Abstract: Atherosclerosis is a complex chronic disease. The accumulation of myeloid cells in the arterial intima, including macrophages and dendritic cells (DCs), is a feature of early stages of disease. For decades, it has been known that monocyte recruitment to the intima contributes to the burden of lesion macrophages. Yet, this paradigm may require reevaluation in light of recent advances in understanding of tissue macrophage ontogeny, their capacity for self-renewal, as well as observations that macrophages proliferate throughout atherogenesis and that self-renewal is critical for maintenance of macrophages in advanced lesions. The rate of atherosclerotic lesion formation is profoundly influenced by innate and adaptive immunity, which can be regulated locally within atherosclerotic lesions, as well as in secondary lymphoid organs, the bone marrow and the blood. DCs are important modulators of immunity. Advances in the past decade have cemented our understanding of DC subsets, functions, hematopoietic origin, gene expression patterns, transcription factors critical for differentiation, and provided new tools for study of DC biology. The functions of macrophages and DCs overlap to some extent, thus it is important to reassess the contributions of each of these myeloid cells taking into account strict criteria of cell identification, ontogeny, and determine whether their key roles are within atherosclerotic lesions or secondary lymphoid organs. This review will highlight key aspect of macrophage and DC biology, summarize how these cells participate in different stages of atherogenesis and comment on complexities, controversies, and gaps in knowledge in the field.  

(Circ Res. 2016;118:637-652. DOI: 10.1161/CIRCRESAHA.115.306542.)

Key Words: atherosclerosis ■ colony-stimulating factors ■ dendritic cells ■ macrophages ■ monocytes
Two Nobel Prizes in Physiology or Medicine awarded just >100 years apart (1908 and 2011) recognized Elie Metchnikoff for elucidating a key aspect of innate immunity—phagocytosis by macrophages—and Ralph Steinman for the discovery of the dendritic cell (DC) and its role in adaptive immunity. Both macrophages and DCs play a critical role in many diseases including atherosclerosis, but the relative contributions of these myeloid cells are still debated. We have known for decades that macrophages are critical to atherogenesis and that monocytes are recruited to atherosclerotic lesions.1,2 On the basis of the foundations of modern macrophage biology laid by van Furth and Cohn,3 including the concept that these professional phagocytes are derived from circulating blood monocytes, a notion has been firmly entrenched that chronic inflammation characterized by monocyte recruitment leads to the accumulation of macrophages in atherosclerotic lesions. More recent studies, however, have revealed a new paradigm related to the ontogeny of tissue-resident macrophages and have conclusively established that these cells can be derived from embryonic or fetal progenitors and self-renew through proliferation.4,5

**Tissue-Resident Macrophages and Their Ontogeny**

Most tissues of the body contain tissue-resident macrophages. The diversity of the tissue environments in which macrophages reside has resulted in considerable phenotypic and functional heterogeneity. Gene expression profiling of macrophage populations from several tissues, for example, has established that only a small number of transcripts (eg, CD64 and MerTK) associate with all macrophage populations.6 In addition to maintaining tissue homeostasis and responding to invading pathogens, macrophages contribute to numerous pathological processes, making them an attractive potential target for therapeutic intervention. However, to do so will require a comprehensive understanding of macrophage origins, the mechanisms that maintain them, and their functional attributes in different tissues and disease contexts.

Macrophage ontology has been debated for decades,7,8 yet the concept of the mononuclear phagocyte system—which asserts tissue macrophages develop exclusively from circulating bone marrow–derived monocytes—has prevailed for nearly a half century.9 However, recent studies using sophisticated fate-mapping approaches have determined that some tissue macrophages and their precursors are established embroyonically in the yolk sac and fetal liver before the onset of definitive hematopoiesis.9–14 Specifically, tissue macrophages arise from 2 distinct developmental programs; early yolk sac–derived erythro-myeloid progenitors that give rise to macrophages without monocyte intermediates, and fetal liver monocytes that derive from late c-Myb+ erythro-myeloid progenitors generated in the yolk sac and hemogenic endothelium, a specialized subset of endothelial cells found in the dorsal aorta at the level of the gonad/mesonephros15,16 (Figure 1). These pathways contribute to macrophage development to varying degrees in several tissues, including the brain, skin, heart, liver, and lung.10,13,17–20 Tissue-resident macrophages can also be derived from recruited monocytes in the postnatal period (Figure 1), although the mechanisms that determine the balance between prenatal versus postnatal derivation are not fully understood. Regardless of their origin, resident macrophage populations in many organs maintain themselves in adulthood through local proliferation, and are largely independent of blood monocyte recruitment.17,21 Intestinal macrophages are an exception. Their renewal is constant throughout adulthood and is dependent on circulating monocytes.22 Circulating monocytes in humans and mice consist of at least 2 subpopulations—classical and nonclassical (patrolling), with distinct of cell surface marker and chemokine receptor expression patterns and functions during inflammatory responses.23–25

