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Minireview

Dendritic Cells and Regulatory T Cells in Atherosclerosis

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Although macrophages and other immune system cells, especially T cells, have been shown to play disease-promoting roles in atherosclerosis, less is known about the role of antigen presenting cells. Functional, immune stimulating dendritic cells (DCs) have recently been detected in aortic intima, the site of origin of atherosclerosis. We had compared DCs with macrophages in mice with experimental atherosclerosis, to clearly define cell types by developmental and functional criteria. This review summarizes recent advances in studies of DCs in humans and in mouse models of atherosclerosis, as well as providing a simple strategy to measure regulatory T (Treg) cells in the mouse aorta.

INTRODUCTION

Dendritic cells (DCs) were first identified in 1973 and shown to differ functionally from macrophages (Steinman and Cohn, 1973; 1974). DCs were later shown to be the principal cells involved in mixed leukocyte reactions, being more potent than B cells and macrophages (Steinman and Witmer, 1978). Despite initial reluctance to regard DCs as a separate cell type, DCs are now regarded as central to the initiation of antigen-specific immunity and tolerance (Steinman, 2012). Similar to macrophages, DCs were shown to reside in both lymphoid and nonlymphoid tissues (Geissmann et al., 2010). The presence of functional DCs in the blood vessels of humans and mice suggests that these cells may be involved in the initiation, progression, and/or regression of atherosclerosis, a representative chronic vascular inflammatory disease (Choi et al., 2009; 2011; Hansson and Hermansson, 2011). This review will highlight the role of human and mouse DCs in the steady state and in atherosclerotic aortae. We will also discuss the effects of DC subsets on the homeostasis of regulatory T (Treg) cells in the setting of atherosclerosis.

Dendritic cells in healthy and atherosclerotic human aortae

DCs in healthy human aortae

The presence of DCs in human aortic tissue was first shown by the morphological analysis of S-100 positive intimal cells (Bobryshev and Lord, 1995). These S-100$^+$ DCs were found to be more abundant in atherosclerosis-prone than in atherosclerosis-resistant areas of normal healthy aortae (Bobryshev and Lord, 1995). These cells were found to contain Birbeck granules, which are characteristic of epidermal Langerhans cells (Bobryshev et al., 1997). Importantly, networks of vascular-associated DCs were observed in the aortic intima of healthy young individuals (Millonig et al., 2001).

DCs in atherosclerotic human aortae

Several studies have shown that DCs are involved in the pathology of human vascular diseases. For example, S-100$^+$ DCs in contact with T and B lymphocytes were shown to be present in human aortic aneurysms, especially at the aortic adventitia (Bobryshev et al., 2001). These adventitial DCs were shown to play an important role in the development of giant cell arteritis by inducing T cell self-activation (Weyand et al., 2005). Moreover, DCs were also shown to accumulate and interact with T cells at sites of Takayasu's arteritis (Inder et al., 2000).

Morphological analysis of patients with atherosclerosis showed that vascular DCs accumulated in atherosclerotic lesions and were in contact with T cells and a small number of macrophages in the neovascularized areas of these lesions (Bobryshev and Lord, 1998). An examination of aortae from young (15-34 year old) subjects with early atherosclerotic lesions showed that inflammatory cells, including DCs, were present in the earliest stage of these lesions, thus enhancing our understanding of the initial pathophysiological processes of atherosclerosis (Millonig et al., 2002). During the advanced stage of human atherosclerosis, nodular lymphoid follicles containing several types of immune system cells, including endothelial cells, T cells, B cells, macrophages, and CD21-positive follicular DCs, form at the adventitia (Houtkamp et al., 2001), suggesting that DCs are involved throughout the atherosclerotic process.

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Aortic DCs in human atherosclerotic lesions have been shown to express several characteristic proteins, including heat shock protein 70, DC-SIGN, 5-lipoxygenase, and complement C1q (Bobryshev and Lord, 2002; Cao et al., 2003; Soilleux et al., 2002; Spanbroek et al., 2003), with 5-lipoxygenase and C1q shown to play important roles in the inflammatory processes of atherosclerosis. For example, advanced or vulnerable plaques were found to have higher numbers of DCs, and the DCs that accumulated in rupture-prone areas had a mature phenotype, expressing CD83 and DC-LAMP (Yilmaz et al., 2004). A mechanism by which aortic DCs trigger innate immune responses was revealed by analyzing the level of expression of toll-like receptors (TLR) in human aortic DCs (Pryshchep et al., 2008). TLR 2 and TLR 4 were ubiquitously expressed by DCs, and the resident DCs were shown to have immune sensing properties and to trigger T cell immunity.

