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How dendritic cells shape atherosclerosis

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Abstract

Atherosclerosis is an inflammatory disease of the arteries, which results in major morbidity and mortality. Immune cells initiate and sustain local inflammation. Here we focus on how DC-mediated processes might be relevant to atherosclerosis. Although only small numbers of DCs are detected in healthy arteries, these numbers dramatically increase during atherosclerosis development. In the earliest fatty streaks, DCs are found next to the vascular endothelium. During plaque growth, new DCs are actively recruited, and their egress from the vessel wall is dampened. In the adventitia next to mature atherosclerotic lesions, tertiary lymphoid organs develop, which also contain DCs. Thus, DCs likely participate in all stages of atherosclerosis from fatty streaks to mature lesions.

Induction of immune responses in the artery drives atherosclerosis

Atherosclerosis is a chronic inflammatory disease of large and medium-size arteries and the immune system plays a significant role both in disease initiation and progression [1]. A large body of evidence suggests that both innate and adaptive immune responses are involved in the pathogenesis of atherosclerosis (review in[2]). Disruption of local tissue homeostasis, diet or metabolism-related elevated blood lipoproteins and especially low density lipoprotein (LDL), chronic local inflammation and systemic factors all promote the activation of an immune response to self- and modified-self antigens in the aorta and secondary lymphoid organs. Induction of a local immune reaction is probably essential for atherosclerosis development and progression. Recent data suggest that dendritic cells (DCs) might contribute to local immune responses and disease progression in the artery.

DCs are professional antigen-presenting cells that present various exogenous and endogenous antigens to T lymphocytes, providing an important link between the innate and adaptive immune responses [3, 4]. They play an important role in host defense to pathogens, in cancer, and are required for proper self-tolerance and prevention of autoimmunity [5]. Although the presence of DCs in atherosclerotic plaques suggests a role in disease, details of the heterogeneity and function of DCs in atherosclerosis are only just emerging. Here we discuss the origin, location and function of DCs in atherosclerosis. In comparison to previous reviews in the field [6, 7], we will focus more on mouse models of atherosclerosis, which provide opportunities to use genetic, pharmacologic, live cell imaging and other approaches to interrogate immune cell function in atherosclerosis.

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Characterizing DCs subsets

DCs, together with macrophages, belong to the myeloid lineage of blood cells [8]. Depending on the markers used, functional and phenotypic differences between macrophages and certain DCs may not be obvious [9]. Integrin *Itgax* (CD11c) is referred to in the literature as a marker for mouse DCs, but was recently demonstrated to be upregulated by monocytes and macrophages as well [10, 11]. Several recent studies showed that "specific" surface markers for DCs, including costimulatory molecules CD80 and CD86, are also expressed by many subsets of tissue macrophages, which makes it hard to unequivocally distinguish between these two populations of cells in non-lymphoid tissues, such as atherosclerotic aorta.

Monocytes are mononuclear phagocytes that originate from the bone marrow and circulate in the blood. They are characterized by expression of CD11b, CD115 and variable levels of Ly6C. In the context of hypercholesterolemia these cells can upregulate CD11c [10, 11]. When monocytes migrate into the tissue, they can differentiate into macrophages, which phagocytose and kill pathogens, secrete cytokines, present antigen and regulate inflammation. Most macrophages express CD11b, CD68 and F4/80 surface markers. Resident tissue macrophages express low levels of MHCII, however inflammatory macrophages are known to upregulate MHCII. The functional role of monocytes in atherosclerosis is reviewed elsewhere [12, 13].

In mice, DCs are typically characterized by surface expression of CD11c, the ability to strongly upregulate and express high amounts of MHC II, the co-stimulatory molecules CD80 and CD86 and various members of TNFR superfamily, such as CD40. In addition, DCs have a morphologically distinct "dendritic" like shape. Even though many features of DCs are similar to macrophages, DCs likely represent unique and potent subset of professional antigen-presenting cells (APC) capable of activating naive lymphocytes.