Tissue-resident macrophages densely populate the normal arterial wall, yet their ontology, function, and the mechanisms that sustain them are poorly understood. The arterial wall consists of 3 layers: a thin intima, a thick media, and an outer adventitia. Arterial macrophages reside largely within the adventitia. Through gene expression analysis, we recently demonstrated that arterial macrophages constitute a distinct population among tissue macrophages.26 Using multiple fate-mapping approaches, we showed that arterial macrophages arise embryonically from CX3CR1+ yolk sac erythro-myeloid progenitors and fetal liver monocytes. Macrophage colonization also associated with a period of recruitment of circulating monocytes immediately after birth. The postnatal period of monocyte influx was brief, and corresponded with transient expression of chemokines and cell adhesion molecules implicated in leukocyte recruitment. In this way, development of the arterial macrophage pool is unique among previously described macrophage populations. Arterial adventitial macrophages are maintained in adulthood through local proliferation without further meaningful contribution from blood monocytes. Importantly, proliferation sustains arterial macrophages not only during steady-state conditions but also mediates their rebound after severe depletion after sepsis. Maintenance of the arterial macrophage niche depended on interaction between macrophage CX3CR1 and CX3CL1 expressed on PDGFRα+ mesenchymal cells.
In addition to expressing core signature macrophage markers, including CD64, MerTK, F4/80, and CD11b, arterial macrophages uniquely express lymphatic vessel endothelial hyaluronan receptor-1 (Lyve-1). Historically, Lyve-1 was thought to be a specific marker of lymphatic endothelium; however, it is also expressed on some macrophages in the aorta, heart, eye, and tumors. In the arterial adventitia, Lyve-1 expression is unique to tissue-resident macrophages because macrophages that arise from recruited blood monocytes do not express the receptor.26 Thus, Lyve-1 expression distinguishes resident arterial macrophages from monocyte-derived cells recruited after systemic injection of the bacterial cell wall component, lipopolysaccharide. Lipopolysaccharide exposure induces a reduction of Lyve-1+ macrophages, transient replenishment of the arterial compartment by bone marrow–derived Lyve-1– macrophages and subsequent reestablishment of functional homeostasis by the Lyve-1–resident macrophage population. Lyve-1+ macrophages renew locally through proliferation, but may also partly derive from arterial-resident macrophage progenitor cells. In atherosclerosis, most macrophages that amass in developing intimal lesions are derived from blood monocytes. Whether resident arterial macrophages influence lesion progression, however, remains unknown. The ability to distinguish resident and monocyte-derived macrophages may prove a powerful tool for advancing our understanding of macrophage biology in atherosclerosis progression.

DC Origin and Classification

DCs are subdivided into classical (cDCs) and plasmacytoid (pDCs). These cells are derived from a common DC progenitor and precursors of cDCs (pre-cDCs) form in the bone marrow and disseminate to lymphoid organs (Figure 1). In addition to cDCs derived from common DC progenitors and pre-cDCs, cDCs can originate from blood monocytes. DC developmental pathways have been studied mostly in lymphoid tissues, and additional work will be required to map DC development in other tissues, including the normal aorta. Several excellent reviews provide a comprehensive summary of recent advances in the DC field27,28 and key facts related to their origin, cell surface markers, gene expression, transcription factors, lineage tracing, in vitro derivation from cultured bone marrow, important complexities, and gaps in knowledge are summarized in the Online Data Supplement.