Several recent studies have sought to identify the DC subsets associated with atherosclerosis. For example, plasmacytoid DCs (pDCs) were found to be present in human atherosclerotic lesions and to sense microbial motifs that result in the stimulation of cytotoxic T cells (Niessner et al., 2006). Moreover, BDCA-1^+ myeloid DCs and BDCA-2^+ plasmacytoid DCs were shown to be recruited to neovascularized areas in patients with advanced atherosclerosis (Van Vre et al., 2011). However, it is not feasible to functionally address each DC subset in humans. Thus, to more fully elucidate the function of DC subsets, studies are warranted in atherosclerosis-prone animal models, including *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice (See Fig. 1 for DC subsets).

Understanding the function of dendritic cells in atherosclerosis: lessons from mouse models

The administration of DCs pulsed with β-galactosidase-derived peptide to *Apoe*$^{-/-}$ mice expressing LacZ in aortic smooth muscle cells was found to increase the formation of atherosclerotic lesions, suggesting that DC-mediated presentation of atherosclerosis related-antigens can increase lesion formation (Ludewig et al., 2000). More recently, hyperlipidemia was shown to impair activation of CD8α^- DCs, attenuating Th1-type immune responses (Shamshiev et al., 2007). The antigen presentation capacity of DCs isolated from hyperlipidemic mice was not impaired, however, allowing these cells to induce T cell activation and leading to adaptive immune responses (Packard et al., 2008). In contrast, hyperlipidemic status was reported to inhibit the migration of DCs to draining lymph nodes, probably aggravating local inflammation (Angeli et al., 2004). Therefore, DCs that accumulate locally in the aorta may play an important role in atherosclerosis (Fig. 1).

Defining DCs as an immune component of healthy mouse aortae

Most early studies on DCs in murine aortae utilized morphological analysis or immunostaining of DC-related markers (Bobryshev et al., 1999; 2001; Moos et al., 2005; Ozmen et al., 2002). Later in-depth analysis of immune cells in mouse aortae, as determined by flow cytometry, showed that CD11c$^+$MHCII$^+$ DCs were present in normal aortae prior to the generation of atherosclerotic lesions (Galkina et al., 2006). Analysis of the spatial distribution of aortic immune cells by *en face* immunofluorescence showed that CD11c$^+$CD68$^+$ DCs had accumulated in the aortic intima of atherosclerosis-prone areas of normocholesterolemic C57BL/6 mouse aortae, with fewer of these intimal leukocytes accumulating in atherosclerosis-resistant strains, such as C3H/HeSnJ and BALB/c (Jongstra-Bilen et al., 2006). Moreover, these intimal DCs in normal aorta were found to originate from bone marrow-derived monocytes and to be VCAM-1 dependent. However, these studies did not show whether these aortic DCs could stimulate T cells, an important functional property of DCs. Our previous study was the first to show that aortic CD11c$^+$MHCII$^+$ DCs could take up blood-born protein antigens and present them to T cells, showing that these CD11c$^+$ cells in normal aortae are bona fide DCs (Choi et al., 2009). Moreover, we showed that these aortic intimal DCs are mainly localized to the aortic sinus, the cardiac valves, the lesser curvature of the aortic arch, and the opening of the arterial branches. Taken together, these findings revealed that DCs are an immune component of healthy mouse aortae (Fig. 1).

The involvement of DCs in the development of atherosclerosis

Several studies have shown that alterations in DC populations could affect the formation of atherosclerotic lesions. A deficiency in granulocyte/macrophage colony-stimulating factor (GM-CSF) was found to decrease the number of lesional CD11c$^+$ DCs, leading to an attenuation of atherosclerosis (Shaposhnik et al., 2007). GM-CSF has also been reported to regulate intimal DC proliferation in nascent atherosclerotic lesions (Zhu et al., 2009). Intimal DCs were shown to express the chemokine receptor CX$_3$CR1, and CX$_3$CR1 deficient mice had fewer intimal CD11c$^+$ DCs and fewer atherosclerotic lesions, suggesting that CX$_3$CR1 contributes to the formation of atherosclerotic lesions possibly by mediating the accumulation of CX$_3$CR1$^+$ DCs in aortic intima (Liu et al., 2008). Increases in DC populations induced by the overexpression of human Bcl-2 were found to enhance T cell activation and to increase plasma concentrations of Th1-driven IgG2c autoantibodies directed against oxidation specific epitopes, but to decrease plasma cholesterol concentrations (Gautier et al., 2009).