In secondary lymphoid organs of mice, $CD11b^+CD11c^+$, $CD8\alpha^+$ $CD11c^+$, $CD8\alpha^-$ $CD4^-$ $CD11c^+$, $CD8\alpha^-$ $CD4^+$ $CD11c^+$, PDCA-1^+CD11c^+ (pDC) and $CD103^+$ $CD11c^+$ subsets of DCs have been defined [14]**.** $CD11b^+CD11c^+$ DCs are present under homeostatic conditions in lymphoid organs and are characterized by low TNF expression. During infection or stimulation with pathogen-derived stimuli, $CD11b^+CD11c^+$ cells rapidly accumulate. These cells produce high amounts of TNF and inducible NO synthase (iNOS), an enzyme required for the production of nitric oxide, and have all morphological features of DCs [15]. Classical or conventional DCs (cDCs) such as $CD8\alpha^+CD11c^+$ and $CD8\alpha^-CD11c^+$ mostly specialize in antigen presentation. The main function of pDC is the production of type I IFN [16]. The presence and function of $CD103^+CD11c^+$ cells was first identified in gut-associated lymphoid tissue (GALT) [17] where these cells promote the differentiation of regulatory T cells (Tregs), which are know to suppress the inflammation [18, 19]. More recently, $CD103^+$ DCs were found to play a role in the skin immune response [20]. Recent reports showed that retinoic acid (RA) produced by $CD103^+CD11c^+$ DCs can promote inflammation in the gut due to induction of Th1 and Th17 effector T cells [21, 22].

DC maturation, differentiation and antigen capture

Being professional antigen-presenting cells, DCs are known to capture antigen in peripheral tissues and migrate to specialized secondary lymphoid organs, such as lymph nodes or spleen, where they can continuously scan for antigen-specific T cells [23]. This paradigm has been best demonstrated in skin [24], but is thought to apply generally. After the uptake of the antigen and after receiving of proper "second-signal" through Toll-like receptor (TLR) or Nod-like receptor (NLR) stimulation during their migration to secondary lymphoid

organs, DCs undergo maturation [25]. Maturation of DCs is accompanied by enhanced expression of MHC II, co-stimulatory molecules and the chemokine receptor CCR7, which ensures efficient DC migration toward T cell rich areas and antigen presentation. In secondary lymphoid organs, DCs present the processed antigen in the context of MHC I (for intracellular peptide antigens) or MHC II (for extracellular peptide antigens) or CD1d for glycolipids to T cells and NKT cells respectively [26]. Antigen presentation leads to T cell activation and expansion or, depending on signals in microenvironment, to T regulatory cell differentiation [27].

The pattern recognition receptors of the TLR and NLR families [28, 29] are critical for the ability of DCs to discriminate “self” and “foreign” antigens for presentation to T cells [30]. Engagement of TLRs with their ligands in addition to maturation and enhanced antigen presentation enables DCs to produce a variety of inflammatory cytokines such as IL-6, TNF, IL-12, IL-23, GM-CSF and others with potent effects on T cells and other immune cells.

Origin of DCs in atherosclerosis

The aorta represents the major site for the manifestation of atherosclerosis in mice and consists of three compartments: adventitia (external layer), media (middle muscular layer) and intima (below endothelium). The thickness of both adventitia and intima significantly increases upon atherosclerosis development due to accumulation of various immune cells in both compartments. The atherosclerotic plaque causes massive growth of the intima. The media also increase its thickness, possibly by increased smooth muscle cell (SMC) proliferation and differentiation [31].

DCs were first described in aortas in 1995 [32]. Aortas of healthy C57BL/6 mice already contain $CD11c^+$ DCs. Together with macrophages, DC numbers were found to expand in the arterial wall and in plaques atherosclerotic conditions [33-35]. Aortic $CD11c^+$ DC population has not been analyzed in detail, and it is not known whether all DC subsets found in secondary lymphoid organs are present in the atherosclerotic aorta.

