Plasmacytoid Dendritic Cells Protect Against Atherosclerosis by Tuning T-Cell Proliferation and Activity


Rationale: Unlike conventional dendritic cells, plasmacytoid DCs (PDC) are poor in antigen presentation and critical for type I interferon response. Though proposed to be present in human atherosclerotic lesions, their role in atherosclerosis remains elusive.

Objective: To investigate the role of PDC in atherosclerosis.

Methods and Results: We show that PDC are scarcely present in human atherosclerotic lesions and almost absent in mouse plaques. Surprisingly, PDC depletion by 120G8 mAb administration was seen to promote plaque T-cell accumulation and exacerbate lesion development and progression in LDLr−/− mice. PDC depletion was accompanied by increased CD4+ T-cell proliferation, interferon-γ expression by splenic T cells, and plasma interferon-γ levels. Lymphoid tissue PDC from atherosclerotic mice showed increased indoleamine 2,3-dioxygenase (IDO) expression and IDO blockage abrogated the PDC suppressive effect on T-cell proliferation.

Conclusions: Our data reveal a protective role for PDC in atherosclerosis, possibly by dampening T-cell proliferation and activity in peripheral lymphoid tissue, rendering PDC an interesting target for future therapeutic interventions. (Circ Res. 2011;109:1387-1395.)

Key Words: plasmacytoid dendritic cells ▪ atherosclerosis ▪ immune tolerance ▪ T cells

Plasmacytoid dendritic cells (PDC) are a subset of dendritic cells derived from both myeloid and lymphoid precursors in bone marrow and constitute only 0.1–0.5% of the total leukocyte pool in blood and peripheral lymphoid tissue. As the main type I interferon (IFN)-producing cells, PDC have a critical role in detection of and host defense against bacterial and viral infection but also in sensing RNA/DNA and immune complexes. On stimulation, PDC produce large amounts of type I interferons (IFN-α, IFN-β, IFN-ω, and IFN-λ) in a toll-like receptor (TLR) 7- and 9-dependent manner, thereby inducing effector T-cell and natural killer (NK) cell activation and linking innate and adaptive immunity. PDC differ from conventional dendritic cells (CDC) in that they are poor T-cell activators due to low expression of major histocompatibility complex class II and costimulatory molecules. PDC from atherosclerotic lesions have been documented in 1995 and was subsequently confirmed by Weyand et al. PDC (CD4+CD45RA+IL-3αCD123+) ILT3+ (ILT1CD11) were reported to be expressed in the shoulder region of human plaques, where they are believed to regulate T-cell function, even in the absence of antigen recognition. CpG induced IFN-α release by PDC effected a 10-fold upregulation of tumor necrosis factor–related apoptosis-inducing ligand expression on CD4+ T-cell surface, thereby promoting vascular smooth muscle cell (VSMC) and endothelial cell (EC) apoptosis, processes that are generally deemed deleterious for plaque stability. However, these in vitro data leave unaddressed whether PDC are instrumental in plaque destabilization in vivo.

Whereas viral infection associated acute PDC activation results in massive type I IFN release, chronic activation of PDC in the absence of infection was reported to cause severe autoimmune diseases. For instance, in experimental autoimmune encephalomyelitis, PDC were shown to exert overt pathogenic activity, mainly by enhancing T helper 17 (Th17)-dependent immune responses. In patients with systemic lupus erythematosus, PDC activity and IFN-α/β release...
correlated with disease activity and severity. Conversely, evidence is culminating that immature and alternatively activated PDC have the capacity to dampen chronic low-grade inflammation and autoimmune diseases, including diabetes type I, asthma, and transplant rejection, possibly by suppressing CD8+ effector T cell and inducing CD4+CD25+ regulatory T-cell (Treg) function by the release of tolerogenic molecules such as indoleamine 2,3-dioxygenase (IDO) or programmed death-ligand 1 (PD-L1). Altogether, these observations show that PDC are very plastic cells with the capacity to produce high levels of type I IFN and activate the adaptive immune system in infection on one hand and to regulate inflammation by inhibiting effector T-cell and inducing regulatory T-cell responses on the other hand.

In the present study, we addressed the actual role of PDC in atherosclerosis by a loss-of-function approach, providing evidence for an IDO-dependent, T-cell–suppressive activity of PDC in human and mouse atherosclerosis.

Methods
An expanded Methods section is available in the Data Supplement at http://circres.ahajournals.org.

Atherosclerosis Induction and PDC Depletion in Mice
Female LDLr−/− mice, obtained from The Jackson Laboratory and backcrossed at least 11 times to C57Bl/6, were placed on high-fat diet containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). Two weeks later, atherosclerotic lesions were induced in the carotid artery by bilateral placement of semiconstrictive collars. To study effects of PDC depletion on atherosclerosis development, a PDC-depleting antibody, 120G8 (250 μg per mouse per injection, Bioceros, Utrecht, The Netherlands), was administered intraperitoneally 4 times per week for 3.5 weeks starting at the time of collar placement (n=19), after which mice were euthanized. From these mice, the aortic root was isolated and studied to analyze effects of PDC depletion on natural atherosclerosis development. To study effects of PDC depletion on progression of atherosclerosis, 120G8 (250 μg per mouse per injection, Bioceros, Utrecht, The Netherlands) was administered intraperitoneally 4 times per week for 3 weeks, starting at 4 weeks after collar placement, once initial lesions had formed (n=17). As control antibody, an isotype control (GL113) was used. All animal work was approved by the regulatory authority of Maastricht University and performed in compliance with the Dutch government guidelines.

