CpGs) with an enhanced...
IFN-α expression, which in turn amplified inflammatory cytokine responses and immune cell activation,3,4 suggesting proatherogenic functions of this DC subset. Conversely, pDCs can also exert tolerogenic functions, as shown in a vascular graft model.12 Thus, the role of pDCs in atherosclerosis remains to be conclusively explored.

Methods

Mouse Models
Apolipoprotein E–deficient (ApoE−/−), low-density lipoprotein–deficient (Ldlr−/−), lymphocyte-deficient (Rag1−/−), and wild-type mice (C57BL/6J) were purchased from the Jackson Laboratory. At 6 to 8 weeks, female mice were subjected to a high-fat diet (HFD, 21% fat, 0.15% cholesterol, Altromin). Plasmacytoid DC depletion in ApoE−/− mice was performed by i.v. injection of the monoclonal antibody PDCA113,14 (500 μg/mouse, Miltenyi Biotech) or an isotype control antibody (500 μg/mouse, BioXcell) at day 1 and day 7 after the initiation of HFD feeding. PDCA1 antibody treatment efficiently depleted pDCs in lymph nodes, spleen, and bone marrow while not altering cDC, T-cell or B-cell frequencies, as assessed by fluorescence-activated cell sorting (FACS) analysis (online-only Data Supplement Figure I). Some mice were injected with oligonucleotide CpG 1858 (25 μg/mouse, Invivogen) complexed to DOTAP (15 μg/mouse, Roche) or with DOTAP or PBS alone 3 times/week for 4 weeks. Some mice were i.p. injected with murine IFN-γ (100 000 U, HyCultBiotec) 2 times/week for 4 weeks. Some ApoE−/− mice were i.v. injected with Cramp/DNA complexes15 3 times/week for 4 weeks, together with receiving PDCA1 or isotype control antibody. Female Ldlr−/− mice were irradiated and reconstituted with Cramp+/− or Cramp+/+ bone marrow17 and after recovery were placed on HFD for 4 weeks. Some ApoE−/− mice were treated with Cramp (i.p. 100 μg/mouse), DNase I (i.v., 120 U/mouse, Sigma), or both 3 times/week for 4 weeks. No changes in cholesterol or triglyceride serum levels, as assessed by EnzyChrom high-density lipoprotein and LDL/VLDL Assay Kit, were observed between groups of mice (online-only Data Supplement Table I). Rag1−/−/Ldlr−/− mice were fed a HFD for 12 weeks. High-fat diet–fed lysosome M (lys)–enhanced green fluorescent protein (EGFP) ApoE−/− mice18 were i.v. injected with 250 μL of liposomes containing clodronate19 1 day before and propidium iodide (2 μg/mL final concentration) immediately before imaging. Experiments were approved by local authorities.

Atherosclerotic Lesion Quantification and Immunohistochemistry
The extent of atherosclerosis was assessed in aortic roots and in aortas by staining for lipid depositions with Oil-red-O.17 The relative content of macrophages and the localization of pDCs, Cramp, and plaque immunoglobulin Gs (IgGs) were determined by immunofluorescence staining. Nuclei were counterstained with 4',6-Diamidino-2-phenylindol (DAPI). Human carotid artery specimens were obtained from autopsy or vascular surgery. The location of blood dendritic cell antigen-2 (BDCA2) and LL37 expression in the plaque was determined using standard immunohistochemistry techniques.20

DC Isolation and Stimulation In Vitro
Plasmacytoid DCs were isolated from spleens of C57BL/6J mice using the Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec). Alternatively, pDCs were sorted (FACSARia, BD Bioscience) from bone marrow of C57BL/6J with an antibody cocktail against CD45,SiglecH, and CD45RA (B220). Isolated pDCs were stimulated with oxidatively modified low-density lipoprotein (ox-LDL) or di-ox-LDL (50 μg/mL), Cramp (50 μg/mL, Inovogen), DNA (10 μg/mL, mouse C57Bl6/J female genomic DNA, Zaygen), CpG 1585 (5 μg/mL, Invivogen), or Cramp/DNA complexes15 (10 μg Cramp, mixed with 2 μg of DNA), 4 μL of mouse serum containing hGH (36 μU/mL) or low (1 μU/mL) titers of anti–double-stranded DNA (antidsDNA) antibodies per well, or serum obtained from Rag1−/−/Ldlr−/− mice.

Antigen-Specific T-Cell Proliferation In Vivo
CD4+ T cells were isolated from the spleen of OT-II mice (CD4 T cell isolation kit, Miltenyi), and labeled with CFSE (Fluka). Plasmacytoid DCs prestimulated with oxLDL (50 μg/mL), CpG1585 (5 μg/mL), or a combination of both were pulsed for 45 minutes with OVA232 to 310 peptide (30 μg/mL). Untreated pDCs were used as control. For in vivo studies, 5×105 CD4+ CFSE-labeled OT-II cells were i.v. injected into C57BL/6 recipient mice. One day later, 1×105 untreated or treated OVA-2 peptide-pulsed pDCs were injected into the footpad. After 3 days, T-cell proliferation was analyzed by CFSE dye dilution in peripheral lymph nodes of recipient mice by flow cytometry.

Flow Cytometry and ELISA
Lymph nodes and spleen were dissociated into single-cell suspensions using collagenase D, and aortas digested using Liberase III (Roche). Staining for flow cytometric analysis was conducted using combinations of specific antibodies from eBiosciences, Miltenyi, and BD Bioscience. Data was acquired using a FACSAnaltyt (BD Bioscience), and results analyzed by FlowJo software (Tree Star).

Cell culture supernatants or sera were analyzed by ELISA detecting IFNα (VeriKine Mouse Interferon-Alpha ELISA Kit, PBL Laboratories), dsDNA (Mouse antidsDNA Kit, Alpha Diagnostic), and antidsDNA antibodies (Varelis dsDNA Antibody ELISA, Phadia) according to the manufacturer’s protocol; antidsDNA Ab (+) >55 IU/mL. Reciprocal serum dilutions to determine antinuclear antibodies in humans were performed by indirect immunofluorescence on Hep-2 substrate according to the manufacturer’s instructions (ANA IFT IgG Kit, Viramed); (+) >1:160. Antibodies to malondialdehyde-modified (MDA)-LDL and oxLDL were measured by chemiluminescent ELISA.21

Quantitative Real-Time Polymerase Chain Reaction
RNA was isolated from aortas with the ZR RNA MicroPrep RNA isolation kit (Zymo Research), and complementary DNA was reverse transcribed from total RNA (Promega). Real-time polymerase chain reaction analysis was performed using SYBRGreen (Ferments) and specific primer pairs. Amplification was performed in duplicate using a 7900 HT fast real-time polymerase chain reaction system (Applied Biosystems).

Two-Photon Microscopy
In vivo 2-photon microscopy of exposed carotid arteries was performed as described.22 In short, the exposed left carotid artery was visualized using an upright Olympus FV1000MPF 2-photon microscope system equipped with a 1.05 water dipping objective. A pulsed Ti-Sapphire laser (MaiTai DeepSee, Spectra Physics) was tuned to 810 nm for visualization of second harmonic generation (SHG) signal of collagen, green fluorescent protein (GFP), and propidium iodide. Emitted fluorescent signals were detected with 3 photomultiplier tubes. Series of subsequent xy-sections (256×256 pixels) were obtained over time at an acquisition rate of 4 Hz. All image processing was performed using Image-Pro 3D Analyzer 7.0 (Media Cybernetics).

