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Abstract
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Reference

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Background—Plasmacytoid dendritic cells (pDCs) bridge innate and adaptive immune responses and are important regulators of immuno-inflammatory diseases. However, their role in atherosclerosis remains elusive.

Methods and Results—Here, we used genetic approaches to investigate the role of pDCs in atherosclerosis. Selective pDC deficiency in vivo was achieved using CD11c-Cre × Tcf4–/flox bone marrow transplanted into Ldlr−/− mice. Compared with control Ldlr−/− chimeric mice, CD11c-Cre × Tcf4–/flox mice had reduced atherosclerosis levels. To begin to understand the mechanisms by which pDCs regulate atherosclerosis, we studied chimeric Ldlr−/− mice with selective MHCII deficiency on pDCs. Significantly, these mice also developed reduced atherosclerosis compared with controls without reductions in pDC numbers or changes in conventional DCs. MHCII-deficient pDCs showed defective stimulation of apolipoprotein B100–specific CD4+ T cells in response to native low-density lipoprotein, whereas production of interferon-α was not affected. Finally, the atheroprotective effect of selective MHCII deficiency in pDCs was associated with significant reductions of proatherogenic T cell–derived interferon-γ and lesional T cell infiltration, and was abrogated in CD4+ T cell–depleted animals.

Conclusions—This study supports a proatherogenic role for pDCs in murine atherosclerosis and identifies a critical role for MHCII-restricted antigen presentation by pDCs in driving proatherogenic T cell immunity. (Circulation. 2014;130:1363-1373.)

Key Words: antigen presentation ▪ atherosclerosis ▪ dendritic cells ▪ immunity ▪ lymphocytes

The first suggestion of adaptive immune activity in atherosclerosis came from the observation that human leukocyte antigen (HLA)-DR was abundantly expressed in both innate and vascular cells of human atherosclerotic lesions.1 In the late 1980s, researchers reported that low-density lipoproteins (LDL) undergo oxidative modification in vivo and incite the generation of autoantibodies to modified LDL.2 This was followed in the mid 1990s by the discovery that CD4+ T lymphocytes from human atherosclerotic lesions recognize LDL–derived antigen in an HLA-DR–dependent manner3 and by the identification of vascular dendritic cells (DCs) in human aortic intima.4 These seminal studies generated great interest in the immune mechanisms of atherosclerosis and were followed by 2 decades of intensive research into the roles of adaptive immune responses in disease initiation and progression. The studies defined the distinct roles of T lymphocyte subsets in the disease process5: Th1-biased responses promote atherosclerosis whereas Tregs play a major counter-regulatory role and limit lesion inflammation and development, in part through the antiatherogenic roles of interleukin 10 and transforming growth factor β.5–7 Until recently, however, only a few studies had addressed the contribution of DCs to the immune responses of atherosclerosis.

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DCs are detected in normal vessels preferentially in regions predisposed to atherosclerosis where they accumulate lipid and contribute to the development of early fatty streaks. Besides these lipid scavenging properties, investigators have recently interrogated the roles of DCs in shaping atherosclerotic immune responses. DCs from normal and atherosclerotic vessels are able to process and present model antigens to CD4+ T cells in a major histocompatibility complex (MHC)II-dependent manner. Adventitial DCs, like spleen and lymph node DCs, engage in sustained interactions with T cells, leading to T cell proliferation and cytokine secretion. However, the outcome of these interactions between conventional DCs (cDCs) and T cells on atherosclerosis is still unclear. For example, genetic manipulations to expand or deplete the general pool of cDCs (and CD11c-expressing macrophages) using the CD11c-diptheria toxin receptor mouse did not reduce the development or progression of atherosclerosis. This disappointing and unexpected finding could be attributed either to a dominant role of cDCs in the modulation of cholesterol homeostasis or to the critical role of cDCs in the control of steady-state myelogenesis, blurring any potential role of DCs in adaptive immune responses to atherogenic stimuli.