In Vivo Proliferation Assays
LDLr−/− mice on a high-fat diet treated either with 120G8 or GL113 received intraperitoneal injections of BrdU (0.8 mg/kg) for 5 consecutive days. BrdU incorporation in CD4+ and CD8+ T cells was assessed using a FITC BrdU flow kit (BD) according to the manufacturer’s instructions. Cells were analyzed using FACS CANTO II.

LDLr−/− recipient mice on a high-fat diet received CFSE-labeled purified ovalbumin (OVA)-specific OT-1/CD45.1 or OT-2/CD90.1 cells together with nontransgenic purified CD8−/CD90.1 or CD4+CD25+CD45.1 T cells that served as an internal control. The next day, mice received intravenous irradiated (1500 rad) Kb−/−-actmOVA or C3H-actmOVA cells. Three days later, OT-1/CD90.1 and OT-2/CD45.1 proliferation and expansion were determined, based on CFSE dilution and the ratio of OT-1/CD45.1 to CD90.1 control CD8+ T cells and OT-2/CD45.1 control CD4+ T cells. In parallel, splenocytes were restimulated with OVA 257–264 or OVA 323–339 peptide (A&A Labs, San Diego, CA) in the presence of Brefeldin A for 5 hours. Surface staining for CD8/CD4 (BD) and CD45.1/CD90.1 (eBioscience) was performed.

PDC–T-Cell Coculture
PDC were isolated from spleens of Bl6 FLt3L-treated, high-fat diet fed LDLr−/− mice, using PDCA-1 microbeads (Miltenyi). Splenic CD3+ T cells from PDC-depleted LDLr−/− mice on a high-fat diet were isolated after staining with biotinylated CD4 (Biolegend) and ovalbumin (OVA)-specific OT-1/CD45.1 or OT-2/CD90.1 T cells were cocultured for 3 days at a 1:6 ratio in the presence or absence of 1-MT (Sigma Aldrich, 100 μmol/L) or anti-PD-L1 (20 μg/mL, Bioceros). CD3+ T-cell proliferation was assessed by 3[H]-thymidine incorporation and plotted as relative proliferation index, defined as the ratio of T-cell proliferation in the presence and absence of PDC. In parallel cultures, purified CD4+ T cells were CFSE-labeled before stimulation, and proliferation was determined by flow cytometry.

Human Atherosclerotic Plaque Analysis
Human atherosclerotic lesions were isolated from carotid endarterectomy patients and graded for progression stage according to Virmani et al. All human work was approved by the Ethics Committee of the University Hospital Maastricht. Written informed consent for participation in the study was obtained from all individuals.

Statistical Analysis
Data are expressed as mean±SEM and are considered statistically significant at P<0.05.

Results
PDC Are Scarce in Human and Mouse Atherosclerotic Lesions
Concordant with previous observations, CD123+ PDC were seen to be present in human atherosclerotic lesions, and their presence increases with plaque progression (P<0.05) (Figure 1A). Of note, CD123+ stained cells were found to show considerable colocalization with macrophages (Figure 1B) and VSMC (Figure 1C), supporting earlier findings from van Viré et al showing that CD123 is not a specific marker for human PDC. Next, we considered BDCA-4 as a more selective human PDC marker. Flow cytometry on human whole blood samples confirmed the high specificity of this marker for human PDC, as it was virtually absent on circulating monocyte, B-cell, T-cell, and granulocyte subsets (Online Figure I, A). Surprisingly, BDCA-4+ staining revealed the scanty presence of PDC in human plaques; moreover, BDCA-4+ cell expression did not differ between stable and unstable advanced atherosclerotic lesions (Figure
In agreement, microarray (Figure 1E and Online Figure I, B) and real-time PCR analysis (Online Figure I, C) failed to demonstrate differential expression of established PDC markers during plaque progression. Likewise, PDC were almost absent in mouse carotid and aortic artery lesions of LDLr−/− and ApoE−/− mice as well (data not shown), although we did observe few scattered PDCs in the adventitia (Figure 1F), which is in line with our human data (Online Figure I, D).

**Effective and Selective Depletion of PDC in Mice by 120G8 Antibody Treatment**

Next, we addressed the specificity of PDC depletion by 120G8 mAb, which recognizes PDCA-1 (also referred to as bone marrow stromal cell antigen 2 [BST2]), a marker specifically expressed on mouse PDC. 120G8 significantly depleted (>90%) PDC numbers in blood and spleen (P<0.05) (Figure 2A). PDC repopulation started 24 hours after a single 120G8 administration, and full recovery was obtained after 72 hours (Online Figure II, A), necessitating a 120G8 dose regimen once every 2 days for effective and persistent PDC depletion. It has been reported that in vitro PDCA-1 expression is upregulated at the mRNA level in other cell types in response to viral infection or exposure to inflammatory stimuli, which theoretically could thwart the depletion specificity.17 In our study, 120G8 treatment affected neither CDC (Figure 2B) nor B-cell numbers (Figure 2C). Also, monocyte and granulocyte levels were unchanged (Online Figure II, B and C). Moreover, assessment of PDCA-1 expression by flow cytometry showed that in high-fat diet–fed LDLr−/− mice, PDCA-1 expression is completely restricted to PDC (Figure 2D). These data demonstrate that in our mouse model of atherosclerosis, PDC depletion by 120G8 mAb was effective and...
specific. To investigate whether prolonged antibody administration by itself could modulate immune responses, we compared the T-cell activation status between GL113- and PBS-treated mice and did not find a difference in the number of CD44\(^{\text{high}}\) T cells (Online Figure II, D). At a functional level, 120G8 treatment almost abrogated CpG-induced PDC activation in vivo, as judged by the 6-fold attenuated induction in plasma IFN-\(\gamma\) release on CpG injection in 120G8 treated versus control mice (\(P<0.05\); Figure 2E). Although PDC activity and TLR 7/9 function appear to be intact, under conditions of hyperlipidemia, baseline plasma IFN-\(\gamma\) levels remained unchanged after PDC depletion, both in the plaque initiation and progression study. This suggests that an atherogenic stimulus per se does not increase IFN-\(\gamma\) release by peripheral PDCs and/or that PDC are under these conditions not the major source of circulating IFN-\(\gamma\).