Human Microarray Data Evaluation
Seventeen atherosclerotic tissue samples from atherosclerotic carotid artery segments (9 from early and 8 from advanced lesions) from the Maastricht Pathology Tissue Collection were processed as described.23 Total RNA was isolated and purified and RNA quality determined using a 21000 Bioanalyzer (Agilent Technologies). Samples with an RNA integrity number of 5 were further processed (mean RNA integrity number =7.8). Double-stranded complementary DNA was synthesized (One-Cycle Target Labeling Kit, Affymetrix) and used as a template for the preparation of biotin-labeled complementary RNA using the GeneChip IVT Labeling Kit (Affymetrix).
di-oxLDL uptake in vitro, plaque sizes between IgG- and PDCA1-detected by immunofluorescence in the aortic root of apolipoprotein E– deficient (Apoe<sup>−/−</sup>) mice. Mann Whitney tests were applied to compare pDC frequencies and percentages within quadrants are shown, SSC indicates side scatter (n=5 mice per group). Bars represent median with interquartile range. C, SiglecH (SigH) and interferon α (Ifn-α) messenger RNA (mRNA) expression in aortae of HFD-fed Apoe<sup>−/−</sup> mice (n=5) relative to expression in healthy 6-week-old Apoe<sup>−/−</sup> mice (control, n=6 mice per group). Bars represent mean ± SD. D, Interferon α serum protein levels in HFD-fed Apoe<sup>−/−</sup> mice (n=9) relative to expression in healthy 6-week-old Apoe<sup>−/−</sup> mice (n=5) on NC. Bars represent mean ± SD. **P<0.01, ***P<0.001. Apoe, apolipoprotein E; pDCs, plasmacytoid dendritic cells; NC, normal chow; HFD, high-fat diet; mRNA, messenger RNA; ctrl, control; SiglecH, sialic acid binding immunoglobulin-like lectin H; and IFNα, interferon α.  

**Figure 1.** Plasmacytoid dendritic cells (pDCs) accumulate in atherosclerosis. A, Single SiglecH<sup>+</sup> pDCs (green, red arrow heads) were detected by immunofluorescence in the aortic root of apolipoprotein E– deficient (Apoe<sup>−/−</sup>) mice fed a high-fat diet (HFD) for 12 weeks; cell nuclei are counterstained with DAPI (blue). B, Enzymatic digestion and fluorescence-activated cell-sorting (FACS) analysis of aortae of healthy 6-week-old Apoe<sup>+/+</sup> mice on normal chow (NC) and Apoe<sup>−/−</sup> mice fed a HFD for 12 weeks; representative dot blots and percentages within quadrants are shown, SSC indicates side scatter (n=5 mice per group). Bars represent median with interquartile range. C, SiglecH (SigH) and interferon α (Ifn-α) messenger RNA (mRNA) expression in aortae of HFD-fed Apoe<sup>−/−</sup> mice (n=5) relative to expression in healthy 6-week-old Apoe<sup>−/−</sup> mice (control, n=6 mice per group). Bars represent mean ± SD. D, Interferon α serum protein levels in HFD-fed Apoe<sup>−/−</sup> mice (n=9) relative to expression in healthy 6-week-old Apoe<sup>−/−</sup> mice (n=5) on NC. Bars represent mean ± SD. **P<0.01, ***P<0.001. Apoe, apolipoprotein E; pDCs, plasmacytoid dendritic cells; NC, normal chow; HFD, high-fat diet; mRNA, messenger RNA; ctrl, control; SiglecH, sialic acid binding immunoglobulin-like lectin H; and IFNα, interferon α.

**Study Patients**

A total of 30 patients with advanced carotid artery stenosis (>70%, determined by measuring the peak systolic velocity by ultrasound) were included in this study. Asymptomatic patients (n=15) without neurological symptoms were diagnosed at the Vascular Centre of the Technical University Munich; symptomatic patients (n=15) displaying neurological symptoms (transient ischemic attack or nondisabling stroke) were assigned from the Institute of Neurology of the hospital Klinikum rechts der Isar, Munich. Patients with chronic kidney disease, diabetes mellitus, autoimmune disease, and ongoing steroid or other immunosuppressive medication were excluded from the study. Blood sampling was performed within 2 days preceding surgical intervention.

**Statistics**

Unpaired Student t tests were used to compare Cramp messenger RNA (mRNA) and dsDNA antibody titers between control and HFD-fed Apoe<sup>−/−</sup> mice, dsDNA antibody titers between IgG- and PDCA1-treated Apoe<sup>−/−</sup> mice, and dsDNA antibody titers and plaque size between Cramp<sup>+/+</sup>and Cramp<sup>−/−</sup>mice. Mann Whitney tests were applied to compare pDC frequencies and IFN-α levels between control and HFD-fed Apoe<sup>−/−</sup> mice, Di-oxLDL uptake in vitro, plaque sizes between IgG- and PDCA1-treated Apoe<sup>−/−</sup> mice, and dsDNA antibody titers between patients with asymptomatic versus symptomatic carotid artery stenosis. ANOVA 1-way analysis of variance followed by the Tukey multiple comparison post tests were used for comparing SigH and IFNα mRNA expression between control and HFD-fed Apoe<sup>−/−</sup> mice, CD36 expression, OVAd-488 uptake, and IFN-α secretion by pDCs in vitro, plaque size between controls, CpG, and CpG+PDCA1-treated, or Cramp and Cramp+DNAse-treated Apoe<sup>−/−</sup> mice. Kruskal-Wallis tests were applied to compare T-cell proliferation in vivo and plaque size between controls, Cramp+DNA+isotype, and Cramp+DNA+PDCA1-treated Apoe<sup>−/−</sup> mice. Differences where P was <0.05 were considered statistically significant.

**Results**

Plasmacytoid DCs are not only present in lymph nodes and spleen (online-only Data Supplement Figure II) but could also be detected in atherosclerotic lesions. As shown by staining for Siglec-H<sup>+</sup>, single pDCs were localized to the plaque shoulders and areas bordering the necrotic core in aortic root plaques of Apoe<sup>−/−</sup> mice fed a HFD for 12 weeks (Figure 1A). Compared with healthy wild-type Apoe<sup>+/+</sup> mice or Apoe<sup>−/−</sup> mice on normal chow, diet-fed Apoe<sup>−/−</sup> mice displayed higher frequencies of aortic PDCA1<sup>+</sup>pDCs (Figure 1B, data not shown). In addition, mRNA transcripts of SiglecH and Ifn-α were increased in aortic tissue together with elevated serum protein levels of IFN-α in Apoe<sup>−/−</sup> mice fed a HFD compared with Apoe<sup>−/−</sup> mice on normal chow (Figure 1C and D).

Modified lipoproteins (eg, oxLDL) are deposited in the arterial wall during atherogenesis<sup>1,2</sup> and can be taken up by...
CD11c⁺ DCs in the vessel wall. Moreover, oxLDL has been found to induce the upregulation of scavenger receptors on DCs. We therefore tested the capacity of pDCs to take up and to respond to oxLDL. Indeed, sorted pDCs were capable of taking up di-labeled oxLDL (Figure 2A). Similar to prototypical TLR7/TLR9 activation with CpGs, exposure to oxLDL selectively enhanced surface expression of CD36 (Figure 2A), which has been implicated in its phagocytosis. However, oxLDL did not alter surface expression of CD68, SR-A, MHC-II, and CD86 nor secretion of IFN-α protein, whereas MHC-II and CD86 expression increased after CpG stimulation (online-only Data Supplement Figure IIIA and data not shown).

Mature pDCs are capable of initiating T-cell responses. We therefore tested the capacity of pDCs to take up and to respond to oxLDL. Indeed, sorted pDCs were capable of taking up di-labeled oxLDL (Figure 2A). Similar to prototypical TLR7/TLR9 activation with CpGs, exposure to oxLDL selectively enhanced surface expression of CD36 (Figure 2A), which has been implicated in its phagocytosis. However, oxLDL did not alter surface expression of CD68, SR-A, MHC-II, and CD86 nor secretion of IFN-α protein, whereas MHC-II and CD86 expression increased after CpG stimulation (online-only Data Supplement Figure IIIA and data not shown).