A few studies addressed the role of a distinct DC subset, plasmacytoid DCs (pDCs), in atherosclerosis. pDCs originate in the bone marrow, circulate in the blood and home to secondary lymphoid organs as well as sites of inflammation. pDCs are specialized type I interferon (IFNs) producers in response to virus infection and as such are major players in innate immune responses. As the name suggests, pDCs are also capable of antigen presentation to T cells, a function shown to be critical in some autoimmune disease models, although not in viral infection responses. pDCs are detected in normal and atherosclerotic vessels, both in humans and mice. Reduced blood levels of pDCs in humans are suggested to reflect increased plaque infiltration and correlate with coronary artery disease. Vascular pDCs are able to present antigen to T cells in vitro and can load a model peptide on MHCII in vivo. However, the outcome and relevance to atherosclerosis remains uncertain. Whereas some studies have suggested a proatherogenic role for pDCs, other investigators have reported an atheroprotective effect. The reasons for these discrepancies remain unknown, and the mechanisms through which pDCs alter immune responses in atherosclerosis remain elusive. In particular, each of the above-mentioned atherosclerosis studies used antibody-mediated depletion of pDCs targeting PDCA1 (BST-2/CD317), which is not entirely specific for pDCs, especially in inflammatory settings. Therefore, alternative approaches are required to definitively address the role of pDCs and the mechanisms through which they modulate immune-mediated diseases. Here, we used selective genetic approaches to interrogate the role of pDCs in the development of murine atherosclerosis. We identify a selective regulator of the pDC lineage, and CD11c-restricted deletion in CD11c+ cells, designated Tcf4+/flox (conditional Tcf4 deletion in CD11c+ cells, designated Tcf4-cko thereafter) or CD11c-Cre × Tcf4-/- mice (conditional Tcf4 deletion in CD11c+ cells, designated Tcf4-cko thereafter) or CD11c-Cre × Tcf4-/- mice (conditional Tcf4 deletion in CD11c+ cells, designated Tcf4-ko thereafter) BM. After recovery, mice were put on high-fat diet (HFD) for 8 weeks. Tcf4-/ Tcf4-ko mice displayed marked reduction of pDC (CD11c+ B220+ PDCA1+ cells; see Figure I in the online-only Data Supplement) compared with control Tcf4+/ Tcf4+ WT mice. The depletion was selective for pDCs, as we found no difference in other cell populations (T cells, B cells, monocytes, and neutrophils) in blood or lymphoid organs (Figure IIIA in the online-only Data Supplement). Of note, contrary to the phenotype of cDC-less mice, blockade of pDC...
development did not alter myelogenesis, despite chronic feeding with a HFD (Figure III in the online-only Data Supplement). We also assessed the numbers of other DC subtypes. As previously reported for Tcf4-cKO mice, a B220lo cDC-like (CD11chi MHCII+) population that derives from converted Tcf4–/– pDCs was increased in the spleen and lymph nodes (Figure 1B), consistent with the role of Tcf4 in maintaining the cell fate of mature pDC through active opposition of a cDC default program. We also found increased numbers of CD11b+ and CD8α+ cells within this population were not significantly changed (data not shown). Loss of pDCs and increase of cDCs may have effects on regulatory T cells. However, we found no differences in the levels of spleen regulatory T cells between groups (Figure III in the online-only Data Supplement).

Animal weight (Figure 1D), plasma HDL-cholesterol (2.46±0.36 mmol/L versus 2.81±0.22 mmol/L; P=0.64), and triglycerides (5.51±0.73 mmol/L versus 5.29±0.54 mmol/L; P=0.45) were similar between the 2 groups of mice. However, Ldlr−/− Tcf4-cKO mice showed a significant, although relatively small, increase of plasma total cholesterol levels in comparison with Ldlr−/− Tcf4-WT animals (Figure 1D). A similar phenotype has previously been reported in cDC-depleted Ldlr−/− or Apoe−/− mice, suggesting a similar potential role for pDCs in cholesterol metabolism. Previously, increased cholesterol in cDC-depleted mice was proposed to explain the lack of effect of cDC depletion on atherosclerosis. It is therefore remarkable that despite higher plasma cholesterol levels, pDC-less Ldlr−/− Tcf4-cKO mice showed significantly reduced atherosclerosis compared with Ldlr−/− Tcf4-WT controls (Figure 1E). Reduced lesion development was associated with a substantial reduction in plaque T cell accumulation (Figure 1F). Thus, blockade of PDC development substantially limits proatherogenic adaptive immunity, indicating a prominent role in disease development.
MHCII-Restricted Antigen Presentation to T Cells by pDCs

We next addressed the potential functions of pDCs that may be influencing atherosclerosis. In general, pDCs have so far been found to be less potent stimulators of CD4+ T cells in the presence of cognate antigen than cDCs or inflammatory/BM-derived DCs. Nevertheless, pDCs are capable of antigen presentation in a number of conditions.19,26,30 We therefore addressed the role of MHCII-dependent functions of pDCs. Aortic pDCs from Apoe−/− mice have already been shown to take up injected Eα antigen and present it in the context of MHCII.36 Aortic pDCs from Ldlr−/− mice are also capable of Eα antigen presentation (Figure 2A). Staining with the Y-Ae antibody (which recognizes the Eα peptide specifically in the context of MHCII I-Ab) was readily detectable on aortic pDCs from chow and