**Depletion of PDC Accelerates Atherosclerosis in LDLr\(^{-/-}\) Mice**

To address the role of PDC in atherosclerosis, we examined lesion development and progression in LDLr\(^{-/-}\) mice fed a high-fat diet. For plaque initiation, 120G8 treatment was started at the time of collar placement, whereas for plaque progression, it was started at week 4 after collar placement, once initial lesions had formed. The 120G8 treatment did not affect body weight, nor did it lead to overt pathogenic responses; 120G8 treatment tended to decrease plasma cholesterol levels initially (1384\(\pm\)78.53 versus 1126\(\pm\)46.28 pg/mL in control and 120G8-treated mice, respectively), but this effect was blunted at later stages of plaque development (1097\(\pm\)44.83 versus 1231\(\pm\)72.31 pg/mL in control and 120G8-treated mice, respectively) (Table). To our surprise, atherosclerosis considerably deteriorated after PDC depletion. Plaque volume was 2-fold increased in the plaque initiation study (1.4\(\times\)10\(^7\)\(\pm\)2.6\(\times\)10\(^6\) versus 2.7\(\times\)10\(^7\)\(\pm\)4.7\(\times\)10\(^6\) \(\mu\text{m}^3\) in control and 120G8-treated mice, respectively) (Figure 3A), whereas we observed a 3-fold increase in plaque progression.

**Table. Body Weight and Plasma Cholesterol Levels**

<table>
<thead>
<tr>
<th></th>
<th>Control Ab</th>
<th>120G8 mAb</th>
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<tbody>
<tr>
<td>Body weight initiation study, g</td>
<td>21.10(\pm)0.23</td>
<td>20.11(\pm)0.42</td>
</tr>
<tr>
<td>Body weight progression study, g</td>
<td>21.90(\pm)0.43</td>
<td>22.75(\pm)0.31</td>
</tr>
<tr>
<td>Plasma cholesterol levels initiation study, pg/mL</td>
<td>1384(\pm)78.53</td>
<td>1126(\pm)46.28*</td>
</tr>
<tr>
<td>Plasma cholesterol levels progression study, pg/mL</td>
<td>1097(\pm)44.83</td>
<td>1231(\pm)72.31</td>
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*\(P<0.05\).
compared with plaques at baseline (1.4×10^7 ± 2.5×10^6 versus 5.4×10^7 ± 7.2×10^6 μm³ in control baseline and 120G8-treated mice, respectively) (P<0.0005) versus only 2-fold for the GL113 Ab-treated mice (1.4×10^7 ± 2.5×10^6 versus 3.3×10^7 ± 7.5×10^6 μm³ in control baseline and GL113-treated mice, respectively) (P<0.05) (Figure 3B). Likewise, PDC depletion induced a more unstable plaque phenotype in the progression study, characterized by necrotic core expansion (Figure 3C) and diminished cap VSMC content (Figure 3D). There was no difference in plaque collagen content (data not shown). In addition to the carotid artery, we also examined atherosclerosis development in the aortic root, showing an essentially similar aggravation of lesion formation after PDC depletion (P<0.05; Figure 3E).

Thus, our data point to an unexpected protective role of PDC in atherosclerosis, which is in contrast to the prevailing notion that PDC might promote atherosclerosis by activating T cells in a type I IFN–dependent manner.5,18 Moreover, given the fact that atherosclerotic lesions are virtually devoid of PDC, they probably exert their atheroprotective effect by modulating immune responses in the periphery and/or adventitia.