In the normal mouse aorta, $CD11c^+$ DCs are preferentially found in the adventitia [36]. However, the presence of DCs in the subendothelial compartment has also been described [37, 38], (Figure 1A). Interestingly, DCs can be mainly found in the lesser curvature of the aortic arch of healthy mice, which is one of the main sites of atherosclerosis development. One study estimated that in this location there are four DCs per ten endothelial cells [39]. Under atherosclerotic conditions, large numbers of DC can be found inside the atherosclerotic plaques and in the adventitia. As DCs in the aorta can possibly be comprised of several different subsets with potentially different origins and kinetics of recruitment, the question arises how they might enter different compartments (Figure 1C). It is possible that DCs migrate between the different compartments of the arterial wall and accumulate in the area with the highest concentration of specific chemokines, pathogen associated molecular patterns (PAMP) or antigen (Figure 1B).

Monocytes and atherosclerosis

Several distinct populations of monocytes have been described in the normal blood [12]. The current paradigm suggests that these populations all originate from bone marrow-derived common monocyte precursor (MDP) cells. Functionally, however, these monocyte populations are thought to be diverse, playing different roles in homeostasis versus disease. Mouse monocytes can be divided into two major populations based on their Gr1/Ly6C surface expression. $Gr1^+/Ly6C^{high}$ monocytes (also called “inflammatory” monocytes) can give rise to “inflammatory” macrophages. These cells are important in typical inflammatory responses as they readily produce pro-inflammatory cytokines such as TNF, IL-12, IL-6 and

iNOS, which produces NO, a potent antibacterial effector and blood vessels homeostasis regulator. In the aorta, an important feature of macrophages is their ability to differentiate and give rise to foam cells under atherosclerotic conditions. $CD68^{+}CD11c^{+}$ cells in early atherosclerotic lesions were recently shown to accumulate lipids. These cells have been proposed to originate from $Gr1^{high}$ monocytes [38, 40], but whether $CD11b^{+}Gr1^{high}$ monocytes can give rise to foam cells during atherosclerosis remains to be determined.

On the other hand, $Gr1^{-}/Ly6C^{low}$ monocytes, also known as "patrolling" monocytes due to their ability to scan and patrol the vessel wall in normal conditions [41], could potentially give rise macrophages that are functionally distinct from the "inflammatory" macrophages described above. These cells can participate in phagocytosis, tissue remodeling, wound repair and, again in the context of atherosclerosis, might participate in foam cell formation [12].

Even though, at the early stages of their development, monocytes and DCs both go through a common monocyte dendritic cell precursor (MDP) stage, later on conventional DCs (cDC) differentiate via a common DC precursor (CDP) and pre-cDC stage [42]. This finding, however, applies to spleen DCs under steady state conditions and not necessarily to inflammatory DCs [42]. The lineage of aortic DCs under steady-state conditions is not known. During atherosclerosis, the aorta is infiltrated by inflammatory $CD11b^{+}CD11c^{+}$ DCs that originate from a common monocyte precursor [42, 43]. This $CD11b^{+}CD11c^{+}$ DC subset is close to so-called "M1 type" macrophages by phenotype and function [34]. Plasmacytoid DCs (pDCs) also originate from common DC precursor (CDP) cells, and might have their own intermediate precursor [41].

To summarize, the origin of macrophages and DCs in atherosclerosis *in vivo* has not been investigated directly. Under atherosclerotic conditions, inflammatory macrophages and DCs could differentiate from a common monocyte precursor [43], but some DCs subsets might also derive from other origins (Figure 2). Additional studies utilizing lineage tracking gene-modified mice on atherosclerosis-prone genetic backgrounds will be required to shed light on the origin of various types of DCs in atherosclerosis.