Macrophage and DC Functions

The homeostatic functions of tissue macrophages are common and diverse. Common functions include immune surveillance against invading pathogens, tissue remodeling and regeneration and support of angiogenesis. For example, tissue macrophages play a key role in limb regeneration in lower species such as the salamander29 and regeneration of neonatal myocardium in mouse injury models.30,31 Diverse macrophage behavior is largely determined by tissue context. For example, microglial cells in the brain facilitate neuronal pruning,32,33 macrophages produce catecholamines to sustain adaptive thermogenesis by adipose tissue and orchestrate the development of beige fat,34,35 and bone marrow macrophages are an integral component of the hematopoietic stem cell niche.36,37 The steady-state functions of other tissue macrophage populations, including resident arterial macrophages, remain largely unknown.

DCs induce tolerance against self-antigens and promote antigen-specific immunity on exposure to pathogens.38 Antigen presentation without costimulation leads to T-cell anergy or deletion. A related homeostatic function of DCs is to...
induce CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T cells (Tregs), which are critical for maintenance of peripheral self-tolerance.\(^{39}\) This important function is under control of a positive regulatory feedback loop.\(^{39,40}\) Treg depletion in Foxp3-diphtheria toxin receptor (DTR) mice induces FMS-like tyrosine kinase 3 ligand (Flt3L) secretion from yet unknown cells, which in turn initiates the expansion of pre-cDCs and cDCs in lymphoid tissues.\(^{39}\) Ablation of cDCs in CD11c-DTR mice led to a loss of antigen presentation by macrophages may be significant. where macrophages outnumber DCs by orders of magnitude, though less efficiently than DCs. Yet, in a diseased tissue and in cell culture assays can present antigen to T cells allowing a long-lived antigen-specific memory T cells to traffic through tissues.

In response to pathogens, DCs in tissues upregulate chemokine receptors such as CCR7, migrate via lymphatics into T cell–enriched areas of lymph nodes and undergo maturation by upregulating T-cell costimulatory molecules, including CD40, CD80/B7-1, CD83, and CD86/B7-2. To fulfill their immunogenic functions, DCs possess machinery to capture, process, and present antigens to T cells (ie, pattern recognition receptors/toll-like receptors [TLRs], c-type lectin receptors and Fc receptors, and MHC molecules). Each DC subset expresses differential repertoires of c-type lectin receptors and TLRs, which biases the T-cell response. Splenic CD8\(^{+}\)CD205\(^+\) DCs are superior at cross-presenting antigen by MHC-I and stimulate CD8\(^+\) T cells and CD8\(^{+}\) DCIR\(^+\) DCs are specialized for presentation by MHC-II and stimulate CD4\(^+\) T cells.\(^{41}\) CD8\(^{+}\) DCs express CD205, TLR3 and secrete IL-12p70, whereas monocyte-derived DCs express CD209/DC-SIGN and TLR4.\(^{41,44}\) pDCs express high levels of the nucleic acid sensors such as TLR7 and TLR9 and secrete type I IFNs during viral infection.\(^{47}\) pDCs can also prime and cross-prime antigens and induce Th1 polarization.\(^{46}\)

Recent studies revealed spatio-temporal complexity in DC–T cell interactions in lymphoid tissue that is critical to virus-specific T-cell responses.\(^{47,48}\) Skin CD11b\(^+\) DCs are infected early, migrate to the draining lymph node and present viral antigens to CD4\(^+\) T cells. At this early time point, there is minimal cross-presentation to CD8\(^+\) T cells. Later, noninfected chemokine (C motif) receptor 1\(^+\) (XCR1\(^+\)) DCs that reside in the draining lymph node acquire viral antigen from infected DCs and cross-present to CD8\(^+\) T cells. A prerequisite for this process is licensing of XCR1\(^+\) DCs by CD4\(^+\) T cells that were activated by CD11b\(^+\) migratory DCs. This implies that sequential interactions between DC subsets and T cells in lymph nodes facilitate diversification of T-cell responses.