Dissecting DC lineages in aortae

We recently evaluated DC subpopulations in normal and atherosclerotic aortae (Choi et al., 2011). Normal aortae have at least two primary DC subsets, CD11b$^+$F4/80$^+$CD14$^+$CD209a/DC-SIGN$^+$ Mo-DCs and CD11b$^-$F4/80$^-$CD103$^+$CD207/Langerin$^+$ classical DCs (cDC), with the latter dependent on Flt3L hematopoietin and its receptor Flt3 (See Fig. 1 for an illustration of DC development).

Classical DCs

In the absence of Flt3, the number of cDCs was reduced and mice with an *Ldlr*$^{-/-}$ athero-prone background developed more severe atherosclerosis. *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice showed reduced numbers of Treg cells, both in the aorta and the periphery, indicating that Flt3-dependent cDCs play a protective role in atherosclerosis by regulating the homeostasis of Treg cells.

Monocyte-derived DCs

Mo-DCs, the other major DC subset found in atherosclerotic lesions, have been assumed to enhance atherosclerosis (Weber et al., 2008). For example, plaque deposition was less severe in CX$_3$CR1-deficient athero-prone mice (Liu et al., 2008). In agreement, we found that only Mo-DCs expressed higher levels of CX$_3$CR1 (Choi et al., 2011), but, to date, there is no direct evidence that Mo-DCs have a proatherogenic function. Thus, a mouse model deficient in Mo-DCs, using a diphtheria toxin/diphtheria toxin receptor (DT/DTR) system, will be helpful to understand the function of these cells in atherosclerosis.