DC turnover in atherosclerotic plaques

One way in which DCs might accumulate inside the arterial wall and in the plaque is through direct recruitment of pre-cDCs and monocytes from the circulation. This process may be directed by specific chemokines, produced by endothelial cells, stromal and other immune cells in aorta [44]. Several studies using gene-modified mice show reduced atherosclerosis in the absence of CX3CR1 or CCR2 chemokine receptors, which correlates with decreased DC accumulation [45-47]. Conversely, CCR7-deficient DC show impaired egress from plaque [48]. It is not known which chemokines are important for low-level steady state DC recruitment to the healthy aorta.

Circulating DC (human) or DC precursors (pre-cDCs in mice) are constantly present in blood and under atherosclerotic conditions may start to interact with endothelial cells. Interactions of monocyte-derived DCs with platelets can also influence DC accumulation in the arterial wall. For example, *in vitro* rolling of monocyte-derived DCs on platelets is mediated by PSGL-1; firm adhesion is mediated by the integrin Mac-1, which is expressed on monocytes and therefore may mediate the recruitment of monocyte-derived DCs to the arterial wall. In addition, interaction with platelets also induced maturation of human monocyte-derived DC and upregulation of CD83 [49].

In addition to DC recruitment, local proliferation of recruited or resident $CD11c^{+}$ cells could also contribute to increased numbers of DCs in the arterial wall. Local proliferation of DCs

has been demonstrated in the aorta and in secondary lymphoid organs [50, 51]. Arterial DC numbers are also affected by egress. Monocyte-derived DCs can emigrate from the arterial wall and atherosclerotic plaques at the early stages of atherosclerosis, however their emigration from developed atherosclerotic lesions is significantly impaired in mice with dyslipidemia [52, 53]. Defective egress of DCs from the aorta and their altered trafficking toward lymph nodes can be yet another mechanism of excessive accumulation of monocyte-derived DCs in the atherosclerotic lesion.

Functional role of DCs in atherosclerosis

The function of DCs in atherosclerosis is not well understood, partially due to the lack of adequate experimental approaches. Historically, most of the attention has been devoted to macrophages as the major functionally important subset of myeloid cells in atherosclerosis. However, the importance of DCs for atherosclerosis is supported by several lines of evidence. As already described, mice lacking either CX3CR1, CCL2, or CCR5 demonstrate reduced atherosclerosis, which correlates with decreased DC accumulation [45-47]. CX3CR1 deficiency also decreases survival of $Gr1^{low}$ blood monocytes, which might explain the decrease in macrophage and DC content in aorta, decrease foam cells formation and therefore overall decrease of atherosclerosis [54]. Secondly, studies in which the receptor for diphtheria toxin was expressed under the CD11c promoter (CD11c-DTR) to transiently deplete $CD11c^+$ cells, showed enhanced cholesterolemia, suggesting a role of DC in regulation of cholesterol homeostasis [55]. Third, deletion of molecules involved in antigen presentation and DC migration, decreases atherosclerosis [56, 57]. All these studies point to the importance of DCs in atherosclerosis. Even though the number of DCs in the aorta during the initial stages of disease is very modest, DC accumulation dramatically increases as disease progresses [58].

DCs and accumulation of lipids

Although lipid uptake, foam cell formation and plaque growth in the artery has been attributed mostly to macrophages, recent studies demonstrated that DCs in the subendothelial space of aorta could also efficiently accumulate lipids and therefore contribute to initiation and further progression of the disease [38]. How many foam cells are DC-derived is not clear and awaits experimental investigation, particularly using lineage-tracking systems. It is possible that at least some foam cells can derive from $CD11b^+CD11c^+$ DCs (Figure 3A). Lipid and oxidized low-density lipoprotein (oxLDL) uptake may result in enhanced presentation of lipid- and peptide antigens to natural killer T cells (NKT) and T cells (Figure 3B). One of the major receptors for oxLDL is CD36, which is expressed by macrophages and monocyte-derived DCs [59]. In addition, CD36 mediates oxLDL-induced TLR4/TLR6 activation [60]. Stimulation of DCs by oxLDL through binding of CD36 and TLR4 leads to their activation and can be accompanied by enhanced cytokine production [61] (Figure 3C).