![Image](http://circ.ahajournals.org/)

Figure 2. Antigen presentation by plasmacytoid dendritic cells (pDCs) in vivo and in vitro. A, Chow or high-fat diet (HFD)-fed Ldlr−/− mice were injected with Eα-GFP (or PBS) and whole aortas were digested and analyzed by flow cytometry for pDC uptake of Eα-GFP using Y-Ae antibody. Data are from 4 pooled aortas/group. B, pDCs detected by staining for Siglec-H (red) in the aortic sinus of Ldlr−/− mice were able to uptake and process DQ-OVA [green or yellow (green + red)]. Cell nuclei are stained by DAPI (blue). A indicates adventitia; M, media; and P, plaque. Representative images from analysis of 5 mice. C, Proliferation of OT-II CD4 T cells after incubation with spleen CD11c+ from wild-type (WT) mice or bone marrow (BM) pDCs from WT and MHCII−/− mice incubated with or without 100 µg/mL ovalbumin. Data representative of 2 separate experiments. *P<0.05. D, OT-II CD4 T cell proliferation after incubation with pDCs from µMT or µMT:pIII+IV− pDCs with or without 100 µg/mL ovalbumin continuously. *P<0.05. E, OT-II CD4 T cell proliferation after incubation with pDCs from µMT or µMT:pIII+IV− pDCs preincubated with or without 100 µg/mL ovalbumin or CpG-B (5 µg/mL) before addition of OT-II T cells only. *P<0.05. F, OT-II T cell proliferation in vivo (% of total OT-II) after injection of ova-loaded µMT or µMT:pIII+IV− pDCs in popliteal lymph nodes from the uninjected control (Con) or injected (Inj) hindlimbs. *P<0.05. G, Activation of human ApoB100-specific T cell hybridoma (48-5T), measured by interleukin (IL) 2 secretion, after 15 h coculture with spleen CD11c+ cells or BM pDCs from µMT or µMT:pIII+IV− mice with or without native human low-density lipoprotein (LDL; 25 µg/mL). Data representative of at least 2 experiments in D and E, and 4 experiments in G with similar results. *P<0.05.
HFD-fed Ldlr−/− mice injected with Ea-GFP but not those injected with PBS (Figure 2A). In addition, after injection of DQ-OVA (a self-quenched conjugate of ovalbumin that exhibits bright green fluorescence on proteolytic degradation), cells in the aortic root plaques of Ldlr−/− mice staining positive for the pDC marker Siglec-H were also positive for processed DQ-OVA (Figure 2B).

We then investigated the ability of pDCs to present the model antigen ovalbumin to purified OVA-specific OT-II CD4+ T cells, using Mhcii−/− mice to confirm the antigen deficiency. Both wild-type (WT) splenic cDCs and BM pDCs induced T cell proliferation in the presence of OVA (Figure 2C). As expected, pDCs stimulated OT-II T cells to a lesser extent, but the majority of their effect was dependent on MHCII, because there was significantly less T cell proliferation in the presence of MHCII-deficient pDCs (Figure 2C).

To target MHCII selectively in pDCs, we took advantage of the cell and tissue-specific promoters of the MHCII transactivator (CIITA), pI, pIII, and pIV, which specifically controls expression of MHCII genes and a handful of antigen presentation–related genes. The pI promoter controls MHCII expression in cDCs, macrophages, and microglia, pIII selectively controls MHCII expression in pDCs and B cells, whereas pIV controls MHCII expression by thymic epithelial cells and immune-stimulated nonhematopoietic cells. Therefore, mice receiving BM cells lacking pIII and pIV (pIII+/IV−) allow the study of the role of MHCII-restricted antigen presentation by pDCs and B cells. We backcrossed pIII+/IV− mice with B cell–deficient μMT mice to generate (μMT:pIII+pIV−) mice lacking MHCII-restricted antigen presentation only by pDCs. Compared with μMT controls, μMT:pIII+pIV− pDCs did not express detectable MHCII above isotype control staining in flow cytometry analysis (Figure IVA in the online-only Data Supplement). Firstly, we studied the role of selective MHCII deficiency in pDCs on antigen–specific T cell activation in culture. Importantly, μMT:pIII+pIV− pDCs secreted inflammatory cytokines at normal levels in response to CpG activation (Figure IVB in the online-only Data Supplement), confirming that their innate functions were intact.