**PDC Exert Their Atheroprotective Effect by Regulating CD4⁺ T-Cell Proliferation and Function in Lymphoid Tissue**

As a next step, we examined effects of PDC depletion on plaque composition to address the potential mechanisms responsible for the protective effects of PDC in atherosclerosis. We found that PDC depletion led to an increase in lesional T-cell accumulation (P<0.05; Figure 4A and Online Figure III, A). The scarce presence of PDC in mouse atherosclerotic lesions suggests that the increased T-cell infiltration into the lesions probably reflects peripheral modulation of T-cell function. Indeed, blood and spleen T-cell content was increased in PDC-depleted mice (P<0.05; Figure 4B), suggesting that PDC interfere with T-cell homeostasis. As PDC have been reported to induce regulatory T-cell
expansion,11,19 we investigated whether the increase in plaque CD3 \(^+\) T-cell content is owing to decreased Treg numbers or function and an associated failure to control T-cell responses. However, we did not observe a difference in blood and spleen Treg numbers between 120G8- and GL113-treated LDLr \(^{-/-}\) mice (Online Figure III, B). In keeping, IL-10 plasma levels did not differ as well (Online Figure III, C). To address whether the increase in T cells resulted from increased proliferation or survival, we examined T-cell proliferation in vivo. Spleens of PDC-depleted mice were enriched in BrdU\(^+\) CD4\(^+\) T cells (Online Figure III, D) but not CD8\(^+\) T cells (Online Figure III, C). Moreover, T-cell proliferation appeared to be antigen-dependent in that OVA challenge led to augmented proliferation of OT-2 CD4\(^+\) T cells (P<0.05; Figure 4D) but not OT-1 CD8\(^+\) T cells (Online Figure III, E) in PDC-depleted versus nondepleted mice. In addition, these findings also show that the increased proliferation is not due to intrinsic T-cell effects, as both innate and administered T-cell proliferation was increased, but to changes in the environment in PDC-depleted mice. In parallel to increased T-cell proliferation, IFN-\(\gamma\) (P<0.0005; Figure 4E) as well as IL-6 and MCP-1 plasma levels (Online Figure III, F) were seen to be increased after PDC depletion. FACS-sorted CD3 \(^+\) T cells (purity 95%) from spleens of PDC-depleted LDLr \(^{-/-}\) mice tended to have increased IFN-\(\gamma\) expression (P=0.07; Figure 4F). In addition, CD3 \(^+\) T cells isolated from atherosclerotic (high-fat diet-fed) LDLr \(^{-/-}\) mice expressed higher levels of GATA-3 (Th2 marker) as well as of t-bet (Th1 marker) compared with T cells isolated from nonatherosclerotic mice (chow diet-fed) mice (Online Figure III, G). PDC depletion abrogated this effect for GATA-3 but not t-bet, suggesting that PDC might help to dampen the high-fat diet-induced Th1 shift in LDLr \(^{-/-}\) mice. Overall, these data further substantiate a tolerogenic activity of PDC under atherogenic conditions, probably by suppressing T-cell proliferation and function.

**PDC Suppress CD4\(^+\) T-Cell Proliferation in an IDO-Dependent Manner**

To address the underlying mechanism for the T-cell suppressive capacity of PDC in atherosclerosis, we compared the expression of known key regulators of PDC tolerogenicity, such as IDO, IL-10, PD-L1, and inducible costimulator-ligand (ICOS-L), by PDC isolated from chow (nonatherosclerotic mice) versus high-fat diet-fed (atherosclerotic mice) LDLr \(^{-/-}\) mice (purity, 98.3%). Expression of PD-L1 and IDO (P<0.05; Figure 5A) but not IL-10 and ICOS-L (data not shown), was significantly elevated in PDC from high-fat diet-fed versus chow-fed mice. Importantly, CD3\(^+\) T cells isolated from spleen of PDC-depleted atherosclerotic mice, cocultured with PDC in the presence of 1-methyl-trypthophan (1-MT), an IDO blocker, but not anti–PD-L1, displayed markedly induced T-cell proliferation (P<0.05; Figure 5B), suggesting that PDC suppress T-cell proliferation in an IDO-dependent manner. The 1-MT induced CD4\(^+\) T-cell mitogenic response was confirmed by flow cytometry (Figure 5C), showing a similar increment in CD4\(^+\) T-cell proliferation after coculture with PDC in the presence of 1-MT. Baseline levels of IFN-\(\alpha\) in plasma of high-fat diet-fed versus
chow-fed LDLr<sup>−/−</sup> mice were unaltered (Figure 5D), as well as IFN-γ expression by PDC (Figure 5E), firmly establishing that in mice, proatherogenic conditions per se do not promote IFN-γ release and immunogenic activity. Moreover, we extend these findings to the human context, as in LDLr<sup>−/−</sup> mice, IFN-γ plasma levels were also seen to be unchanged (Figure 5F) and IFN-γ expression by PDC even significantly lowered in atherosclerotic patients versus healthy controls (P<0.05; Figure 5G). Altogether, our data indicate that both in mice and humans, conditions of chronic atherosclerosis do not trigger PDC immunogenic activity, IFN-α release, and ensuing T-cell activation and proliferation. Rather, this milieu may even consolidate PDC’s innate tolerogenic capacity to suppress T-cell proliferation.20

Discussion
In the present study, we are the first to demonstrate a contributory role for PDC in atherosclerosis. Despite the scarce presence of PDC in mouse atherosclerotic lesions, depletion of PDC in LDLr<sup>−/−</sup> mice by 120G8 mAb aggravated atherosclerosis development and progression. Lesions of PDC-depleted mice were characterized by increased T-cell accumulation and a more unstable plaque phenotype, which, as we show, probably is linked to a deficiency in PDC-associated, epitope-specific dampening of T-cell response.

We demonstrate selective and almost complete PDC depletion by the use of 120G8 mAb in LDLr<sup>−/−</sup> mice. PDCA-1 expression was exclusively restricted to the PDC population, and no other leukocyte subsets other than PDC were depleted.
These findings confirm previous reports that highlight the specificity of the 120G8 mAb, all showing selective depletion of PDC in blood, bone marrow, lymph node, thymus, and nonlymphoid organs of C57Bl6 mice but not of CD4/CD8 T cells, DX5+ NK, and CD19+ B cells. Alternative PDC ablation or depletion models currently available such as iKAROS and IRF8 (mutant) all have major effects on non-PDC subsets, whereas the CD11c.CRE/E22 and the BDCA-2.5dTR mice are interesting new models for future ablation studies.