DC and antigen presentation in atherosclerosis

When considering how DCs might contribute to development of atherosclerosis, several studies imply that antigen-presentation plays a major role. Lack of the co-stimulatory molecules CD80 and CD86, which are known to be involved in immunological synapse formation and activation of T cells, reduces atherosclerotic plaque size [56]. Interestingly, mice lacking CD74, (MHCII-associated protein invariant chain, which regulates antigen processing and inhibits DC motility *in vivo* [62]), also demonstrate marked reduction of atherosclerosis [57]. Imaging of fixed aortic samples demonstrated that T cells could be found in close proximity to DCs in atherosclerotic plaques, implying potential DC-T cell interactions [63, 64].

Under atherosclerotic conditions, some DCs may take up “atherosclerosis-specific antigens” [65], become locally activated, and may migrate to paraaortic lymph nodes, where they may present the antigen to naïve T cells, leading to T cell activation and proliferation. During progression of atherosclerosis, the emigration rate of DCs from aorta to secondary lymphoid organs is decreased, which could lead to accumulation of antigen-loaded DCs in the plaque area [53]. DCs may also interact locally with T cells, resulting in their activation in the arterial wall. It is also possible that T cells, which were originally primed in secondary lymphoid organs can be re-stimulated by DCs locally in the aortic wall and atherosclerotic plaque. Overall, such mechanisms might participate in increased of local inflammation and atherosclerotic plaque growth.

DCs and local cytokine production may maintain low-grade chronic inflammation

Another important but largely overlooked function of DCs in atherosclerosis is related to their ability to produce various cytokines. TLR engagement can trigger cytokine production by DCs in addition to antigen presentation. DCs are capable of producing a number of pro-inflammatory cytokines, including TNF, IL-6 and IL-12, which are pro-atherogenic. For example, atherosclerosis-prone ApoE deficient mice that lack TNF expression show reduced atherosclerotic lesion size [66]. Likewise, in ApoE and IL-6 double deficient mice plaque size is reduced [67]. IL-6 has been shown to enhance lesion development in mice, because treatment of wild type mice fed high fat diet with recombinant IL-6 resulted in significant increase of lesion size [68]. However, analysis of old ApoE and IL-6 double deficient mice showed enhanced plaque formation [67]. The differences in these studies could be explained by a dual role for IL-6 in the control of inflammation at different stages of atherosclerosis.

IL-12 is a heterodimer, which consists of two subunits: p35 and p40. It drives Th1 differentiation. Studies so far have demonstrated unequivocally that IL-12 is a pro-atherogenic. *Il12p40*−/− *Apoe*−/− double knockout mice lack the p40 subunit common to IL-12 and IL-23 and show decreased lesion formation [69], while injection of recombinant IL-12 into $Apoe^{-/-}$ mice increases lesion size [70]. On the other hand, pharmacological blockade of IL-12 signaling reduced plaque size [71]. IL-12 seems to be important for both Th1 differentiation within the plaque and for overall T cell recruitment into the plaque [72]. DCs also can produce other cytokines of the IL-12 family, including IL-23 and IL-27, which are known to regulate Th17 and Th1 lineage differentiation and activation [73]. However, the function of these cytokines in atherosclerosis remains to be investigated (Figure 3C). Plasmacytoid DCs (pDC) are an important source of interferons α and β. Recent study demonstrated that IFNβ promotes atherosclerosis development by stimulation of macrophage recruitment (Figure 3D), [74].