Antigen presentation can also occur in peripheral tissues, but is stochastic because of a lack of a full T-cell repertoire. However, re-exposure to a previously encountered antigen significantly increases the chances of DC antigen presentation to a memory T cell in peripheral tissues because the initial exposure will produce long-lived antigen-specific memory T cells that traffic through tissues.

It is interesting that the abundance of tissue macrophages rapidly decreases in the early stages of inflammation, that macrophages express MHC-II and costimulatory molecules, and in cell culture assays can present antigen to T cells although less efficiently than DCs. Yet, in a diseased tissue where macrophages outnumber DCs by orders of magnitude, local antigen presentation by macrophages may be significant.

In vitro studies established that macrophages can be classified into functionally distinct subsets.\(^{49}\) Functional polarization is also observed in vivo under physiological and pathological conditions.\(^{50}\) Macrophages undergo either classical M1 or alternative M2 activation, although other subsets have been described.\(^{51-54}\) In fact, M1 and M2 can be viewed as the ends of a spectrum and this nomenclature may be misleading.\(^{55,56}\) M1 macrophages express high levels of inflammatory cytokines, increased production of reactive nitrogen and oxygen intermediates, and promote Th1 responses.\(^{49,57}\) M2 macrophages participate in tissue remodeling, wound healing, and immune regulation\(^{51,58}\); are highly phagocytic; express high levels of scavenging molecules, mannose and galactose receptors; and produce ornithine and polyamines through the arginase pathway.\(^{50}\) Both M1 and M2 macrophage phenotypes are found in atherosclerotic lesions. The phenotypic profile of macrophages at different stages of atherosclerosis is poorly understood,\(^{54,59}\) but is likely influenced by both lineage commitment and responsiveness to the local microenvironment.

Although macrophages and DCs have distinct origins and specialized functions, it is important to remember that the biology of these myeloid cells overlaps and is not fully understood in the context of complex diseases. In light of the well-documented fact that the phenotype of differentiated cells can be modulated by environmental cues (eg, plasticity of T-cell subsets and epithelial/endothelial–mesenchymal transdifferentiation), it is possible and even likely that similar phenotypic modulation may occur in macrophages and DCs during the pathogenesis of complex diseases, such as atherosclerosis.

**Stages of Atherogenesis**

Atherosclerotic lesions in mouse models progress through stages where intimal myeloid cell biology and molecular mechanisms may be distinct (Table). Nascent lesions begin in specific regions of the arterial wall early after initiation of a cholesterol-rich diet (CRD) in low-density lipoprotein (LDL) receptor–deficient (Ldlr\(^{-/-}\)) mice. During this stage, myeloid cells that normally reside in the intima in regions predisposed to atherosclerosis begin accumulating intracellular lipid.\(^{59}\) Increased expression of endothelial cell adhesion molecule and chemokactic cytokines, monocyte recruitment and the proliferation of intimal myeloid cells begin after 1 to 2 weeks of hypercholesterolemia.\(^{61}\) This combined with continued accumulation of intracellular lipid results in early lesions that are composed almost entirely of myeloid foam cells. The abundance of myeloid cells in the intima continues to increase and their numbers stabilize over weeks to months. Advanced lesions progress through phases that include atheroma with large necrotic areas, fibro-fatty nodules containing chondrocyte-like cells and calcified acellular regions. Smooth muscle cells migrate from the media into the intima, deposit matrix, and contribute to the formation of a fibrous cap.\(^{62}\) The biology of this stage is complex. Macrophage abundance in advanced lesions remains stable. They turn over within weeks, and proliferation, not monocyte recruitment, is the primary mechanism of macrophage renewal.\(^{63}\) These findings suggest that the local environment in advanced lesions is distinct from early lesions where monocyte recruitment is a significant
component of local myeloid cell expansion. Intra-plaque hemorrhages occur in advanced lesions located in the innominate artery of apolipoprotein E−deficient (ApoE−/−) mice. These lesions exhibit fibrotic conversion of necrotic zones, accompanied by loss of the fibrous cap. Hemorrhages are associated with fissures through fatty streaks that form adjacent to or on top of established plaques.64 This suggests that foam cell lesions can form over established plaques possibly through foam cell lesions with hemorrhage (in the innominate artery of old ApoE−/− mice).