Figure 2. Oxidatively modified low-density lipoprotein (oxLDL) can be taken up by plasmacytoid dendritic cells (pDCs) and enhances pDC-driven T-cell responses. A, The uptake of Di-labeled oxLDL (left) and expression of CD36 (right) after exposure to oxLDL, type-A oligonucleotides (CpGs), or PBS over 12 hours by sorted plasmacytoid dendritic cells (pDCs) assessed by fluorescence-activated cell-sorting (FACS) analysis; representative histograms with fluorescence minus 1 (FMO) controls and quantifications of mean fluorescence of 3 independent experiments are shown. Bars represent median with interquartile range. B, Sorted pDCs (unpulsed control), OVA-2–pulsed pDCs pretreated with or without oxLDL or CpGs over 12 hours were transferred into wild-type mice transfused with CFSE⁺ OT-II T cells. After 3 days, T-cell proliferation was quantified by CSFE dilution and FACS analysis; representative dot plots and percentage of CFSE⁺ CD4⁺ T cells (red markers) within gates are shown, n=4 independent experiments. Bars represent median with interquartile range. C, The uptake of 488-labeled OVA peptide pretreated with PBS, oxLDL (left) or CpGs (right) over 12 hours was assessed in sorted pDCs by FACS analysis; representative histograms with FMO controls and quantifications of mean fluorescence of 3 independent experiments are shown. Bars represent mean±SD. *P<0.05, **P<0.001. oxLDL indicates oxidatively modified low-density lipoprotein; and CpG, type-A oligonucleotide.
weeks compared with vehicle-treated mice (online-only Data Supplement Figure IVB). To further assess sequelae of pDC activation, diet-fed Apoe/H/H/H mice were repetitively injected with CpGs over 4 weeks. Lesion formation and macrophage accumulation were exacerbated in the aortic root and aorta in CpG-injected Apoe/H/H/H mice compared with control-treated mice (Figure 3B, online-only Data Supplement Figure IVC). These effects were abrogated in Apoe/H/H/H mice additionally depleted of pDCs by PDCA1 antibody injections (Figure 3B, online-only Data Supplement Figure IVC). These findings clearly demonstrate that pDCs and their effector cytokine IFN-α drive early lesion development.

Breakdown of tolerance to otherwise inert self-DNA fragments released by dying cells6 (eg, in psoriasis) can be triggered by Cramp, an antimicrobial peptide rapidly released from neutrophil granules,28,29 which binds self-nucleic acids to form complexes that trigger TLR7/TLR9 activation and type-I IFN secretion by pDCs.11,15 Also, NETs containing self-DNA and antimicrobial peptides released by activated neutrophils during cell death, a process termed “NETosis,” can trigger pDC activation.7,10,30 Like CpGs and dsDNA containing CpG motifs15 (online-only Data Supplement Figure VA), wild-type Bl6 DNA complexed with Cramp (but not DNA or Cramp alone) induced IFN-α production by murine pDCs (Figure 4A), without affecting MHC-II expression (online-only Data Supplement Figure VB). Likewise, a pDC-dependent increase in IFN-α serum levels was observed in Bl6 mice infused with Cramp/DNA complexes (online-only Data Supplement Figure VC), confirming pDC responses toward DNA-containing complexes also in vivo.

An increased expression of Cramp mRNA (Figure 4B) and Cramp protein (23.1±6.9 versus 0% staining/plaque area) was observed in the vessel wall of diet-fed Apoe−/− mice compared with healthy Apoe−/− controls. Cramp was detected in the vicinity of segment-nucleated neutrophils within atherosclerotic lesions (Figure 4C), likely reflecting neutrophil accumulation during early lesion development,17 and in colocalization with small DNA fragments in necrotic core areas (Figure 4C). Using 2-photon microscopy of atherosclerotic carotid arteries, we furthermore revealed the presence of NETs in atherosclerotic carotid arteries and within plaques of monocyte-depleted LysmEGFP/EGFP Apoe−/− mice carrying only fluorescent neutrophils (Figure 4D, data not shown). Extracellular NETs were detected by use of propidium iodide, a vital cell-impermeable nuclear marker that stained clouds of DNA in the vicinity of EGFP neutrophils at atherosclerotic sites and could be observed as early as 2 weeks after the initiation of diet (online-only Data Supplement Table II). Notably, the injection of Cramp/DNA complexes into Apoe−/− mice promoted an enhanced atherosclerotic lesion formation in the aortic root when compared with control-injected Apoe−/− mice, which was averted in mice additionally depleted of pDCs by PDCA1 antibody treatment (Figure 4E). These data clearly indicate that Cramp/self-DNA complex-mediated pDC activation drives atherosclerotic plaque development. Thus, Cramp-complexed self-DNA derived from neutrophil-expelled NETs and dying plaque cells may contribute to breakdown of tolerance to self-DNA.7–10,31

In systemic lupus erythematosus, patients were found to develop autoantibodies to self-DNA and antimicrobial peptides in NETs, indicating that these complexes serve as auto-antigens to trigger B-cell activation and the generation of anti-DNA–specific antibodies.7 We here demonstrate that serum anti-dsDNA antibody titers were increased in hyperlip-
Figure 4. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells (pDCs), and Cramp and NETs are detectable in atherosclerotic arteries. A, Interferon α (IFN-α) protein secretion by sorted pDCs stimulated overnight as indicated (Bars represent mean±SD, n=3–5 independent experiments). B, Cramp messenger RNA (mRNA) expression in aortas of atherosclerotic high-fat diet (HFD)–fed apolipoprotein E–deficient (Apoε−/−) mice (n=9) relative to expression in healthy 6-week-old Apoε−/− mice (n=5). Bars represent mean±SD. C, Cramp staining (red) detected by immunofluorescence in the aortic root in diet-fed Apoε−/−; cell nuclei and DNA are stained by DAPI (blue); arrowheads indicate segment-nucleated neutrophils. D, Luminal presence of propidium iodide–positive extracellular DNA (red) in colocalization with enhanced green fluorescent protein–positive neutrophils (EGFP−) neutrophils (green) in atherosclerotic LysmEGFP preparations. Apoε−/− mice; collagen of the vessel wall was visualized by second harmonic generation (SHG, blue); autofluorescence of elastin bands of the vessel wall appears in aquamarine. E, Oil-red O lesions in the aortic root in diet-fed Apoε−/− mice treated with PBS (control), or Cramp/DNA complexes together with isotype control or PDCA1 antibody for 4 weeks; representative images of the aortic root are shown. Bars represent median with interquartile range. *P<0.05, **P<0.001. IFN-α indicates interferon α; mRNA, messenger RNA; HFD, high-fat diet.

Notably, diminished antisDNA antibody titers were observed in Ldlr−/− mice reconstituted with Cramp−/− versus Cramp+/+ bone marrow (Figure 5C) together with a protection from atherosclerotic lesion formation (Figure 5D) and a reduction in macrophage content in the aortic root (19.2±2.4 versus 30.2±3.5%, respectively, P<0.05). Conversely, treating mice with Cramp promoted atherosclerotic plaque growth compared with control-treated Apoε−/− mice but was ineffective in mice additionally treated with DNAse to degrade extracellular DNA (Figure 5E), indicating that proatherogenic functions of Cramp require the association with extracellular self-DNA.