To confirm a defect in antigen presentation by pDCs in the absence of pIII+pIV, we repeated the OT-II stimulation experiments in the presence of either μMT:pIII+pIV− or control μMT BM were analyzed after 4 weeks recovery followed by a HFD for 6 weeks. Animal weights (29.69 ±0.94 versus 30.77 ±0.97) and total plasma cholesterol (7.49±0.79 g/L versus 7.71±0.52 g/L, in μMT:pIII+pIV− → Ldlr−/− and μMT:pIII+pIV− → Ldlr−/− mice, respectively, P=0.82) were similar between groups. Numbers of blood monocytes and neutrophils were also comparable between the 2 groups of mice (Figure VA in the online-only Data Supplement). Unlike Tc4+cKO mice, pIII+pIV deletion had no effect on the distribution of pDCs (Figure 3A) or cDCs (Figure 3B). However, μMT:pIII+pIV− → Ldlr−/− mice displayed a selective abrogation of MHCII expression on pDCs (Figure 3C). MHCII expression on cDCs was unaltered (Figure VB in the online-only Data Supplement) and there were no differences in cDC activation markers, including CD40, CD80, and CD86, between the 2 groups of mice (data not shown). Interestingly, aortic root lesion size was significantly reduced in μMT:pIII+pIV− → Ldlr−/− mice (Figure 3E). We therefore assessed the effect of this pDC-restricted MHCII deficiency on T cell responses. pIII+pIV deletion had no impact on Tregs levels in the spleen and did not alter their suppressive potential (Figures VC and VD in the online-only Data Supplement). However, we found a significant reduction of proatherogenic IFN-γ-producing CD4+ T cells (but no differences in interleukin 17+ T cells) in μMT:pIII+pIV− → Ldlr−/− compared with μMT:pIII+pIV− → Ldlr−/− mice, using intracellular flow cytometry staining on freshly isolated spleen T cells (Figure 3D and Figures VE and VF in the online-only Data Supplement). Importantly, there was a substantial decrease of vascular T cell infiltration in lesions of μMT:pIII+pIV− → Ldlr−/− mice (Figure 3F). Thus, MHCII expression by pDCs is required to drive a proatherogenic T cell immunity.
The Proatherogenic Effect of pDC-Selective MHCII Expression Requires the Presence of CD4+ T Cells

To further substantiate the T cell–dependent effects of pDC MHCII, we repeated the experiment with additional groups of μMT: pIII+/IV+/+ → Ldlr–/– and μMT: pIII+/IV–/– → Ldlr–/– mice receiving a depleting anti-CD4 antibody (see Methods in the online-only Data Supplement) during 8 weeks of HFD. As expected, μMT: pIII+/IV–/– mice displayed a selective abrogation of MHCII expression on pDCs (Figure 4A), and T cell depletion was substantial in anti–CD4-treated mice (Figure 4B) and maintained throughout the experiment (data not shown). Animal weights were similar between groups (Figure 4C). pII+IV deficiency had no effect on serum cholesterol, whereas CD4+ T cell depletion led to a 25% decrease (Figure 4D), as previously reported in Apoe−/−/Rag1−/− and Ldlr−/−/Rag1−/− mice.31 CD4 depletion led to a 50% decrease in atherosclerosis in μMT: pIII+/IV−/→ Ldlr−/− mice (Figure 4E), which is consistent with the phenotype of Rag1−/− deficient animals31,32 and the proatherogenic role of CD4+ T cells.33 Remarkably, CD4 depletion did not reduce lesion development in μMT: pIII+/IV−/→ Ldlr−/− mice (despite reduced cholesterol), indicating that pDC MHCII deficiency had abrogated the proatherogenic properties of CD4+ T cells (Figure 4E). The results strongly support an MHCII-CD4+ T cell dependent pathway for the proatherogenic effect of pDCs.

Selective MHCII Expression on pDCs Promotes Atherogenesis in the Presence of B Cells

B cells are known to significantly regulate atherosclerosis,14–36 and pDCs might influence B cell responses. Because the above pIII+/IV−/− experiments were performed in B cell–deficient animals, we generated B cell–sufficient mice with selective abrogation of MHCII in pDCs. To this aim, lethally irradiated Ldlr−/− mice were reconstituted with a mixture of 80% BM from μMT: pIII+/IV−/− mice and 20% BM from WT mice. In this case, B cells only develop from the 20% WT BM and are MHCII+. However, 80% of pDCs will be generated...
from the μMT:pIII+IV− BM and should therefore be deficient in MHCII. Control Ldlr−/− mice were reconstituted with a mixture of 80% BM from μMT mice and 20% BM from WT (all B cells and pDCs are MHCII+). After recovery, mice were fed a HFD for 8 weeks. Proportions of pDCs, cDCs, and T and B cells were similar between the 2 groups of mice (Figure VI in the online-only Data Supplement) and only pDCs were defective in MHCII expression (Figure 5A–5C). This pDC-specific MHCII deficiency again resulted in a significant reduction of lesion size (Figure 5D) and 70% reduction of vascular T cell
infiltration (Figure 5E) along with reduced systemic levels of IFN-γ (Figure 5F) despite no change of plasma cholesterol levels (µMT/WT: 10.96±0.61 g/L, µMT:pIII+IV−/−pDC: 9.91±0.58 g/L, P=0.22).