Chemokine production by DCs is involved in regulation of immune cell migration to the arterial wall

DCs in the aorta also can produce various chemokines, which influence further recruitment of monocytes and lymphocytes into the plaque. In particular, DC-derived chemokines are important for recruitment of T cells. For example, both CCL17 (also known as TARC) and CCL22 were found in human atherosclerotic plaques [75]. These chemokines induce chemotaxis of T cells and elicit their effects by interacting with the chemokine receptor CCR4 on the T cells. CCL2 (MCP-1) is secreted by various myeloid cells (DC in particular) and is known to recruit monocytes, memory T cells and DC to the site of inflammation and tissue injury [76]. CCL4 is a chemoattractant for NK cells, monocytes and a variety of other immune cells [77]. All of these chemokines are produced by DCs in an atherosclerotic environment and may influence increased cell recruitment into the lesion site [78].

DCs and tertiary lymphoid organ formation

The appearance of arterial tertiary lymphoid organs (ATLOs) in mouse aorta was shown at the late stages of atherosclerosis development [58, 79]. These structures in the arterial wall have all signatures of specialized lymphoid organs, including the presence of follicular dendritic cells (FDCs), high endothelial venules (HEV), peripheral-node addressin-positive (PNAd+) vessels as well as T and B cell zones [79]. An important question pertains to the specific signals that initiate the formation of arterial TLO. In embryogenesis as well as during chronic inflammation, de novo lymphoid organ formation depends on the specific cell type called lymphoid tissue inducer (LTi) cells. These cells are characterized by $CD3^{-}CD4^{+}LT\beta^{+}$ surface phenotype and, although of lymphoid lineage, are distinct from T lymphocyte [80, 81]. In atherosclerosis SMC can serve as inducer cells for ATLO formation [82]. However, in atherosclerosis previous studies implicated a possible role of DCs interacting with $CD3^{+}CD4^{+}$ T cells in the initial stages of tertiary lymphoid organ formation in the thyroid gland by regulation of chemokine and adhesion molecule expression [83]. Therefore, interactions of DCs with CD4 T cells could potentially influence TLO development in atherosclerosis. Whether DC or DC-T cell interactions and their influence on chemokine production are also important for LTi recruitment into the atherosclerotic aorta remains to be elucidated.

Future directions and remaining questions

Although experiments with gene-modified mice have demonstrated that reduction of atherosclerosis correlates with decreased number of DCs in the mouse aorta when chemokine and integrin expression is manipulated, the exact functions of DCs under atherosclerotic conditions is unclear. In this review, we made an attempt to delineate several DC-mediated processes that are potentially relevant to atherosclerosis, namely presentation of atherosclerosis-relevant antigens to lymphocytes, production of chemokines and cytokines to sustain local inflammation and a potential for lipid uptake and differentiation to foam cells. With regard to antigen presentation by DCs to T cells in the atherosclerotic aorta, new *in vivo* imaging technologies may be useful to study DC-T cell interactions, similar to studies performed for DC-T cell interactions in secondary lymphoid organs [23]. It remains an open question whether specific targeting of DCs or DC-mediated functions will be beneficial to curb atherosclerosis in humans.

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Box 1

Immune recognition and potential “atherosclerosis-specific” antigens

Oxidized LDL (oxLDL)

Autoantibodies to oxLDL are found in both mice and humans with atherosclerosis [84]. Depending on isotype, these antibodies can be protective, neutral or disease-exacerbating.

Heat shock protein 60 (HSP60)

Soluble HSP60 levels in serum are elevated in humans with atherosclerosis [86] and correlate with carotid intima-media thickness, a measure of atherosclerosis. Antibodies to HSP60 in plasma also correlate with atherosclerosis progression [85].

ApoB100 (the main apolipoprotein of LDL)

T cell hybridomas cloned from human ApoB100 transgenic mice immunized with human oxLDL respond to native LDL and human ApoB100, but not to oxidized LDL [65].