Plasmacytoid DCs

Less is known about the function of pDCs, another Flt3/Flt3L

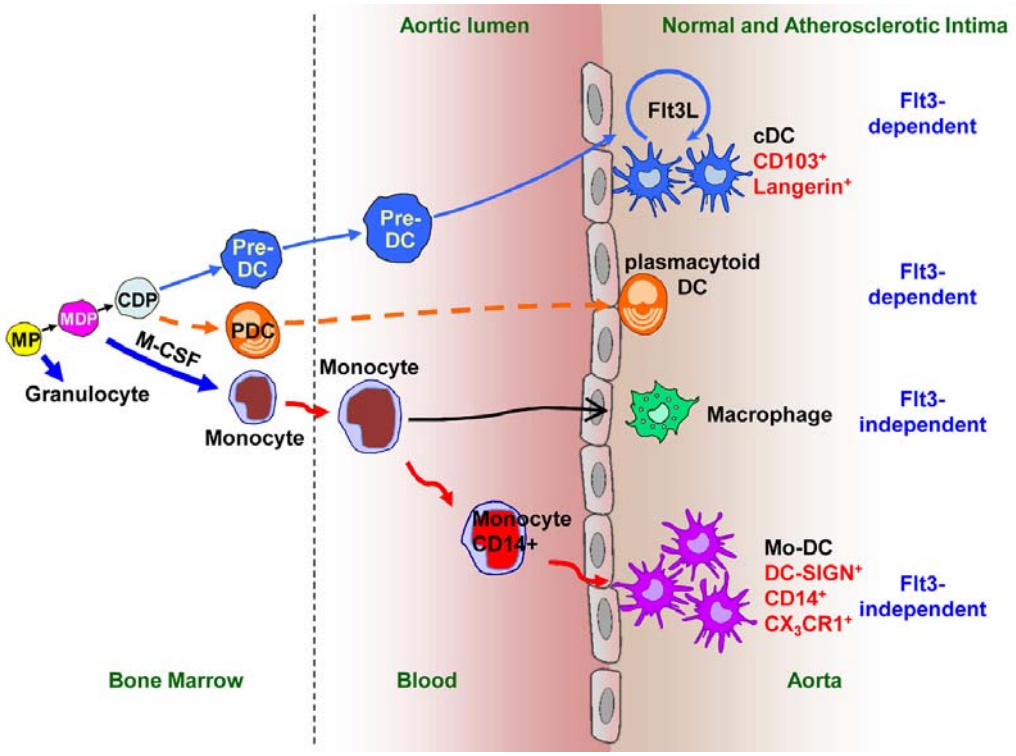

Fig. 1. The development of aortic DCs and macrophages. All DC lineage precursors except monocytes express a receptor (Flt3/Flk2/CD135) for Flt3L hematopoietin. Studies on mice deficient in M-CSF or Flt3L or their respective receptors indicate that M-CSF and Flt3L guide the steady state in vivo development of monocytes/macrophages and DCs (Ginhoux et al., 2006; McKenna et al., 2000; Waskow et al., 2008; Witmer-pack et al., 1993). Although pDCs have been observed only in atherosclerotic aortae, all remaining populations are observed in both healthy and diseased aortae. Abbreviations: MP, common myeloid progenitor; MDP, macrophage-DC progenitor; CDP, common DC progenitor; pre-DC, DC precursor; pDC, plasmacytoid DC; M-CSF, macrophage-colony stimulating factor.

dependent DC subset in atherogenesis. Previous studies have suggested that pDCs may be involved in either reducing or promoting atherosclerosis. These pDCs have been observed in human atherosclerotic lesions and have been found to stimulate cytotoxic T cell function by secreting interferon-α, probably leading to the exacerbation of atherosclerosis (Niessner et al., 2006). Moreover, auto-antigenic protein-DNA complexes were found to stimulate pDCs, whereas injection of anti-PDAC-1 antibody was shown to reduce the number of pDCs, decreasing the formation of atherosclerotic lesions and suggesting that pDCs have a proatherogenic function (Doring et al., 2012), However, injection of 120G8 antibody, which also decreased the number of pDCs, was found to increase atherosclerotic lesion formation in a mouse model by abrogating the indoleamine 2,3-dioxygenase (IDO)-mediated suppression of T cells (Daissormont et al., 2011). In these studies, pDC functions in atherosclerosis were explored by *in vivo* depletion of this cell type using pDC-depleting antibodies (the anti-SiglecH monoclonal antibody 440c; or the anti-BST2 monoclonal antibody 120G8 or PDCA1). However, this approach remains problematic because SiglecH and BST2 are constitutively expressed on other immune cells and are expressed on most cell types after activation, suggesting that injection of pDC-depleting antibodies may also deplete additional cell types (Blasius et al., 2006; Swiecki et al., 2010; Zhang et al., 2006). Alternative approaches are therefore needed to validate the role of pDCs in atherosclerosis. For example, two transgenic models are currently available, BDCA2-DTR and *Siglech*-DTR mice, in which pDCs can be selectively and inducibly depleted by injection of DT during inflammatory immune responses (Swiecki et al., 2010; Takagi et al., 2012).

CCL17$^+$DCs

CCL17-expressing DCs were recently shown to accumulate in atherosclerotic lesions and to exacerbate atherosclerosis by limiting the expansion of Treg cells (Weber et al., 2011). These CCL17$^+$ DCs expressed CD11c and CD11b, with high levels of MHCII and costimulatory molecules, including CD40, CD80, and CD86. These cells, however, did not express surface CD8α, CD115, F4/80, SiglecH, or PDCA-1, indicating that these CCL17$^+$ DCs may be distinct from previously defined Mo-DCs and pDCs (Choi et al., 2011; Daissormont et al., 2011; Doring et al., 2012). Further studies are needed to elucidate the lineage of this DC population.

Regulatory T cells in atherosclerosis

Treg cells are known to play a pivotal role in the control of a variety of immune-mediated pathologies, such as autoimmunity, colitis, and chronic inflammation including atherosclerosis (Hansson and Hermansson, 2011; Josefowicz et al., 2012). An in-