**Discussion**

Atherosclerosis development is driven by both innate and adaptive immune responses. Recent studies have further highlighted the role played by LDL in driving antigen-specific proatherogenic T cell immunity. T cell–mediated responses and disease severity were shown to be highly dependent on cDC subtype. CCL17-expressing DCs restrain Treg responses and promote atherosclerosis, whereas Flt3-dependent CD103+ DCs and CD11c-restricted MyD88 signaling sustain atheroprotective Tregs, as do DCs that were manipulated to exert tolerogenic activity. However, whether these distinct effects require antigen presentation by DC subsets remains elusive. Reduction of atherosclerosis in mice lacking MHCII-associated invariant chain CD74 is frequently cited as evidence for a potential role of antigen presentation in atherosclerosis. However, CD74-deficient mice display defective CD4+ T cell selection and massive reduction of thymic and spleen CD4+ T cells already in the absence of atherosclerosis, precluding any direct conclusion regarding the distinct role of antigen presentation in disease development. Therefore, the in vivo role of MHCII-restricted antigen presentation by cDCs in the development of atherogenic immunity remains unknown. In addition, as mentioned above, sustained total cDC depletion did not result in atheroprotection.

Recent studies therefore focused on the pDC subset and its potential role in atherosclerosis, but discrepant results and mechanisms were reported. As an alternative to the
antibody depletion strategy, used in all 3 previous studies that addressed the role of pDCs in atherosclerosis, we used genetically-modified mice with selective deficiency in pDCs. Our results clearly show that the development of atherosclerosis is reduced in pDC-less mice, which strongly argues in favor of a major role of pDC-mediated immunity in driving the atherogenic process.

A limitation of the depleting strategies mentioned above and the use of pDC-less mice is that they allow no conclusion about innate versus adaptive functions of pDCs in atherosclerosis. Indeed, besides their major role in shaping innate immune responses, pDCs have also been suggested to function as antigen presenting cells (APCs). They are capable of antigen cross-presentation to CD8+ T cells,44,45 express MHCII molecules, and acquire a mature phenotype to internalize, process, and present antigen to CD4+ T cells.17,18 However, such APC function could not be observed in vivo using models of virus infection and antibody-mediated pDC depletion.46 It appears that under conditions of acute viral infection, pDCs mainly act via type I IFN production,20 whereas the contributions of innate versus adaptive immune functions of pDCs to chronic immune diseases require more investigation. An APC function was recently demonstrated in a model of experimental autoimmune encephalomyelitis, in which pDCs inhibited T cell–mediated autoimmunity.19 Whether this result could be translated to other (auto)immune-mediated diseases was still unknown. Here, we addressed this question in the context of atherosclerosis by generating mice with selective abrogation of MHCII expression in pDCs and provided strong evidence for a critical role of MHCII-restricted antigen presentation by pDCs in driving proatherogenic T cell responses. The results are of high importance and should prompt a reassessment of the differential roles of pDCs and cDCs in shaping adaptive immune responses during atherogenesis.

Our results might appear in contradiction with the tolerogenic role assigned to pDCs in other settings. However, previous studies on the role of pDCs in antigen-specific CD4+ T cell responses in vivo used a disease-unrelated model antigen (ie, OVA30), which might not faithfully reproduce the regulation of disease-specific immune responses. In other studies, Irla et al15 reported an inhibitory role of MHCII-restricted antigen presentation by pDCs in a mouse model of experimental autoimmune encephalomyelitis. However, in the experimental autoimmune encephalomyelitis model, the disease process is initiated after active immunization with antigen in association with adjuvants, which is different from the spontaneous development of adaptive immune responses to endogenous LDL-derived antigens in the atherosclerosis model. APC function of pDCs might differ between these 2 different ways of induction of adaptive immunity. Finally, the outcome of antigen presentation by pDCs might depend on the nature of the presented antigen and the local microenvironment where presentation occurs. For example, exposure to oxidized LDL selectively enhanced the surface expression of the scavenger receptor CD36, with enhanced phagocytic function of pDCs and increased capacity to prime antigen-specific T-cell responses.23 It is conceivable that under basal noninflammatory conditions, LDL presentation by pDCs induces tolerogenic adaptive immune responses, which then gradually switches toward effector responses with the progressive high load of cholesterol and environmental inflammatory stimuli. This hypothesis merits further investigation.