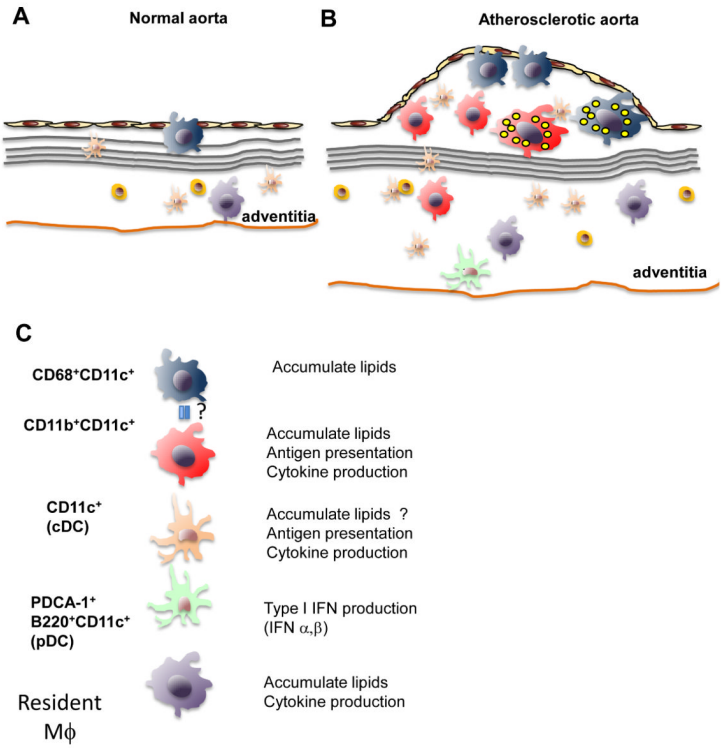

Figure 1. DCs in normal and atherosclerotic aorta in mice

a) Structure of normal arterial wall of mouse aorta. A few macrophages (Mφ) (purple), T cells (yellow) and DC can be found mainly in the adventitial compartment. $CD68^+CD11c^+$ (blue) can be found in subendothelial space even in naïve mice. **b)** Atherosclerotic aorta. In atherosclerosis the thickness of intima increases due to extensive atherosclerotic plaque growth. Significant numbers of $CD11b^+CD11c^+$ cells (red) accumulate mainly in the plaque, but also in adventitia. Foam cells are formed and accumulate in large numbers in the plaque. Various types of DC (tan, green) also appear in the plaque and aorta adventitia. The number of T cells also significantly increases. **c)** Potential functions of myeloid cells subsets in atherosclerosis are depicted. $CD68^+CD11c^+$ cells may be identical with $CD11b^+CD11c^+$ cells