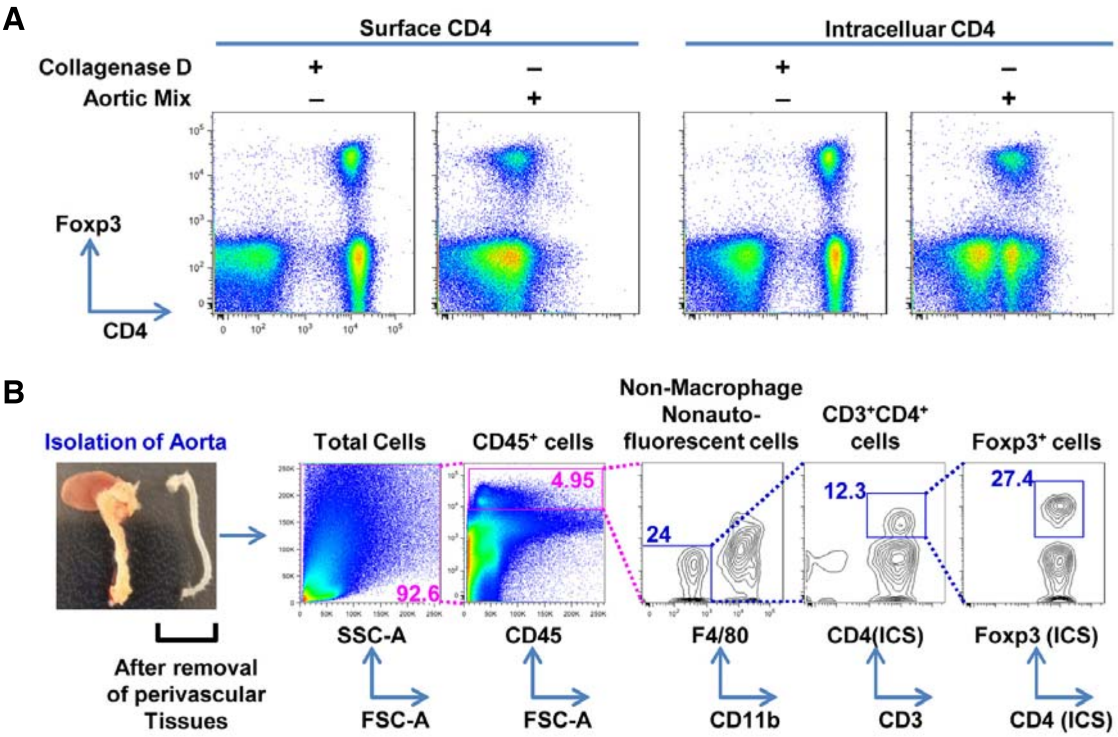

Fig. 2. Simple method of measuring Foxp3$^+$ Treg cells in the aortae of *Ldlr*$^{-/-}$ mice. Single aortic cell suspensions were prepared as described (Choi et al., 2011). Briefly, mouse aortae were harvested, and perivascular fat tissues were further processed under a microscope. The remaining clear blood vessels, including the thoracic aorta, aortic arch and aortic sinus (see pictures of aortae before and after removal of perivascular tissues), were digested with a mixture of collagenase types I and XI, hyaluronidase and DNase I (aortic mix) to yield single cell suspensions. While trying to optimize Treg cell staining, we found that the surface expression of CD4 was lost during digestion with the aortic mix. Using splenic single cell suspensions, we compared the effects of collagenase D and the aortic mix on surface CD4 staining, finding that the aortic mix removed CD4 from the surface of splenocytes. Since CD4 and Foxp3 staining are critical to define Foxp3$^+$ Treg cells in CD4$^+$ T cells, we incubated fix-permeabilized splenocytes with antibody against CD4 after digestion with the aortic mix. Intracellular CD4 proteins were well-retained and sufficient to determine the numbers of CD4$^+$Foxp3$^+$ T cells in the spleen. Using this method, the aortic cells from *Ldlr*$^{-/-}$ mice fed a Western diet for 12 weeks were analyzed for the presence of Treg cells. (A) Aortic mix cleaves surface CD4 but has no effect on intracellular staining. After lysing red blood cells, splenocytes were incubated for 45 min with collagenase D or aortic digestion enzyme mix. Fc-blocked cells were surface-stained for CD4 or fix-permeabilized with Foxp3 staining buffer to stain intracellular CD4. Foxp3-staining was performed according to the manufacturer's protocol (eBioscience Foxp3 staining Kit). (B) Measurement of Foxp3$^+$ Treg cells in *Ldlr*$^{-/-}$ aortae. Aortic cells were surface stained for CD45, CD11b, F4/80, and CD3, fixed and permeabilized and stained for intracellular CD4 and Foxp3. To analyze Treg cells, CD45$^+$ leukocytes were gated; non-macrophages and non-autofluorescent cells were selected; and CD3$^+$CD4$^+$ T cells were gated out. The CD4$^+$ T cells were further analyzed to determine the number positive for Foxp3.