It should be noted that the present work addressed the role of pDCs in early atherosclerosis, at which point proatherogenic T cell immunity greatly influences atherosclerosis development in mice.32 Additional studies are needed to determine the contribution of pDC-mediated immunity at later stages of disease development. Because pDCs and T cells infiltrate both early and advanced atherosclerotic lesions in humans,21–25,47,48 we speculate that our results will also bear relevance to the human disease. However, direct testing of this hypothesis is still required.

In conclusion, we present new evidence that MHCII-restricted antigen presentation by pDCs drives proatherogenic T cell immunity. The results shed new light on the role of adaptive immune responses in atherosclerosis and may have implications for the design of specific therapeutic strategies.

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Disclosures
None.

References


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CLINICAL PERSPECTIVE

Atherosclerosis is an inflammatory vascular disease driven in part by adaptive immune responses to low-density lipoprotein (LDL) cholesterol–derived antigens. The subtype of antigen-presenting cells responsible for activation of LDL-specific pro-atherogenic T cell responses has remained elusive. Plasmacytoid dendritic cells (pDCs) bridge innate and adaptive immune responses and are important regulators of immuno-inflammatory diseases. Here, we used genetic approaches to investigate the role of pDCs in atherosclerosis. We show that selective pDC deficiency in vivo reduces atherosclerosis in Ldlr−/− mice. To examine the role of antigen presentation by pDCs in atherosclerosis, we generated Ldlr−/− mice with selective MHCII deficiency in pDCs. Remarkably, these mice also developed reduced atherosclerosis compared with controls. The atheroprotective effect of selective MHCII deficiency in pDCs was associated with significant reductions of proatherogenic T cell–derived interferon-γ and lesional T cell infiltration, and was abrogated in CD4+ T cell–depleted animals. Because pDCs and T cells infiltrate both early and advanced atherosclerotic lesions in humans, we speculate that our results will also bear relevance to the human disease. However, direct testing of this hypothesis is still required, and additional studies are needed to determine the contribution of pDC-mediated immunity at later stages of disease development. In conclusion, we present new evidence that MHCII-restricted antigen presentation by pDCs drives proatherogenic T cell immunity. The results shed new light on the role of adaptive immune responses in atherosclerosis and may have implications for the design of specific therapeutic strategies.
MHC class II-restricted antigen presentation by plasmacytoid dendritic cells drives pro-atherogenic T cell immunity

Supplemental Material
Supplemental Methods

Mice

All experiments were approved by the Home Office, UK and were performed under PPL 80/2426. Ldlr⁻/⁻ and μMT mice were purchased originally from Jackson labs and were on a C57Bl/6 background. CD11c-Cre x Tcf4⁻/⁻ and CD11c-Cre x Tcf4⁺/⁺ control littermates were originally generated in the lab of Prof. B. Reizis. Mice lacking the pIII and pIV promoter elements of Class II transactivator (CIITA) (pIII+IV⁻/⁻) were maintained on a μMT (B cell deficient) background and μMT mice were used as controls. MHCII-deficient bone marrow was kindly provided by the lab of Sebastian Amigorena (INSERM U932, Institut Curie, Paris, France). For atherosclerosis experiments, male 6-8 week old Ldlr⁻/⁻ mice were lethally irradiated (9.5 Gy) then injected i.v. with 1x10⁷ bone marrow cells from donor mice. After 4 weeks recovery, mice were fed a high fat diet (21% Fat, 0.15% Cholesterol) for 6 or 8 weeks. In order to assess the effects of selective deficiency of pIII+IV on pDCs but not B cells, lethally irradiated Ldlr⁻/⁻ mice were reconstituted with a mixture of 80% bone marrow from either μMT:pIII+IV⁻/⁻ or control μMT mice and 20% bone marrow from WT C57BL6 mice. In this case, all B cells derive from the WT cells whereas 80% of all other leukocytes derive from μMT or μMT:pIII+IV⁻/⁻ cells. In some experiments, 100 µg of anti-CD4 depleting antibody (clone YTS 191.1) was injected every 10 days starting coincident with the start of HFD feeding.