Immunogenic DNA-containing immune complexes have been shown to contain LL37 and to further enhance pDC activation.6–10 This concurs with results that serum of Apoε−/− mice containing high levels of antisDNA antibody titers but not serum containing low titers significantly increased IFN-α production in pDCs (Figure 5F), leaving MHC-II expression unaltered (online-only Data Supplement Figure VE). Conversely, serum derived from atherosclerotic Rag1−/−Ldlr−/− mice did not elicit IFN-α responses in pDCs (data not shown). Although we refrained from purifying idemic Apoε−/− mice compared with healthy control Apoε−/− mice (Figure 5A). Conversely, antisDNA antibody titers were reduced in Apoε−/− mice treated with PDCA1 antibody to deplete pDCs compared with IgG-injected controls. Furthermore, antisDNA titers were elevated in IFN-α– and CpG-treated Apoε−/− mice but not in CpG-injected mice depleted from pDCs (online-only Data Supplement Figure VI). Interestingly, humoral responses to other known auto-antigens in atherosclerosis, namely MDA-LDL and oxLDL, were not affected by pDC depletion, as shown by unaltered IgG antibody concentrations toward these antigens in PDCA1 versus control-treated Apoε−/− mice (online-only Data Supplement Figure VI). This indicates that pDCs specifically promote dsDNA antibody formation in early atherosclerosis. However, increased MDA-LDL and oxLDL–specific IgG antibody levels were observed in CpG-treated Apoε−/− mice but not in mice additionally treated with PDCA1 antibody (online-only Data Supplement Figure VI). This suggests that strong pDC activation also promotes unspecific amplification of humoral immune responses to antigens in atherosclerosis, likely because of abundant type-I IFN production.32,33
IgG-containing immune complexes because of the limited amount of serum available from mice, these data suggest that IgG complexes containing DNA in serum may contribute to pDC activation. Interestingly, abundant IgG deposits could be detected in atherosclerotic plaques of diet-fed Apoe/H11002 mice, predominantly in the vicinity of necrotic plaque areas (Figure 5G). Thus, auto-immunogenic complexes deposited within lesions in vivo may contribute to the pathogenic insult in atherosclerosis.

To assess whether these mechanisms may also be relevant to human disease, differential expression analysis was performed on gene expression microarrays from human atherosclerotic lesions. Comparisons of early and advanced carotid artery specimens revealed an increase in expression of pDC markers in advanced versus early lesions (online-only Data Supplement Table III). Accordingly, BDCA2+ pDCs and LL37 were detected in advanced human carotid plaques but less frequently in early lesions (Figure 6A). Negative controls showed no staining (data not shown). This suggests that the presence of pDCs correlates with plaque progression in human atherosclerosis. Furthermore, reciprocal serum dilutions to determine auto-antibodies directed against nuclear antigens revealed average titers of 1:190.0±99.1 in asymptomatic patients but 1:871.1±548.4 in symptomatic patients (n=8–9, P=0.1336). Moreover, elevated levels of circulating antidsDNA antibodies were observed in patients with symptomatic compared with asymptomatic carotid artery stenosis (Figure 6B); age, risk factors, and medication were not different between patient groups (online-only Data Supplement Table IV).
Discussion

In recent years, it has become evident that a number of specialized immune cell populations contribute to atherogenesis. Besides monocytes/macrophages, several T-cell subsets, conventional DCs, and neutrophils have emerged as critical players in atherogenesis. The role of pDCs, however, has remained elusive to date. Here we provide the first evidence that a pDC-dependent pathway of autoimmune activation critically drives early atherosclerotic lesion formation.

Plasmacytoid DCs have previously been found in carotid artery plaques, primarily in the plaque shoulder region. In addition, reduced circulating pDC counts have been described in patients with coronary artery disease, which may correspond to their enhanced accumulation within atherosclerotic lesions. Accordingly, we observed an accumulation of pDCs in murine and human atherosclerotic plaques but not in other secondary lymphoid organs (not shown) and an increased expression of pDC markers in advanced versus early human atherosclerotic carotid artery lesions.

Described as inflammatory amplifiers, plaque-residing pDCs were shown to respond to CpGs (containing motifs typically found in microbial DNA) with an enhanced IFN-α expression, which in turn amplified inflammatory TLR4, TNF-α, IL-12, and matrix metalloproteinase-9 expression by myeloid DCs and cytolytic T-cell functions, and which correlated with plaque instability. In order to scrutinize the role of pDCs in atherosclerosis, we employed a specific pDC-depleting antibody. In line with the notion that pDCs exert proinflammatory effects, we here demonstrate that pDCs and their activation critically drive atherosclerotic lesion formation. Administration of a PDC-A1 antibody to deplete pDCs protected from lesion formation. Conversely, stimulation of Apoe/−/− mice with type-B CpGs, known to activate B cells rather than pDCs, was previously shown to not affect atherosclerotic lesion formation. These findings, furthermore, corroborate that activating pDCs rather than other cell types by type-A CpGs (used in our study) mediates enhanced plaque growth. Although reduced IFN-α serum levels could be observed in pDC-depleted mice, administration of IFN-α aggravated lesion formation and macrophage plaque content in Apoe/−/− mice, extending findings that treatment with IFN-α or IFN-β accelerates atherosclerotic lesion formation and that IFN-β promotes macrophage accumulation in plaques. Plasmacytoid DC–derived type-I IFNs can likewise also activate other immune cells, such as cDCs and B cells, to promote autoimmunity, which may also be relevant in atherosclerosis.

Plasmacytoid DCs have also been shown to exert tolerogenic functions; in a vascularized graft model, pDC depletion or prevention of pDC lymph-node homing inhibited regulatory T-cell differentiation and tolerance induction whereas adoptive transfer of tolerized alloantigen-presenting pDCs prolonged graft survival. In the present study, we clearly demonstrate that pDCs exert proatherogenic functions during early lesion formation.

The responsiveness of pDCs to viruses or bacterial infections, as epitomized by treatment of Apoe/−/− mice with CpGs, may corroborate the link between atherosclerosis and chronic infection burdens and possibly the inflammatory activation of vulnerable plaques in response to acute infections. However, nucleotides released from necrotic or apoptotic cells may also be prone to binding TLR7/TLR9 and to inducing IFN-α production by pDCs in the presence of antimicrobial peptides released from inflammatory cells. For instance, self-DNA fragments released by dying cells (eg, in psoriasis) can be bound by Cramp, an antimicrobial peptide rapidly released from neutrophil granules to form complexes that trigger TLR7/TLR9 activation and type-I IFN secretion. Similarly, NETs,
web-like structures containing self-DNA and antimicrobial peptides, can trigger pDC activation.\textsuperscript{10,11,30}

These factors may also be present in atherosclerosis plaques. Extending findings in humans, where an increased expression of LL37 has been described in atherosclerotic carotid artery plaques compared with normal arteries in colocalization with macrophages and endothelial cells,\textsuperscript{40} we here observed increased Cramp expression in atherosclerotic Apoe\textsuperscript{−/−} arteries. Cramp protein was detected in colocalization with DNA fragments in plaque necrotic core areas, suggesting that Cramp/self-DNA complexes may form in atherosclerosis. Notably, injection of Cramp promoted atherosclerotic plaque growth dependent on the presence of extracellular self-DNA. Thus, the formation of Cramp/self-DNA complexes may contribute to pDC activation and enhanced atherosclerosis, as exemplified by their capability of triggering IFN-α secretion by pDCs in vitro and their pDC-dependent acceleration of lesion formation in Apoe\textsuperscript{−/−} mice in vivo. Cramp may be released by neutrophils recruited during early lesion development,\textsuperscript{17,29,41} as implied by its detection in the vicinity of segment-nucleated neutrophils in plaques. In addition, we unprecedentedly observed the formation of NETs in atherosclerotic carotid arteries. These Cramp/self-DNA complexes contained in neutrophil-processed NETs may further trigger pDC activation.\textsuperscript{10,11} Cytokines including IFN-α may reciprocally prime neutrophils to allow the formation of NETs,\textsuperscript{42} thus driving positive feedback amplification.