crease in Treg cells in lymphoid tissues has been correlated with reduced atherosclerosis (Ait-Oufella et al., 2006; Hermansson et al., 2011; Mor et al., 2007), with Treg cells playing a critical role in several mouse models of atherosclerosis. For example, mice deficient in CD80 and CD86 showed defects in the homeostasis of Treg cells, resulting in severe atherosclerosis when reconstituted on an *Ldlr*$^{-/-}$ athero-prone background. In addition, transfer of Treg-poor *CD28*$^{-/-}$ splenocytes into *Apoe*$^{-/-}$ *Rag2*$^{-/-}$ mice resulted in an increase in lesion size. Finally, depletion of Treg cells by anti-CD25 antibody aggravated atherosclerosis in *Apoe*$^{-/-}$ mice. However, the protective effect of Treg cells on atherosclerosis was marginal in *Apoe*$^{-/-}$ *dnTGFβ*$^+$ mice, suggesting the involvement of T-cell TGFβ signaling. Furthermore, in the absence of inducible costimulatory molecule (ICOS), an increase in splenic pathogenic CD4 T cells, but a decrease in Treg cells, in *Ldlr*-deficient mice resulted in an increased atherosclerotic burden (Gotsman et al., 2006).

Feedback control of Treg cell homeostasis by DCs in vivo

A growing body of evidence suggests that DCs play an important role in the induction of antigen-specific unresponsiveness or tolerance in peripheral T cells (Hawiger et al., 2001; Kretschmer et al., 2005; Steinman and Nussenzweig, 2002). In mice, DCs play a critical role in maintaining Treg cells (Darrasse-Jéze et al., 2009). Importantly, MHCII-expressing CD11c$^+$ DCs were required for the homeostatic maintenance of naturally occurring Treg cells. This dynamic feedback link between DCs and Treg cells was further supported by the amelioration of autoimmunity and inflammation following Flt3 ligand treatment, with an increase in the number of DCs leading to an increase in the number of Treg cells. Moreover, we found that the number of Treg cells in the aorta was reduced in Flt3/Flt3L-dependent classical DC-deficient athero-prone mice (Choi et al., 2011).

Measuring Treg cells in the mouse aorta
Most previous attempts to quantitate mouse Foxp3$^+$ Treg cells in atherosclerosis have involved the quantification of Treg cells in the spleen and peripheral lymph nodes, not in the aorta itself (Gotsman et al., 2006; Sasaki et al., 2009). Alternatively, quantitative PCR has been used to measure Foxp3 mRNA in the aorta (Weber et al., 2011). Recent attempts have utilized Foxp3-reporter mice crossed with $\mathit{Ldlr}^{-/-}$ mice (Maganto-García et al., 2011; Weber et al., 2011). To determine the number of Treg cells in the aorta by flow cytometry, we had to overcome several technical challenges (Choi et al., 2011). These include the extremely small number of Foxp3$^+$ T cells in the aorta, the loss of surface CD4 expression during the process of cell isolation (likely due to enzymatic digestion) and the presence of autofluorescent cells that interfered with the counting of the small number of Foxp3$^+$ T cells. After overcoming these challenges, we optimized our assays and successfully determined the numbers of Treg cells in mouse aortae, as shown in Fig. 2.

Harnessing dendritic cells as a therapeutic target in atherosclerosis

Several attempts to treat atherosclerosis have involved modulating DC function or the delivery of atherosclerosis-related antigens to DCs. Oral administration of the active form of vitamin D3 (calcitriol) was found to induce tolerogenic DCs and Treg cells, leading to the attenuation of atherosclerosis in mice (Takeda et al., 2010). In addition, the administration of DCs pulsed with oxidized low density lipoprotein (LDL) to mice deficient in LDL receptors reduced atherosclerosis and increased plaque stability (Habets et al., 2010). More recently, the injection of IL-10 was found to induce tolerogenic DCs loaded with apolipoprotein B100, which attenuated atherosclerosis possibly by inducing the generation of antigen-specific Treg cells (Hermansson et al., 2011). Taken together, these results suggest that tolerogenic DCs may be a promising target for the treatment of atherosclerosis, and that direct antigen delivery to tolerogenic DCs may prevent atherosclerosis. Lastly, Flt3 ligand may have important implications in the development of new interventions to target this mechanism of atherosclerosis.

CONCLUSIONS

Recent studies have begun to address the role of DC subsets in experimental atherosclerosis. It is not surprising that each DC subset has different effects on this disease. Current understanding of DC subsets in the aorta may be enriched in two ways: by developing mice in which each subset is selectively labeled with a reporter gene such as green fluorescent protein (GFP) and by developing transgenic mice in which each DC subset is specifically eliminated, with DTR or GFP expression under the control of DC subset-specific genes. These genetic mouse models may set the stage for future work on, for example, the mechanistic function of cDCs and Mo-DCs in the aorta, both during steady state and during the development of atherosclerosis.

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