**Topographical Predisposition to Atherosclerosis and Intimal Myeloid Cells in the Normal Intima**

In all species, regions of the arterial tree have a distinct predilection to atherosclerosis. Atherosclerotic lesions form in curvatures, branch points, and bifurcations, whereas straight segments of arteries are protected. This topographical pattern is related to the unique hemodynamic profile of arterial regions.65 In straight segments, blood flow is uniform laminar. It has a parabolic vector profile and a forward direction throughout the majority of the cardiac cycle.66 Disturbed laminar flow (frequently referred to as disturbed flow) is found in lesser (inner) curvatures, bifurcations, and branch points. Its direction and velocity during the cardiac cycle is complex, predictable, and includes directionality that is perpendicular to the mean bulk flow.67 Furthermore, during systole disturbed flow is usually in the forward direction and reverses during diastole, with the location of the stagnation point (the site that momentarily has no flow) changing during the cardiac cycle. Turbulent flow has chaotic fluctuations, is irregular and unpredictable. Turbulent flow occurs rarely in humans and very rarely in mice because flow is readily stabilized by a small arterial diameter.

Wall shear stress is the frictional force per unit area exerted by flowing blood on endothelial cells, and is dependent on fluid viscosity and velocity adjacent to the wall.65 Shear stress varies throughout the cardiac cycle. In straight segments of arteries, shear stress is high during systole and low during diastole, with a time-averaged shear stress typically 10 to 20 dynes/cm². Arterial endothelial cells sense shear stress, and this is essential for regulating flow in arteries and normalizing shear stress in situation when the requirement for oxygen changes. For example, a sudden increase in oxygen demand results in local dilatation of arterioles and increased tissue blood flow. This in turn increases the blood velocity and shear stress in the corresponding conduit artery. In response to the increased shear stress, endothelial cells produce more nitric oxide, which mediates dilatation of the conduit artery. Arterial dilatation maintains increased delivery of blood while reducing the velocity, and this normalizes the wall shear stress. If blood flow is altered for a prolonged period, arteries undergo outward or inward remodeling that permanently normalizes wall shear stress. In regions with disturbed flow, the time-averaged shear stress is low; however, the artery does not remodel probably because disturbed flow regions usually encompass only a portion of the arterial circumference, such as the inner but not the outer curvatures.

Uniform laminar flow induces the expression of endothelial cell genes that are considered to be anti-inflammatory and antiatherogenic, such as Krüppel-like factor 2, Krüppel-like factor 4, and endothelial nitric oxide synthase.65,66 In response to disturbed laminar flow endothelial cells exhibit unique homeostatic properties. This includes a polygonal or randomly elongated morphology, as opposed to cells elongated uniformly in the direction of uniformly laminar blood flow. Endothelial cell gene expression, signal transduction, and production of matrix and proinflammatory molecules are also unique in regions with disturbed flow.65,66 Several mechanisms contribute to distinct gene expression patterns in disturbed flow regions, including gene transcription, chromatin-based/epigenetic mechanisms, mRNA processing, and post-transcriptional regulation.69 The lesser curvature of the mouse aortic arch experiences disturbed flow and the biology of this region has been studied extensively70,71 (Figure 2). Even in the absence of hypercholesterolemia, unique endothelial properties including expression of leukocyte adhesion molecules and chemokines promote low-grade chronic inflammation in the lesser aortic curvature characterized by continuous recruitment of circulating monocytes.72 Myeloid cells accumulate in the intima of the lesser curvature and reside immediately below the luminal endothelium. Their numbers are relatively small in comparison with adventitial macrophages that populate all regions of the artery wall.26,72,73 Interestingly, myeloid cells also reside in the intima of the mouse aortic sinus, which is a region highly predisposed to atherosclerosis.73 Intimal myeloid cells have also been reported in atherosclerosis-susceptible regions of the rabbit aorta74 and are found in human arteries.75,76