Interestingly, oxLDL enhanced phagocytosis of pDCs and their capacity to prime antigen-specific T-cell responses while not directly contributing to IFN-α responses or co-stimulatory molecule upregulation. Exposure to oxLDL within plaques\textsuperscript{12} may thus boost uptake of antigenic complexes by pDCs and their activation in atherosclerosis.

Interestingly, diminished antidsDNA antibody titers were observed in Ldlr\textsuperscript{−/−} mice reconstituted with Cramp\textsuperscript{+/−} versus Cramp\textsuperscript{+/+} bone marrow, together with a protection from atherosclerotic lesion formation. Moreover, antidsDNA antibody titers but not antibodies toward MDA- or oxLDL-atherosclerotic lesion formation. In accordance with this, serum of Apoe\textsuperscript{−/−} mice containing high levels of antidsDNA antibody titers but not serum containing low titers significantly increased IFN-α production in pDCs in vitro whereas serum derived from atherosclerotic Rag1\textsuperscript{−/−}Ldlr\textsuperscript{−/−} mice did not elicit IFN-α responses in pDCs. Thus, antidsDNA antibodies, generated as a consequence of pDC activation, may contribute to the pathogenic insult in atherosclerosis.

Although values may be of limited clinical relevance in a rheumatological context, increased levels of circulating antidsDNA antibodies in atherosclerotic Apoe\textsuperscript{−/−} mice, as well as in patients with symptomatic and more advanced atherosclerosis, may support a pathogenic role of antidsDNA antibodies. Importantly, elevated anti-nuclear antibody titers were associated with decreased carotid elasticity in young Finns and were postulated to participate in the development of early atherosclerosis.\textsuperscript{44} Moreover, in an animal model of systemic lupus erythematosus with enhanced antibody titers against dsDNA, atherosclerotic lesion formation was accelerated.\textsuperscript{45}

Chronically increased IFN-α levels and circulating antids-DNA antibody titers in patients with psoriasis and systemic lupus erythematosus\textsuperscript{5} may likewise predispose for an increased risk to develop hyperlipidemic atherosclerosis.\textsuperscript{46}

Collectively, our data provide evidence for a hitherto unrecognized pathway of autoimmune activation in atherosclerosis, driven by pDCs that sense self-DNA fragments complexed to Cramp and contribute to the formation of anti-nuclear antibodies. Exposure to oxLDL during atherogenesis may amplify the immunogenicity of pDCs by enhancing phagocytosis and priming of antigen-specific T-cell responses. Targeting pDC activation may thus constitute a promising approach to limit atherosclerotic lesion development.

**Acknowledgments**

We thank Melanie Garbe, Yvonne Jansen, Theresa Moritz, Silvia Roubrocks, Susanne Schmitz, Melanie Schott, Roya Sojtan, and Stephanie Wilbertz for excellent technical assistance.

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**Disclosures**

None.

**References**

CLINICAL PERSPECTIVE

Atherosclerosis remains the number 1 cause of death in the Western world, and the therapeutic options currently available are limited. Chronic inflammation of the vessel wall has been closely linked to autoimmunogenic processes in atherosclerosis. The role of the specialized subset of plasmacytoid dendritic cells present in human atherosclerotic plaques, however, has not been addressed previously. We provide evidence for a hitherto unrecognized plasmacytoid dendritic cell–driven pathway of autoimmune activation in atherosclerosis that critically amplifies early atherosclerotic lesion formation. Self-DNA (eg, released from dying cells or in neutrophil extracellular traps) and an increased expression of the antimicrobial peptide Cramp/LL37 in atherosclerotic lesions was shown to stimulate breakdown of tolerance to self-DNA and promote interferon-α production by plasmacytoid dendritic cells, aggravating early atherosclerosis and the formation of anti–double-stranded DNA antibodies in apolipoprotein E–deficient mice. Notably, anti–double-stranded DNA antibodies were also found to be elevated in patients with symptomatic versus asymptomatic carotid artery stenosis. Although possibly of limited clinical relevance in a rheumatological context, moderately increased levels of circulating anti–double-stranded DNA antibodies may predispose to atherosclerosis, in line with findings showing elevated anti-nuclear antibody titers to be associated with decreased carotid elasticity and early atherosclerosis. More importantly, chronically increased interferon-α levels and circulating anti–double-stranded DNA antibody titers may provide an explanation for the increased risk of atherosclerosis well recognized in patients with psoriasis and systemic lupus erythematosus.
Auto-Antigenic Protein-DNA Complexes Stimulate Plasmacytoid Dendritic Cells to Promote Atherosclerosis

Yvonne Döring, Helga D. Manthey, Maik Drechsler, Dirk Lievens, Remco T.A. Megens, Oliver Soehnlein, Martin Busch, Marco Manca, Rory R. Koenen, Jaroslav Pelisek, Mat J. Daemen, Esther Lutgens, Martin Zenke, Christoph J. Binder, Christian Weber and Alma Zernecke

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Supplemental Methods

Mouse Models

Apoe^{−/−}, Ldlr^{−/−} and Rag1^{−/−} and C57BL/6J wild type mice were purchased from the Jackson Laboratory. Six to eight weeks old female Apoe^{−/−} mice were fed an atherogenic diet (21% fat, 0.15% cholesterol, Altromin). For in vivo stimulation of pDCs, mice were injected with the oligonucleotide (ODN) CpG 1585 (25 µg/mouse, Inovagen) complexed to DOTAP (15 µg/mouse, Roche) in 200 µl PBS, or with DOTAP or PBS (Gibco) alone 3 times/week for 4 weeks. Efficiency of pDC stimulation was confirmed by determining IFNα concentrations in serum (data not shown). pDC depletion in Apoe^{−/−} mice was performed by intravenous injection of the monoclonal antibody PDCA-1\(^1\) (500 µg/mouse, Miltenyi Biotech) or an isotype control antibody (500 µg/mouse, BioXcell) at day 1 and day 7 after the initiation of high fat diet feeding. Some Apoe^{−/−} mice were injected with murine IFNα (100,000 U, HyCult Biotec) twice a week i.p. for 4 weeks. Some Apoe^{−/−} mice were i.v. injected with Cramp/DNA complexes\(^*\) (100 µg Cramp, Innovagen, mixed with 20 µg of DNA, ODNs, Integrated DNA technologies, in 200 µl of PBS/mouse) 3 times/week for 4 weeks, together with receiving PDCA1 or isotype control antibody (see injection schema below).
Six to eight weeks old female \textit{Ldlr}^{-/-} mice (Jackson laboratory) were irradiated and reconstituted with \textit{Cramp}^{-/-} or \textit{Cramp}^{+/+} bone marrow as described\textsuperscript{4}. Briefly, bone marrow cells \((5\times10^6\) in PBS) were administered to \textit{Ldlr}^{-/-} mice by intravenous tail vein injection 24 h after an ablative dose of whole-body irradiation \((2\times6.5\) Gy). After recovery, transplanted \textit{Ldlr}^{-/-} mice were placed on high fat diet for 4 weeks. Another group of female \textit{Apoe}^{-/-} mice was treated with Cramp (i.p., 100 µg/mouse) with or without DNase I (i.v., 120 U/mouse, Sigma Aldrich) 3 times/week for 3 weeks. No changes in cholesterol or triglyceride serum levels, as assessed by EnzyChrom\textsuperscript{TM} HDL and LDL/VLDL Assay Kit (BioAssay Systems), were observed between groups of mice (Supplemental Table 3). \textit{Rag1}^{-/-} mice were crossed with \textit{Ldlr}^{-/-} mice (\textit{Rag1}^{-/-}\textit{Ldlr}^{-/-} mice) and fed an atherogenic diet for 12 weeks. Lysozyme M (\textit{lys})-enhanced green fluorescent protein (EGFP) mice\textsuperscript{5}, kindly provided by Dr. T. Graf (Albert Einstein College, New York), were i.v. injected with 250 µl of liposomes containing clodronate\textsuperscript{6} one day and PI \((2\mu g/ml\) final concentration) immediately before imaging. Efficient monocyte depletion and the identification of remaining circulating EGFP\textsuperscript{+} cells as SSC\textsuperscript{hi} CD115\textsuperscript{-} CD11b\textsuperscript{+} Gr-1\textsuperscript{+} neutrophils was confirmed by FACS analysis (data not shown). Experiments were approved by local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany) to comply with German animal protection law.