Our data point to an unexpected atheroprotective activity of PDC, which is in contrast to previous findings pointing toward a proatherogenic function. This notion was largely based on guilt by association, in that (1) plaques were seen to express CD123 and IFN-α cells, in particular when progressed to an unstable phenotype, (2) CpG induced PDC activation in vitro led to type I IFN release, and (3) type I IFN were recently reported to contribute to atherosclerosis in ApoE−/− and LDLr−/− mice by stimulating macrophage recruitment. The data presented in this study justify a minor adjustment of this assumption. First, unlike BDCA-4, CD123 staining may not be entirely reflective of the plaque’s PDC content because macrophages and VSMCs appear to express this marker as well and as CD123 cells often lack characteristic plasmacytoid morphology. This observation concurs with recent findings by van Vre et al, showing that CD123 is not a specific PDC marker staining also for endothelial cells in human atherosclerotic lesions. As a result, the actual plaque PDC content may not only be lower than originally envisioned but also does not markedly increase with progression of disease. This also implies that PDC effects may be precipitated primarily in the periphery rather than within the plaque itself. Second, we show that PDC are not the prime source of plasma IFN-α at baseline and that IFN-α release by PDC into the circulation is boosted by CpG treatment but not by atherogenic conditions. Apparently, atherogenic stimuli per se do not induce PDC activation. Moreover, in atherosclerotic mice, circulating IFN-α originates from cell types other than PDC but may be derived from macrophages. Third, we failed to demonstrate progressively increased expression of IFN-α (by microarray or real-time PCR analysis) by circulating PDC from atherosclerotic mice and by human PDC from patients with stable versus unstable disease and by unstable versus stable endarterectomy lesions, confirming that in chronic inflammatory processes such as atherosclerosis TLR7/9 activation of PDC is not very prominent. Collectively, our data indicate that PDC exert their atheroprotective effect primarily by modulating extravascular immune responses.

Our studies also provide a plausible mechanism by which PDC suppress CD4+ T-cell proliferation under conditions of atherosclerosis. PDC isolated from spleens from atherosclerotic mice had a 2-fold increase in expression of tolerogenic molecules IDO and PD-L1 compared with PDC isolated from nonatherosclerotic mice. IDO is an intracellular tryptophan catabolizing enzyme that has been attributed suppressive activity on CDCs and stimulatory activity on Tregs. PD-L1 is an inhibitory costimulatory molecule that interacts with programmed death-1 (PD-1) on CD8+ T cells to suppress their viability and activity.

Moreover, coculture of PDC with T cells in the presence of 1-MT, an IDO blocker, but not anti–PD-L1, showed increased T-cell proliferation, suggesting that PDC suppress T-cell proliferation in an IDO-dependent manner. These observations correspond with previous reports in which PDC were shown to induce tolerance in other low-grade chronic inflammatory and autoimmune diseases. The tolerogenic function of PDC was seen to depend on cytokine/ligand activation. For instance, B7–1 (CD80) engagement by Cytotoxic T-lymphocyte Antigen-4 (CTLA-4Ig), that of CD200R1 by CD200Ig, and B7–1/B7–2 (CD80/CD86) by CD28Ig all have been shown to be able to induce the release of IDO by PDC, leading to the suppression of T cells. It remains to be established which activation pathway is involved in atherosclerosis. Thus, in analogy, during atherosclerosis, PDC not only maintain their immature tolerogenic state but even invigorate their inborn dampening activity so that they can control T-cell activity. It remains to be established whether the same holds for brief episodes of fulminant plaque inflammation (acute myocardial infarction).

In conclusion, this report is the first to unveil a protective role for PDC in an established mouse model of atherosclerosis, throughout disease progression. Given the virtual absence of PDC in the plaque itself, PDC probably exert their activity extravascularly by dampening T-cell proliferation and function in an IDO-dependent manner. Although these findings identify PDC as an interesting new target for therapeutic intervention studies, they warrant further study to elucidate the actual pathways underlying the augmented tolerogenic activity of PDC under conditions of atherosclerosis.

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Disclosures

None.

References

Plasmacytoid Dendritic Cells and Atherosclerosis

Novelty and Significance

What Is Known?

- Plasmacytoid dendritic cells (PDCs) are present in human atherosclerotic lesions.
- PDCs are involved in host defense against bacterial and viral infection via the release of type I interferon. Also, this cell type induces immune tolerance in chronic low-grade inflammation through the release of tolerogenic molecules.

What New Information Does This Article Contribute?

- PDCs differ from classic conventional dendritic cells in that they are poor T-cell activators and are critical for type I interferon responses. Although PDCs are present in human atherosclerotic lesions, their role in atherosclerosis has not been determined. Unexpectedly, we show that PDCs are scarcely present in both human and mouse atherosclerotic lesions. However, PDC depletion by administration of 120G8 increases atherosclerosis development and progression in LDLr\(^{-/-}\) mice. We found that PDC reductions of atherosclerosis we associated with suppression of CD4\(^{+}\) T cell proliferation and activity through the release of tolerogenic molecule indoleamine 2,3-dioxygenase–dependent manner.