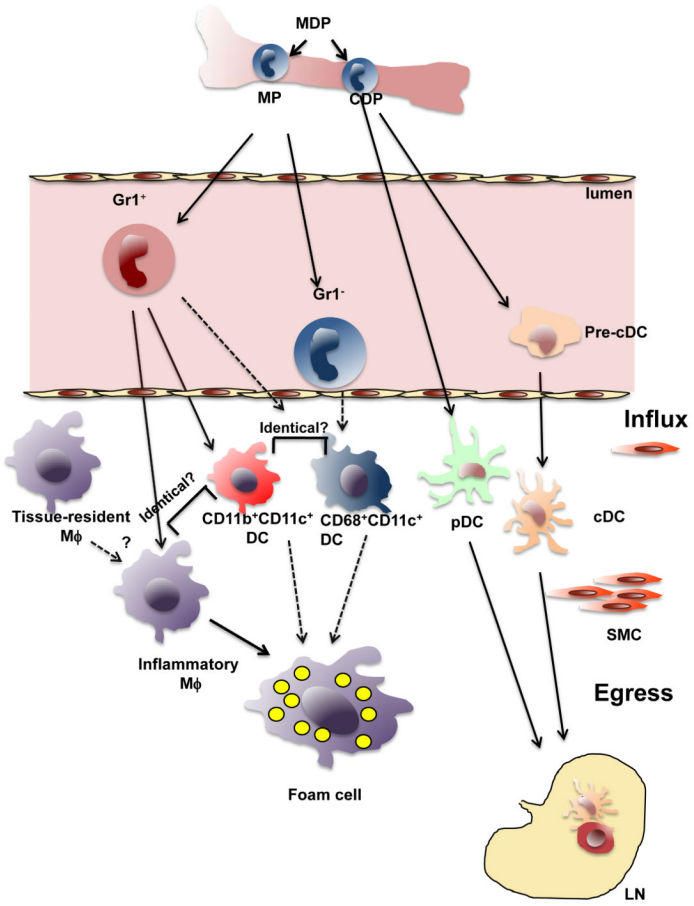

Figure 2. Origin of DCs and macrophages in atherosclerotic aorta

Common monocyte-DC precursor (MDP) gives rise to monocyte precursor (MP) and common DC precursor (CDP). MP gives rise to two functionally different populations of monocytes $Gr1^{+}$and $Gr1^{-}$. $Gr1^{high}$ monocytes can differentiate in tissue into inflammatory macrophages, CD11b+CD11c+ DCs and phenotypically similar $CD68^{+}CD11c^{+}$ cells. Inflammatory macrophages, $CD11b^{+}CD11c^{+}$ cells and $CD68^{+}CD11c^{+}$ cells all give rise to foam cells. CDP can further differentiate into pDC and pre-cDC, which become pDC and cDC, respectively. DC could either accumulate in arterial wall or/and migrate to draining lymph nodes for antigen presentation.

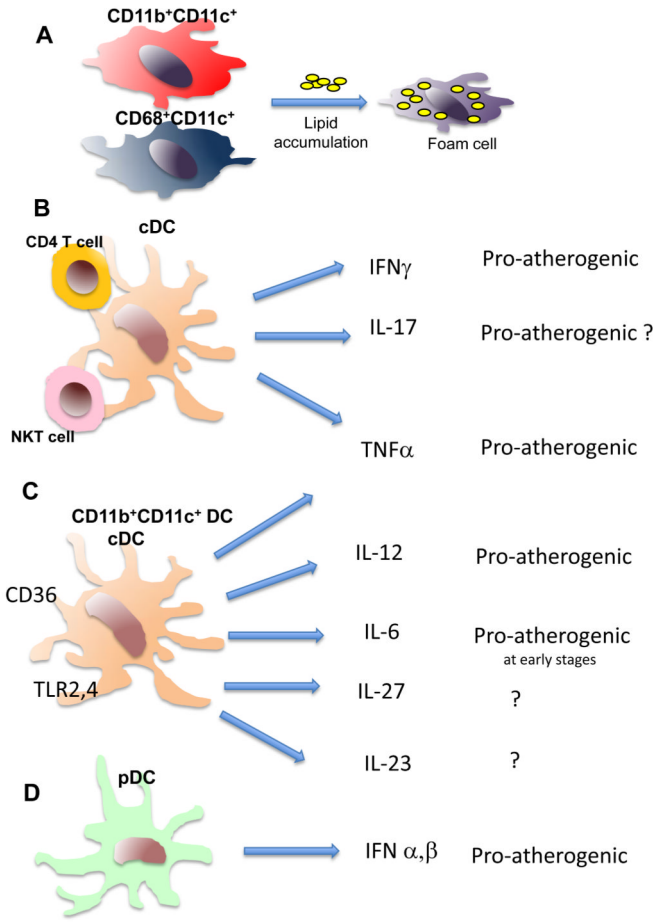

Figure 3. Potential functions of various DC subsets in atherosclerosis

A. $CD11b^{+}CD11c^{+}$ cells can participate in lipid accumulation and foam cell formation. **B**. cDC can participate in interaction with T cells and NKT cells, which results in increased production of IFNγ, IL-17 and TNFα by T cells. **C**. Activation of CD36 and TLRs in $CD11b^{+}CD11c^{+}$ and cDC by lipids results in increased secretion of various DC-derived cytokines. **D**. pDC were shown to produce IFN α,β, which play pro-atherogenic role.