\textit{Atherosclerotic lesion quantification and immunohistochemistry}

The extent of atherosclerosis was assessed in aortic roots and in aortas by staining for lipid depositions with oil-red-O \textsuperscript{4} and quantified by computerized image analysis (Diskus
Software, Hilgers, Königswinter) and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Briefly, atherosclerotic lesions were quantified in 5 μm transverse sections through the heart and the aortic roots. The thoraco-abdominal aorta was opened longitudinally, and the percentage of lipid deposition was calculated by dividing the stained area by total aortic surface. The relative content of macrophages was determined by mAb staining for MOMA-2 (MCA519, AbD Serotec) or Mac-2 (M3/38, Cedarlane). pDCs were detected by staining for Siglec-H (440c, HyCult Biotech). A rabbit anti-Cramp antibody (Innovagen) was used to detect Cramp on acetone fixed fresh frozen sections. Plaque IgG was detected using a biotinylated rabbit anti-mouse IgG (Vector Laboratories) following by a streptavidin-conjugated Alexafluor-555 (Molecular Probes) on 4% paraformaldehyde-fixed frozen sections. Nuclei were counter-stained by 4',6-Diamidino-2-phenylindol (DAPI). Images were recorded with a Leica DMLB fluorescence microscope and CCD camera. Human carotid artery specimens containing all stages of atherosclerosis (from intimal xanthoma to fibrous cap atheroma with plaque rupture) were obtained from autopsy or vascular surgery (IMCAR tissue collection; n=3-6 per plaque type). Atherosclerotic plaques were fixed o/n in 4% paraformaldehyde, processed, embedded in paraffin and sectioned (4 μm). The location and intensity of BDCA2 (mAB, Clone AC144, Miltenyi Biotec) and LL37 (mAB, Clone 3D11, Hycult Biotech) expression in the plaque was determined using standard immunohistochemistry techniques.
**pDC isolation and stimulation in vitro**

pDCs were isolated from spleens of C57BL/6J mice using the Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec) according to the manufactures’ protocol. Alternatively pDCs were sorted (FACSAria, BD Bioscience) from bone marrow of C57BL/6J with an antibody cocktail against CD45, SiglecH and CD45RA (B220). Purity of isolated pDCs was consistently above >90-95% (Miltenyi) and >90% (sorting), as assessed by FACS analysis (data not shown). Isolated pDCs were seeded at 1 x 10^5 cells/200µl into flat bottom 96-well plates in RPMI-1640 medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin/streptomycin (100 U/ml), β-mercaptoethanol (50 µM, Sigma) and 25 ng/ml FLT3-L (Peprotech) and were stimulated with Cramp (50 µg/ml, Innovagen), DNA (10 µg/ml µg, mouse C57Bl6/6J female genomic DNA, Zyagen, or ODNs, Integrated DNA technologies), CpG 1585 (5µg/ml, Invivogen), oxLDL or di-oxLDL (50 µg/ml), and 4 µl of mouse serum containing high (36 Units/µl) or low (1 Unit/µl) titers of anti-dsDNA antibodies per well, and serum obtained from Rag1^-/-Ldlr^-/- mice. For the generation of complexes, 10 µg of Cramp was mixed with 2 µg self-DNA in 20 µl of PBS and incubated for 15 min at room temperature.

**Antigen-specific T-cell proliferation in vivo**

CD4^+^ T cells were isolated from the spleen of OT-II mice by negative selection of CD4^+^ T cells (CD4 T cell isolation kit, Miltenyi), and labeled with CFSE (Fluka). 1x10^5 pDCs were pre-stimulated with oxLDL (50 µg/ml), CpG 1585 (5 mg/ml) or in combination with both and were pulsed for 45 min with OVA_323-339_ peptide (30µg/ml) (ISQAVHAAHAEINEAGR, OVA-2, ANASPEC) for MHC class II-restricted T-cell
stimulation. Untreated pDCs were used as control. For in vivo studies, \(5 \times 10^6\) CD4\(^+\) CFSE-labeled OT-II cells were injected into the tail vein of C57BL/6 recipient mice. One day later, \(1 \times 10^5\) untreated or treated OVA-2 peptide-pulsed pDCs were injected into the footpad. After 3 days, single-cell suspensions were prepared from peripheral LNs of recipient mice to analyze T-cell proliferation by CFSE dye dilution by flow cytometry.

*Enzymatic tissue digestion and flow cytometry*

For FACS analysis of tissues: lymph nodes and spleen were dissociated into single-cell suspensions using collagenase D (Roche) for 30 min at 37\(^\circ\)C. Aortas were excised, flushed in PBS and digested using Liberase III (Roche). Staining for flow cytometric analysis was conducted using combinations of antibodies from eBiosciences (CD11c, CD11b, CD115, B220, CD8a, 440c, CD36, CD68, SR-A, CD80, CD86, CD40, CD3, CD4, CD45), Miltenyi (PDCA-1) and BD Bioscience (MHC II, Gr-1) in HBSS with 0.3 mM EDTA and 0.1% BSA. Data were acquired using a FACSCanto II (BD Bioscience), and results analyzed by FlowJo software (Tree Star).

*Quantitative real time-polymerase chain reaction (PCR)*

RNA was isolated from aortas with the ZR RNA MicroPrep RNA isolation kit (Zymo Research) according to manufacturer’s protocols. cDNA was reverse transcribed from 2 \(\mu\)g of DNase-treated total RNA (Promega). Real time-PCR analysis was performed using SYBRGreen (Fermentas) and specific primer pairs (Sigma) according to manufacturer’s protocols:

\[\text{Ifn-\(\alpha\):} 5'\text{-TCTGATGCAGCA}G\text{TGGG-3', 5'\text{-AGGGCTCTCCAGACTTCTGCTTG-3'}}\];

\[\text{SiglecH:} 5'\text{-GCTGGGATGCTGCTGCTCCCG-3', 5'\text{-GGTGAGGCAAGACAAAGGACA-3'}}\];
Cramp: 5'-TCCCAAGTCTGTGAGGTTCC-3', 5'-CCCATACACTGCTTCACCAC-3',

Gapdh: 5'-CCATCACCATCTTCCAGGAG-3', 5'-GTGGTTCACACCCATCACAA-3'.

Amplification (45 cycles, annealing at 58°C) was performed in duplicate using a 7900 HT fast real time PCR system (Applied Biosystems, USA).