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Supplemental Material

Detailed Methods

Atherosclerosis induction and pDC depletion in mice. Female LDLr\(^{-/-}\) mice, obtained from The Jackson Laboratory and backcrossed at least 11 times to C57Bl/6, were placed on high fat diet containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). Two weeks later, atherosclerotic lesions were induced in the carotid artery by bilateral placement of semi-constrictive collars. To study effects of pDC depletion on atherosclerosis development, a pDC depleting antibody, 120G8 (250µg/mouse/injection, Bioceros, Utrecht, The Netherlands), was administered 4 times per week i.p. for 3.5 weeks starting at the time of collar placement (n=19) after which mice were sacrificed. From these mice, also the aortic root was analyzed to study effects of pDC depletion on natural atherosclerosis development. To study effects of pDC depletion on progression of atherosclerosis, 120G8 (250µg/mouse/injection, Bioceros, Utrecht, The Netherlands) was administered 4 times per week i.p for 3 weeks starting at 4 weeks after collar placement, once initial lesions had formed (n=17). As control antibody, an isotype control (GL113) was used. All animal work was approved by the regulatory authority of Maastricht University and performed in compliance with the Dutch government guidelines.

Histology of mouse atherosclerotic lesions. Mice were euthanized by an overdose of pentobarbital (115mg/kg) and perfused through the left cardiac ventricle with PBS (NaCl/Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\), pH 7.4) containing sodium nitroprusside (0.1mg/ml, Sigma) and 1% paraformaldehyde (PFA). The right common carotid artery and the heart were removed, fixed overnight in 1% PFA and paraffin-embedded sections (4 µm) were cut. To determine plaque volume in the carotid artery, plaque area was measured for consecutive cross sections at 100 µm intervals over a carotid artery segment that covered the entire plaque. To measure plaque volume in the aortic root, plaque area was measured for each valve for consecutive sections at 20 µm intervals that covered the entire lesion. Collagen content was detected by Sirius red staining and expressed as a percentage of plaque area. Slides were analyzed in a blinded manner using a Leica DM3000 light microscope (Leica Microsystems) coupled to a computerized morphometric system (Leica Qwin 3.5.1).

Immunohistochemistry. Immunohistochemical stainings were performed on paraffin carotid artery sections for CD3 (DAKO), Siglec-H/440c (Hycult Biotechnology) and \(\alpha\)-smooth muscle actin (ASMA) (DAKO). Slides were analyzed blindly using Leica Qwin program. The number of positive cells was measured and expressed as a percentage of total number of cells in the plaque. For ASMA, the number of positive cells was expressed as a percentage of plaque area.

Flow cytometry. Blood, spleen and peripheral LN were removed before perfusion and used for FACS analysis of pDC (CCR9\(^{-}\)PDCA-1\(^{-}\), eBioscience), cDC (CD11c\(^{-}\), eBioscience), T cells (CD3\(^{+}\), Miltenyi), T helper cells (CD4\(^{+}\), BD), effector T cells (CD8a\(^{+}\), BD), activated T cells (CD44\(^{+}\), eBioscience), regulatory T cells (CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\), eBioscience), B cells (B220\(^{+}\), BD), monocytes (CD11b\(^{-}\)Ly6G\(^{-}\), BD), granulocytes (Ly6G\(^{+}\)CD11b\(^{-}\)) and NK cells (NK1.1\(^{-}\)CD3\(^{-}\)). For pDC and cDC analysis, spleen and peripheral LN were enzyme...
treated (liberase (0.2 mg/ml, Gibco) and DNAse (0.2 mg/ml, Roche)) for 30 minutes in RPMI medium, before antibody incubation to release DC from the tissue. FACS analysis was performed with FACS Canto II (BD).

**Plasma cytokine and lipid analysis.** Plasma cholesterol levels were measured in duplicate using a colorimetric assay (CHOD-PAP, Roche). Mouse and human plasma levels of IFN-α were measured by sandwich ELISA (PBL Interferon Source). Cytokine analysis of mouse IFN-γ was performed using the Bio-Plex Pro assay (Bio-Rad). To verify functional ablation of pDC after 120G8 treatment, control and 120G8 treated mice (*n* =5 per group) received subcutaneous injection with CpG (CpG-ODN 1585, type A, 40 µg/mouse, Invivogen) and were sacrificed 6 hours later to measure IFN-α plasma levels.

**Mouse pDC and T cell isolation by Fluorescence Activated Cell Sorting (FACS).** Splenic pDC (PDCA-1<sup>high</sup> CCR9<sup>high</sup>) and T cells (CD3<sup>+</sup>) were isolated from LDLr<sup>−/−</sup> mice, either on high fat diet (0.25% cholesterol) (group of atherosclerotic mice, *n* =8) either on normal diet (group of non-atherosclerotic mice, *n* =8), by FACSAria. The purity of the sorted pDC (PDCA-1<sup>high</sup> CCR9<sup>high</sup>) was 98.3%, for the CD3<sup>+</sup> T cells 95%.

**RNA isolation and Quantitative real-time PCR.** RNA from sorted cells was isolated using the ®Trizol (Invitrogen) method. Isolated pDC were analyzed for gene expression of mouse *PD-L1* (5'-ccatcctgttgttcctcattg-3' and 3'-gttcactcttacgatctacacct-5'), mouse *IDO* (5'-actgtgtcctgcaaactggaag-3' and 3'-gagataaccaccttttagcgtcga-5') and mouse *IFN-α* (5'-cattctgcaatgacctccac-3' and 3'-cacgtccttaaaggggact-5'). Isolated CD3<sup>+</sup> T cells were analyzed for expression of mouse *IFN-γ* (5'-tggctgtttctggctgttactg-3' and 3'-actgtacttttagcgtcga-5'). Isolated human pDC from blood were analyzed for gene expression of human *IFN-α* (5'-aatggccttgacctttgctt-3' and 3'-cgttcagttcgacgagac-5').