Cell preparation from aorta

Cell suspension from aorta was prepared by enzyme digestion as previously described. Briefly, Ldlr⁻/⁻ mice were placed under terminal anesthesia and perfused with 2 mmol/L EDTA (Sigma-Aldrich, Gillingham, UK) in PBS via cardiac puncture to remove blood contamination from vascular tissue. After removal of aortas, a single cell suspension was obtained by incubation of aortic segments in an enzymatic suspension containing 450 U/mL collagenase type I, 125 U/mL collagenase type XI, 60 U/mL hyaluronidase, and 60 U/mL DNase (all from Sigma-Aldrich) in PBS containing 20 mmol/L Hepes at 37°C for 1 hour. Digested aortas were then mechanically disrupted through a 40-µmol/L cell strainer to release a single cell suspension. All the Abs used
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for flow cytometry analysis of mouse aorta had been validated on cell suspensions from spleen/lymph nodes untreated or treated with the enzyme digestion cocktail.

**Flow cytometry**

Single cell suspensions of bone marrow, spleen, lymph node, blood and aorta were stained with fluorophore-conjugated antibodies (Supplemental Table 1) and analyzed using LSRII Fortessa (BD) or CyAN ADP (Beckman Coulter) flow cytometers. For intracellular staining, cells were activated with leukocyte activation cocktail (BD) for 4 h. Cells were fixed with IC fixation buffer (eBioscience) before intracellular staining. Cells were processed with Foxp3 buffer set (eBioscience) before staining with Foxp3. Data was analysed using FlowJo software (TreeStar, OR, USA). Dead cells were excluded based on FSc, SSc and positive staining for Live/Dead Aqua (Life Technologies). pDCs were defined as CD11c<sup>lo</sup> PDCA1<sup>hi</sup> B220<sup>+</sup> CD11b<sup>-</sup>, cDC as CD11c<sup>hi</sup> MHCII<sup>+</sup>, B cells as B220<sup>+</sup> IgM<sup>+</sup> or CD19<sup>+</sup> lymphocytes, T cells as CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes, Treg as CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> GITR<sup>+</sup>, monocytes as CD11b<sup>+</sup> Ly6G<sup>-</sup> and Ly6C low, intermediate or high, neutrophils as CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup>. Representative plots are shown in Supplemental Figure 1. In some experiments, Siglec-H (Miltenyi) was used as an additional pDC marker.

**Analysis of in vivo antigen uptake/processing**

To study the ability of aortic pDCs to present systemic antigen, we used the Ea-GFP/Y-Ae system as described previously<sup>7,8,9</sup>. Briefly, Ldlr<sup>−/−</sup> mice fed chow or HFD were i.v. injected either with 1 mg of Ea antigen or PBS, and were killed 4 hours later for FACS analysis. The APCs take up the Ea antigen, and the Ea peptide in the context of MHC (I-A<sup>b</sup>) can be recognized by the Y-Ae mAb.

To determine the anatomical location of the antigen processing pDCs, Ldlr<sup>−/−</sup> mice fed HFD for 28 days were injected i.v. with 2.5 mg of DQ ovalbumin (DQ-OVA; Molecular Probes). After 1 h the aortic sinus was harvested, embedded in Tissue-Tec OCT (Tissue Tek, Sakura Finetek Europe, Zoeterwoude, the Netherlands) and snap frozen for immunohistochemical analysis. For staining, sections were fixed in acetone for 10 mins, air dried, and rehydrated with PBS before incubation in serum-free Protein Block (DakoCytomation) for 30 mins. pDCs were detected by staining for Siglec-H (440c, HyCult Biotech, Uden, The Netherlands). The primary antibody was detected using a Texas Red conjugated donkey anti-rat IgG (Jackson ImmunoResearch). Images
were taken by a Leica DFC340 FX video-camera (Leica Microsystems) connected to a fluorescence microscope (Leica DMRB) using the LAS software (vers. 2.8.1, Leica). Dapi was used to identify nuclei.

For in vivo OT-II stimulation, C57BL6 mice (4/group) were injected with 5 x 10^6 CFSE (Life Technologies)–labeled OT-II T cells. After 24h mice were injected into the left footpad with 1 x 10^5 µMT or µMT:pIII+IV−/− pDCs pre-incubated with ovalbumin (100 µg/ml) for 3h. OT-II T cell proliferation (CFSE dilution) was assessed after 3 days by flow cytometry in the popliteal lymph node, using spleen and the contralateral popliteal lymph node as internal controls.

**In vitro dendritic cell culture**

Bone marrow pDCs and spleen CD11c+ cells were isolated by negative and positive magnetic selection, respectively, according to the manufacturer’s instructions using an AutoMACS Pro separator (Miltenyi). For cytokine production, purified pDCs (2 x 10^4) were treated with type A or B CpG or control GpC oligonucleotides (10 µg/ml; Invivogen). IFNα levels in supernatants were quantified by ELISA (eBioscience).