Two-photon microscopy

In vivo two-photon microscopy of exposed carotid arteries was performed as described\(^1\). In short, the exposed left carotid artery was visualized using an upright Olympus FV1000MPE two-photon microscope system equipped with a 1.05; water dipping objective (Olympus GMBH). A pulsed Ti-Sapphire laser (MaiTai DeepSee, Spectra Physics) was tuned to 810 nm for visualization of second Harmonic Generation (SHG) signal of collagen, GFP, and PI. Emitted fluorescent signals were detected with three photomultiplier tubes (PMTs) tuned to corresponding parts of the emission spectra: SHG, 400-470 nm (PMT 1); GFP, 500-550 nm (PMT 2); PI, 560-660 nm (PMT 3); autofluorescent signals from elastin bands was detected mainly in PMT 2 and PMT 3. Series of subsequent xy-sections (256x256 pixels) were obtained over time at an acquisition rate of 4Hz. Only comparable xy-sections without severe motion artefacts (due to heart- and respiration cycle\(^1\), were selected for further analyses. Overall image quality was improved by 2D Gaussian weighted filtering\(^1\) by resizing the pixel matrix two times (bilinear scaling). All image processing was performed using Image-Pro 3D analyzer 7.0 (Media Cybernetics).
ELISA

Cell culture supernatants or mouse sera were analyzed by ELISA detecting IFNα (VeriKine™ Mouse Interferon-Alpha ELISA Kit, PBL laboratories), and double stranded DNA (Mouse anti-dsDNA Kit, Alpha Diagnostic) according to the manufacture’s protocol. Anti-dsDNA antibodies in human serum were analyzed by Varelisa dsDNA Antibody ELISA (Phadia); anti-dsDNA Ab (+) >55 IU/mL. Reciprocal serum dilutions to determine autoantibodies directed against nuclear antigens (ANAs) in humans were performed by indirect immunofluorescence on HEp-2 substrate according to the manufacturer’s instructions (ANA IFT IgG Kit, Viramed). Positive ANA titers (>1:160) were revealed to be present in 25% of asymptomatic subjects, but 56% of symptomatic patients. In all cases of positive titers, either homogeneous or speckled staining patterns could be observed, in line with the recognition of nuclear antigens. Antibodies to malondialdehyde-modified (MDA)-LDL and oxLDL were measured by chemiluminescent ELISA. Briefly, sera were diluted 1:100 and binding of IgG to MDA-LDL and oxLDL were measured using AP-conjugated rat-anti-mouse IgG, followed by detection with LumiPhos.

Human microarray data evaluation

17 atherosclerotic tissue samples from atherosclerotic carotid artery segments, 9 from early and 8 from advanced lesions, have been retrieved from the Maastricht Pathology Tissue Collection (MPTC). Tissue was obtained during autopsy (Dept of Pathology, Maastricht University Medical Centre) and extracted from carotid arteries, as during carotid endarterectomy procedures. Collection, storage in the Maastricht Pathology...
Tissue Collection (MPTC) and use of tissue and patient data were performed in
agreement with the "Code for Proper Secondary Use of Human Tissue"
(http://www.federa.org/?s=1&m=99). Samples were processed as described previously. In brief, carotid artery specimens were divided into parallel sections for RNA isolation and histological analysis immediately after resection. Carotid artery segments were snap frozen for RNA isolation and adjacent sections were fixed and embedded in paraffin. Based on 4 μm haematoxylin-eosin stained sections, segments were classified according to Virmani et al. Total RNA was isolated using the guanidine isothiocyanate/CsCl method followed by further purification and concentration using RNease mini columns (Qiagen). RNA quantity and quality were determined using a nanodrop spectrometer (Witec AG) and a 20100 Bioanalyzer (Agilent Technologies) respectively. Only samples with a RNA Integrity Number of at least 5 were further processed (mean RIN=7.8). Double stranded cDNA was synthesized from about 2 μg of total RNA using the One-Cycle Target Labeling Kit (Affymetrix), and used as a template for the preparation of biotin-labeled cRNA using the GeneChip IVT Labeling Kit (Affymetrix). Biotin-labeled cRNA was hybridized in duplicate (15 hybrids from early, 14 hybrids from advanced samples) to the Human Genome U133 Plus 2.0 Array (Affymetrix), washed, stained with phycoerythrin-streptavidin conjugate (Molecular Probes) and the signals were amplified by staining with biotin-labeled anti-streptavidin antibody (Vector Laboratories) followed by phycoerythrin-streptavidin. Arrays were laser scanned with the GeneChip Scanner 3000 (Affymetrix) according to the manufacturer's instructions. Data were saved as raw images and quantified using GCOS 1.2 (Affymetrix). The resulting .CEL files have been processed in R (R Development Core). Uploading, background adjustment and quantile
normalization have been performed by affy\textsuperscript{18} according to the vignette's instruction. The resulting expression set has been analyzed by limma\textsuperscript{19}, combining the technical replicates and treating patients IDs as random factors. After Bayesian smoothing of the standard errors of the estimated log-fold changes, the transcripts of interest have been identified within the top list and are reported as reported as log2 fold changes, average expression, and Benjamin-Hochberg false discovery rate adjusted p-values. Housekeeping genes (HPS90 and ACTB) were unaltered in advanced versus early plaques (HSP90AB6P, fold change 0.990109516457994, adj.p.val 0.150944030384547; HSP90B1, fold change 0.97436370698835, adj.p.val 0.627452445273284; HSP90AA1, fold change 1.02995157375258, adj.p.val 0.724731894920322; ACTB, fold change 1.00904889957475, adj.p.val 0.915087042539356). Data have been uploaded to the GEO database (accession: http://www.ncbi.nlm.nih.gov/geo, GSE28829).

Study patients
A total of 30 patients with advanced carotid artery stenosis (>70%, determined by measuring the peak systolic velocity by ultrasonography) were included in this study. All subjects gave informed consent and the study was approved by an institutional review committee. Asymptomatic patients (n=15) without neurological symptoms were diagnosed within the scope of a clinical work-up in consequence of arterial hypertension and/or diabetes mellitus type 2 or by random screenings at the Vascular Centre of the Technical University Munich; Symptomatic patients (n=15) displaying neurological symptoms (transient ischaemic attack or non-disabling stroke) were assigned from the Institute of Neurology of the hospital Klinikum rechts der Isar, Munich. Patients with
chronic kidney disease (eGFR < 60 ml min⁻¹), diabetes mellitus, autoimmune disease, and ongoing steroid or other immunosuppressive medication were excluded from the study. Blood sampling was performed within two days prior to surgical intervention by vein puncture. Venous blood was centrifuged at 2,000 x g for 10 minutes at 20°C and serum stored at -70°C until analysis.

Statistics of Supplemental Data

Unpaired Student \( t \) tests were used to compare dsDNA-antibody titers between control and IFN-\( \alpha \)-treated Apoe\(^{-/-} \) mice. Mann Whitney tests were applied to compare pDC frequencies between isotype and PDCA1-treated mice, plaque size and macrophage content between IgG and PDCA1, and PBS and IFN-\( \alpha \)-treated Apoe\(^{-/-} \) mice. ANOVA 1-way analysis of variance followed by Tukey's multiple comparison post tests were used for comparing plaque size between control, CpG and CpG+PDCA1-treated Apoe\(^{-/-} \) mice, IFN-\( \alpha \) secretion \textit{in vitro} and \textit{in vivo}, and IgG antibody titers to MDA- and CuoxLDL. Kruskal-Wallis tests were applied to compare IFN-\( \alpha \) secretion by pDCs co-cultured with T cells \textit{in vitro}, macrophage content between controls, CpG and CpG+PDCA1-treated Apoe\(^{-/-} \) mice. Differences where \( P<0.05 \) were considered to be statistically significant (Prism 4.0 software, GraphPad).
### Supplemental Table 1

Serum lipid levels in experimental groups of mice.