**BrdU labeling and cell analysis.** LDLr<sup>−/−</sup> mice on high fat diet treated either with 120G8 (*n* =4) or GL113 (*n* =4) received BrdU i.p. injections (0.8mg/day) for 5 consecutive days. BrdU incorporation in CD4<sup>+</sup> and CD8<sup>+</sup> T cells was assessed using a FITC BrdU flow kit (BD) according to manufacturer's instructions. Cells were analyzed using FACS CANTO II.

**OT-1 and OT-2 antigen specific T cell proliferation experiment.** LDLr<sup>−/−</sup> recipient mice (*n* =4 120G8 versus *n* =4 GL113 treated mice) on high fat diet received i.v. 1x10<sup>6</sup> CFSE-labeled purified OT-1/CD45.1 or OT-2/CD90.1 cells together with 2x10<sup>6</sup> non-transgenic purified CD8<sup>+</sup>/CD90.1 or CD4<sup>+</sup>/CD45.1 T cells that served as an internal control. The next day, mice received i.v. 10<sup>6</sup> irradiated (1500 rad) Kb<sup>−/−</sup>-actmOVA or C3H-actmOVA cells. Three days later, OT-1/CD90.1 and OT-2/CD45.1 proliferation and expansion were determined based on CFSE dilution and the ratio of OT-1/CD45.1 to CD90.1 control CD8<sup>+</sup> T cells and OT-2/CD90.1 to CD45.1 control CD4<sup>+</sup> T cells. In parallel, 5x10<sup>5</sup> splenocytes were restimulated with OVA 257-264 or OVA 323-339 peptide (A&A labs, San Diego, CA) in the presence of Brefeldin A for 5 hours. Surface staining for CD8/CD4 (BD) and CD45.1/CD90.1 (eBioscience) was performed.
PDC-T cell co-culture. PDC were isolated from spleen of a Bl6 FLt3L (3×10^6) treated LDLr^{−/−} mouse on high fat diet using PDCA-1 microbeads (Miltenyi). CD3^{+} T cells were isolated from spleen of a pDC depleted LDLr^{−/−} mouse on high fat diet by staining T cells with a biotinylated CD4 (Biolegend) and CD8 (Biolegend) antibody and using a biotin isolation kit (Miltenyi). PDC and CD3^{+} T cells were co-cultured in a 96 well plate coated with anti-CD3/CD28 (0.1µg/ml) in a 1:6 ratio (1×10^5 PDC and 6×10^5 T cells, respectively) for 3 days in iDMEM medium supplemented with either 1-MT (Sigma Aldrich, 100µM) or anti-PD-L1 (20µg/ml, Bioceros). Seventy-two hours later, CD3^{+} T cell proliferation was assessed by 3[H]-thymidine incorporation and plotted as relative proliferation index, being the ratio of T cell proliferation in the presence and absence of pDC. In parallel cultures, purified CD4^{+} T cells were CFSE labeled prior to stimulation and proliferation was determined by flow cytometry 72 hours later upon.

Human plaque analysis. For micro-array analysis, total RNA was extracted from carotid artery sections obtained from autopsy (Department of Pathology, University Hospital Maastricht, Maastricht, the Netherlands) (early (n=13) and advanced stable (n=16) lesions) and surgery (Department of Surgery, Maasland Hospital Sittard, Sittard, the Netherlands) (advanced stable (n=21) and advanced unstable (n=23) lesions). For real-time PCR analysis, RNA was isolated from carotid artery sections obtained from surgery (Department of Surgery, Maasland Hospital Sittard, Sittard, the Netherlands) (advanced stable (n=12) and advanced unstable (n=12) lesions). Plaques were staged by histological analysis of adjacent slides according to Virmani et al. at which intimal thickening (IT) were characterized as early, thick fibrous cap atheroma (TkfcA) as advanced stable, and intraplaque hemorrhaged lesions (IPH) as advanced unstable lesions, respectively. Immunohistochemical stainings were performed on paraffin carotid artery sections from advanced stable (n=22) and ruptured lesions (n=22) from surgery for CD123 (Sigma Prestige), BDCA-4 (R&D Systems), ASMA (Dako) and CD68 (Dako). The purity of the BDCA-4 antibody was checked by flow cytometry by comparing mean fluorescence intensity (MFI) of BDCA-4 expression on pDC versus B cells, T cells and monocytes. Double staining slides were analyzed with the Nuance spectral imaging system (Caliper Life Science / Cambridge Research Instrumentation, Woburn, MA, USA). Spectral imaging data sets were taken from 420-720 nm at 20 nm intervals using a DM-5000 Leica microscope system at 20x (plan apo). Spectral libraries of single-red (Vector Red), single-blue (Vector Blue), and natural occurring iron pigments were obtained from the control slides. The resulting library was applied to the double stained slides to spectrally dissect/unmix them into individual component images using the Nuance™ 3.0 software. Pseudo-color images showing co-localization, as well as an exclusive image of co-localization, were generated with the Nuance 3.0 software. Quantitative assessment of co-localization and pixel-based measurement of the individual markers per microscopic field was done with the same software.