**Antigen-specific T cell stimulation in vitro**

OT-II CD4+ T cells were incubated with cDCs or pDCs preincubated with 100 µg/ml ovalbumin (Sigma) and proliferation quantified after 3 days by ³H thymidine incorporation over the final 18h. An I-A^b^ restricted murine T cell hybridoma (48-5T) recognising human ApoB100 was added to pDCs or CD11c+ cells preincubated for 4h with native human LDL (50 µg/ml; Intracel) in 0.5% serum-DMEM and incubated overnight. Supernatants were analysed for IL-2 levels by ELISA (Peprotech).

**Analysis of atherosclerotic lesions**

Total plasma cholesterol was quantified using a Cholesterol RTU kit (Biomerieux).

Aortic root atherosclerotic lesions were analysed by Oil Red O and CD3 staining as previously described. Images were captured and analysed using a Leica DM6000B microscope and accompanying software.

**Statistics**

Results were presented as mean ± S.E. They were analyzed in GraphPad Prism (La Jolla, CA, USA) using unpaired t-test, non-parametric Mann Whitney U test, one way analysis of variance or two-way analysis of variance, as appropriate. A P value (two-sided) of <0.05 was considered significant. For analysis of
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atherosclerotic lesions in the aortic root, the entire 10-section profile was analyzed by repeated measures two-way analysis of variance and the p value for between groups displayed.
Supplemental methods references

Figure S1

Plots pre-gated on FSc vs SSC and Live/Dead staining

Supplemental Figure 1. Representative plots of gating strategies used. Plasmacytoid dendritic cells (pDCs): CD11c+ PDCA1+ CD11b- B220+. This population is also SiglecH+ (98.1±1.0 %) and CD8+ (88.7±1.7). Conventional dendritic cells (cDCs) were defined as CD11chi MHCII+ and either B220b or negative. B cells were defined as CD19+ MHCII+, CD4+ T cells as CD3+ CD4+ and regulatory T cells as CD4+ Foxp3+ CD25+ GITR+. Monocytes were defined as CD11b+ CD115+ and Ly6G high, intermediate or low. Neutrophils were CD11b+ Ly6G+ Ly6C+.
Supplemental Figure 2: Purity of bone marrow pDCs isolated by negative magnetic selection and expression of MHCII in µMT or µMT:pIII+IV+ pDCs.
Supplemental Figure 3. Plasmacytoid dendritic cell, lymphocyte and monocyte levels in Ldlr−/− mice transplanted with Tcf4-WT or Tcf4-cKO mice. *p<0.05 vs WT. Data representative of 2 separate experiments. A. Aortic pDCs. B. B cells. C. CD4+ T cells. D. Ly6C monocyte subsets and neutrophils in blood and bone marrow (BM). E. Spleen regulatory T cells.
Supplemental Figure 4. A. Anti-MHCII staining on pDCs from μMT or μMT;pIII+IV "−" mice compared to isotype control staining. B. IFNα levels in conditioned medium from bone marrow pDCs from μMT or μMT; pIII+IV "−" mice untreated or treated with CpG control (GpC) or CpG "−"A (10µg/ml) for 24h. Data representative of 2 separate experiments performed in triplicates.
**Supplemental Figure 5.** Immune cell levels and functions in Ldlr<sup>−/−</sup> mice reconstituted with µMT or µMT;plIII+IV<sup>−/−</sup> bone marrow. A. Ly6C monocyte subsets and neutrophils in blood and bone marrow (BM). Data representative of 3 separate experiments. B. MFI for MHCII in cDCs. Pooled data from 2 experiments. C. Spleen regulatory T cells. Representative of 3 separate experiments. D.Suppressive capacity of spleen CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells on CD4<sup>+</sup> CD25<sup>−</sup> effector T cell proliferation to anti-CD3 in the presence of WT spleen CD11c<sup>+</sup> cells expressed as % of effector T cell proliferation alone. Representative of one experiment performed in triplicates with cells pooled from 5 animals/group. E. IFN-γ<sup>+</sup> CD4<sup>+</sup> T cells. Pooled data from 3 experiments. F. IL-17<sup>+</sup> CD4 T cells. Representative of 3 experiments.
Supplemental Figure 6. Lymphoid tissue and blood levels of pDCs (A), monocytes and neutrophils (B), B cells (C) and T cells (D) in Ldlr−/− mice reconstituted with μMT (80%)/WT (20%) or μMT; plll+IV−/− (80%)/ WT (20%) bone marrow. See methods for gating strategy. Data pooled from 2 experiments with at least 5 animals per experiment and per group.
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