<table>
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<th><strong>cholesterol</strong> (mg/dL)</th>
<th><strong>triglycerides</strong> (mg/dL)</th>
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<tr>
<td><strong>Apoe-/-</strong></td>
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<td></td>
<td></td>
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<tr>
<td>IgG</td>
<td>1145.0±233.6</td>
<td>335.4±93.5</td>
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<tr>
<td>PDCA1</td>
<td>1047.0±299.9</td>
<td>275.9±71.1</td>
<td>5</td>
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<tr>
<td>Control</td>
<td>1157.0±108.1</td>
<td>311.3±51.9</td>
<td>3</td>
</tr>
<tr>
<td>CpG</td>
<td>1110.0±69.3</td>
<td>338.0±82.1</td>
<td>4</td>
</tr>
<tr>
<td>PDCA1 + CpG</td>
<td>1025.0±119.5</td>
<td>342.6±77.8</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>1173.0±141.9</td>
<td>285.0±52.4</td>
<td>6</td>
</tr>
<tr>
<td>IgG + Cramp/DNA</td>
<td>1060.0±124.6</td>
<td>334.1±124.8</td>
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<tr>
<td>PDCA1+ Cramp/DNA</td>
<td>1187.0 ±160.7</td>
<td>364.0±65.0</td>
<td>10</td>
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<tr>
<td>Control</td>
<td>794.7±102.8</td>
<td>208.3±56.3</td>
<td>4</td>
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<tr>
<td>Cramp</td>
<td>718.6±118.2</td>
<td>181.3±41.2</td>
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<td>709.7±94.2</td>
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<tr>
<td>Ldlr-/-</td>
<td>259.3±95.6</td>
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<tr>
<td><strong>Cramp-/-</strong></td>
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<tr>
<td>Ldlr-/-</td>
<td>266.0±31.5</td>
<td>36.2±28.5</td>
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</tr>
</tbody>
</table>
**Supplemental Table 2**

Quantification of luminal PI⁺ extracellular DNA (NETs) in atherosclerotic Apoε⁻/⁻ mice.

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<tr>
<th>Western Diet</th>
<th>2 wks</th>
<th>4 wks</th>
<th>6 wks</th>
<th>normal chow</th>
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<tr>
<td>n = mice</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td># mice with NETs (%)</td>
<td>1 (20)</td>
<td>2 (50)</td>
<td>1 (33)</td>
<td>0 (0)</td>
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<tr>
<td># luminal NETs in group</td>
<td>4</td>
<td>15</td>
<td>1</td>
<td>0</td>
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**Supplemental Table 3**


<table>
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<th>gene symbol</th>
<th>log2 fold change</th>
<th>log intensity</th>
<th>adjusted P-value</th>
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<tr>
<td>CLEC4C (BDCA2)</td>
<td>2.530811401</td>
<td>3.14</td>
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<tr>
<td>FCGR2A (CD32)</td>
<td>1.465154699</td>
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<td>4.68E-03</td>
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<tr>
<td>BST2 (CD317)</td>
<td>1.457550632</td>
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<tr>
<td>TCF4 (E2-2)</td>
<td>1.291250583</td>
<td>9.92</td>
<td>6.98E-03</td>
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<tr>
<td>CD83</td>
<td>1.165029107</td>
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<td>IL3RA (CD123)</td>
<td>1.175892357</td>
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**Supplemental Table 4**

Characteristics of asymptomatic and symptomatic patients

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<th>symptomatic (n=15)</th>
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<td><strong>Age, years</strong></td>
<td>69.7±8.1</td>
<td>69.9±10.4</td>
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<td><strong>Sex, females/males</strong></td>
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<td><strong>Smokers (%)</strong></td>
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<td><strong>ACE-inhibitor</strong></td>
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Supplemental Figure 1

Efficiency and specificity of pDC depletion. Frequencies of CD3^+ T cells, B220^+ B cells, CD11c^{high} MHCII^+ cDCs, and PDCA1^+ B220^+ pDC were as assessed among CD45^+ leukocytes by FACS analysis in Apoe^-/- mice 24 hours after i.v. injection of PDCA1 or isotype control antibody in lymph node, spleen and bone marrow. Bars represent median with interquartile range (n=4 each). Representative dot plots of cell distributions in bone marrow are shown; boxed regions indicate pDCs.
Supplemental Figure 2

Localization of pDCs in LN and spleen. SiglecH⁺ pDCs (green) can be detected by immunofluorescence and FACS analysis in lymph nodes and spleens of wild type mice.
Supplemental Figure 3

Expression of surface markers and IFN-α by pDCs in response to different stimuli. (A) Expression of CD68, SR-A, MHCII and CD86 in sorted pDCs stimulated with/without oxLDL overnight, assessed by FACS analysis; representative histograms with FMO/fluorescence minus one controls of 3 independent experiments are shown. (B) IFNα secretion in supernatants of OT-II T cells left untreated or co-cultured with unpulsed pDCs (control), OVA-2-pulsed pDCs overnight pretreated with or without oxLDL or CpG over 3 days. Bars represent median with interquartile range, n=4 independent experiments. *P<0.05.
Atherosclerotic lesion formation in the aorta and plaque macrophage content in Apoe\(^{-/-}\) mice treated with PDCA1 antibody, recombinant IFN-\(\alpha\) or CpG. (A) Atherosclerotic lesions were quantified in the aorta after staining with oil-red O in Apoe\(^{-/-}\) mice fed a high fat diet and treated with IgG or PDCA1 antibody. The relative content of MOMA-2\(^+\) macrophages per total plaque cells was analyzed by quantitative immunofluorescence. Bars represent median with interquartile range. (B) Quantification of atherosclerotic lesions in the aorta after staining with oil-red O and of the relative frequencies of MOMA-2\(^+\) macrophages per total plaque cells in the aortic root in Apoe\(^{-/-}\) mice fed a high fat diet and treated with PBS (control) or IFN\(\alpha\). Bars represent median with interquartile range. (C) Quantification of atherosclerotic lesions in the aorta after staining with oil-red O and of the relative frequencies of MOMA-2\(^+\) macrophages per total plaque cells in the aortic root of Apoe\(^{-/-}\) mice fed a high fat diet and treated with DOTAP (control), CpG or CpG and PDCA1 antibody for 4 weeks. Bars represent mean ± SD (plaque area) and median with interquartile range (macrophage content). *\(P<0.05\). **\(P<0.01\)
Supplemental Figure 5

pDC stimulation with Cramp/DNA complexes or serum, and anti-ds-DNA antibody titers in IFN-α-treated Apoe<sup>-/-</sup> mice. (A) IFN-α protein secretion by sorted pDCs stimulated overnight as indicated (n= 3-4). *P<0.05, ***P<0.001 vs. untreated, #P<0.05 vs. Cramp; †P<0.05 vs. DNA. (B) Expression of MHC-II in sorted pDCs stimulated overnight with Cramp, Cramp+DNA or DNA alone, assessed by FACS analysis; representative histograms with FMO/fluorescence minus one controls of 3 independent experiments are shown. (C) Bl6 mice were injected as indicated and IFN-α serum concentrations were assessed after 4 hours (n= 9-10). *P<0.05 vs. IgG controls, #P<0.05 vs. Cramp/DNA + IgG. (D) Anti-ds-DNA antibody titers were quantified in serum of Apoe<sup>-/-</sup> mice fed a high-fat diet for 4 weeks and injected with PBS (control) or IFNα. n=6 mice per group. *P<0.05. (E) Expression of MHC-II in sorted pDCs stimulated overnight with serum with low (▼) or high (▲) anti-dsDNA antibody titers, assessed by FACS analysis; representative histograms with FMO/fluorescence minus one control of 5 independent experiments are shown. Bars represent mean ± SD.
Supplemental Figure 6

Quantification of IgG autoantibodies directed against MDA-modified (left panel) and oxidized LDL (right panel) in serum of Apoe\(^{-/-}\) mice fed a high-fat diet for 4 weeks and treated with IgG or PDCA1 antibody, or DOTAP (control), CpGs or CpGs+PDCA1 for 4 weeks. Bars represent mean ± SD, n=5-12 mice per group, *P<0.05. **P<0.01.
Supplemental References