Micro-array analysis of human atherosclerotic lesions. RNA was isolated using the Guanidine Thiocyanate (GTC)/CsCl gradient method and a NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG). RNA concentration was determined using the Nanodrop ND-1000 and the RNA quality obtained using the RNA 6000 Nano/Pico LabChip (Agilent 2100 Bioanalyzer) where the RIN (RNA integrity number) was determined. Samples where the RIN number was lower than 5.6 were excluded from the study. The mean RIN was 7.2±0.49. Samples from autopsy were individually hybridized to HGU133 2.0 Plus arrays (Affymetrix, Santa Clara, USA, California) and samples from surgery to Illumina Human Sentrix-8 V2.0 BeadChip® (Illumina Inc., San Diego, USA, California). Analysis has
been performed in R.\textsuperscript{6} For autopsy samples, preprocessing was performed with affy using the Robust MultiArray (RMA) normalization.\textsuperscript{7} For surgery samples, preprocessing was performed by lumi using the Variance Stabilizing Transform (VST) normalization.\textsuperscript{8} Differential expression has been assessed separately for the two sets of data by fitting each gene against a linear model across the given arrays with limma.\textsuperscript{9} Empirical Bayesian shrinkage of the standard errors have been used to moderate t-statistics, F-statistics, and log-odds of differential expression. The resulting ordered dataset has been queried for the set of genes of interest and their results are hereby presented. The adjusted p-values presented in the results are corrected according to the Benjamini and Hochberg False Detection Rate method.\textsuperscript{10}

\textbf{PDC isolation from human blood.} PDC were collected from healthy controls (n=15) and patients with carotid endarterectomy (n=14). PDC were isolated from blood (Lymphoprep\textsuperscript{TM} Isolation). From this, the B220 positive fraction was depleted with magnetic beads (Miltenyi Biotec) after which pDC were isolated from the negative fraction using a BDCA-4 isolation kit (Miltenyi Biotec). The purity for BDCA-4\textsuperscript{+} pDC, as assessed by flow cytometry (CD123\textsuperscript{high} BDCA-2\textsuperscript{high}) was 96\%. RNA was isolated using the Trizol method. Gene expression profile was analyzed by real-time PCR. Patients for this study were recruited at the Department of Cardiology, University Hospital Oslo, Oslo, Norway. The study included 14 patients (7 male, 7 female, average age of 67) with high-grade internal carotid stenosis (≥70\%), diagnosed and classified by precerebral color duplex and CT angiography according to consensus criteria. For comparison, blood was collected from 15 healthy controls, comparable to the patient group for age, sex and smoking habits. The controls were health care workers, consecutively recruited from the same area of Norway as the patients. All human work was approved by the Ethical Committee of the University Hospital Oslo. Signed informed consent for participation in the study was obtained from all individuals.

\textbf{Statistical analysis.} Data are expressed as mean ± SEM and Mann-Whitney U test was used to compare individual groups of animals. Data are considered statistically significant at P<0.05.
Online Figures

Online Figure I. Specificity BDCA-4 antibody and human plaque analysis. A, Depicted are the results of flow cytometry from human peripheral blood mononuclear cells (PBMC). BDCA-4 expression on pDC (CD123$^+$), T cells (CD3$^+$), B cells (CD19$^+$) and monocytes (CD14$^+$). B, Micro-array analysis on human plaque tissue samples from carotid artery. To compare gene expression between early ($n=13$) and advanced stable ($n=16$) lesions, plaque tissue was isolated from autopsy. To compare gene expression between advanced stable ($n=21$) and advanced unstable ($n=23$) lesions, plaque tissue was isolated from surgery. Described are gene name, Probe/Nu ID, fold change and adjusted P-value for the genes that have been analyzed. C, Real-time PCR analysis of human advanced stable ($n=12$) versus vulnerable ($n=12$) carotid atherosclerotic lesions. D, BDCA-4 staining in the adventitia of a human carotid artery lesion.
Online Figure II. Efficiency and specificity of pDC depletion by 120G8 mAb. **A**, Time-span of pDC depletion by 120G8 mAb. LDLr<sup>−/−</sup> mice on high fat diet received one single dose of 120G8 (250µg/mouse) via i.p. injection and pDC recovery was analyzed 24, 48 and 72 hours later in blood (n=3 per group). **B**, Flow cytometry analysis of circulating monocytes (CD11b<sup>high</sup>Ly6G<sup>−</sup>), **C**, granulocytes (CD11b<sup>high</sup>Ly6G<sup>high</sup>) and **D**, activated (CD44<sup>high</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T cells from LDLr<sup>−/−</sup> mice on high fat diet treated with GL113 (n=6) versus 120G8 (n=6) for 3 weeks.
Online Figure III. T cell analysis of atherosclerotic mice treated with GL113 versus 120G8. A, CD3+ T cell content in mouse atherosclerotic lesions (aortic root) (GL113 (n=9) vs 120G8 (n=10) treated mice, $P=0.08$). Data are represented for the plaque initiation study. B, Depicted are the results of flow cytometry analysis for regulatory T cell numbers in blood and spleen from LDLr-/- mice on high fat diet treated with GL113 (n=9) and 120G8 (n=10). C, IL-10 plasma levels. D, BrdU staining of CD8+ T cells in spleen from LDLr-/- mice on high fat diet treated with GL113 (n=4) versus 120G8 (n=4). E, OT-1 CD8+ T cell proliferation in spleen in response to ovalbumin challenge (n=4/group). F, IL-6 and MCP-1 plasma levels (n=8 GL113 vs n=8 120G8). G, Real-time PCR analysis of CD3+ T cells sorted from spleen from LDLr-/- mice on chow diet (n=5) and high fat diet treated with GL113 (n=5) and 120G8 (n=5).
Supplemental References