Myeloid cells in the normal mouse arterial intima express both myeloid and DC markers such as CD68, CD11b, F4/80, CD11c, class II MHC, DCIR2/33D1, and αEβ7 integrin (CD103), and exhibit long dendritic processes, a morphological feature typical of DCs.72,73 It is likely that the local arterial
intimal microenvironment influences this phenotype. Intimal cells are as effective as splenic DCs in antigen handling, processing, and presentation to T cells.\(^7\) In this study, CD11c promoter–enhanced yellow fluorescent protein transgenic mice were injected intravenously with ovalbumin, DCs were isolated by fluorescence-activated cell sorting from the aorta and spleen, and antigen presentation to transgenic OT-I and OT-II T cells was monitored in vitro. T cells are rarely found in the normal aortic intima,\(^7\) although their numbers increase significantly during atherogenesis. It remains to be determined if antigen presentation is a physiological function of intimal myeloid cells in situ in the normal intima. Recently, we obtained data showing that they can protect the intima from infection by intracellular pathogens that are imported within recruited blood monocytes.

The ontogeny of intimal myeloid cells has not yet been determined using fate-mapping strategies that have been used to distinguish fetal from definitive hematopoietic origins, as is reviewed above for tissue-resident macrophages. This is an important endeavor because it may provide clues related to their function and why the local microenvironment can sustain a population of myeloid cells in the absence of disease. Although classical (Ly6Chi) monocytes are constantly recruited to tissues\(^7\) and have the capacity to migrate away or undergo apoptosis. It is also possible that a subpopulation of intimal myeloid cells resides in the intima. Monocytes do not necessarily transform to macrophages after recruitment into tissues\(^7\) and have the capacity to migrate away or undergo apoptosis. The abundance of intimal myeloid cells seems to be tightly regulated. In the lesser curvature of the ascending aortic arch, their abundance increases with age and plateaus in the range of 450 to 650 cells after 12 to 16 weeks of age. Intimal myeloid cell abundance in the thoracic aorta may increase significantly during atherogenesis.

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intermediate, low and very low-density lipoproteins, decreases high-density lipoprotein, and induces lesion formation. The introduction of a CRD to Ldlr−/− mice induces within days the accumulation of intracellular lipid in intimal myeloid cells and converts them into foam cells. During the first week, intimal cell proliferation remains low (as in the normal aorta) and monocyte recruitment is not significantly different from baseline. Thus, initial foam cells in nascent lesions are derived primarily from preexisting intimal myeloid cells rather than recently recruited blood monocytes.

The uptake of lipoproteins by intimal myeloid cells seems to be an efficient process because the majority of intimal lipid is accumulated in foam cells that contain multiple cytoplasmic cholesteryl ester droplets. The response to retention hypothesis proposes that foam cell formation is dependent on the retention and modification of proatherogenic plasma lipoproteins (LDL and very low-density lipoprotein) in the arterial subendothelial matrix. Potential modifications of the retained lipoproteins include oxidation, aggregation, glycation, immune complex formation, proteoglycan complex formation, and conversion to cholesterol-rich liposomes. Many studies have provided circumstantial evidence in support of the response to retention hypothesis, but perhaps the most convincing are functional data from transgenic mice showing that ApoB100 mutations that inhibit proteoglycan but not LDLR binding result in reduced arterial lipid accumulation. Myeloid cells may contribute to LDL modification in the intima by producing reactive oxygen species or enzymes, such as 15-lipoxygenase, phospholipases, and myeloperoxidase. Internalization of oxidatively modified lipoproteins is mediated by scavenger receptor-mediated endocytosis. Macropinocytosis is an alternative mechanism for lipoprotein internalization, and intimal myeloid cells may also extend dendrites through the endothelial monolayer into the artery lumen to capture lipoproteins directly from the circulation, analogous to DC in the gut and the respiratory mucosa that extend dendrites through the lining epithelial layer. The latter may explain how intravenously injected antigens, such as ovalbumin, accumulate in intimal cells. Efficient conditional deletion of intimal myeloid cells in CD11c-DTR transgenic mice immediately before induction of hypercholesterolemia in the Ldlr−/− background results in a paucity of foam cells, significantly reduced intimal lipid accumulation and readily detectable retention of lipoprotein-like particles in the intimal extracellular matrix after 5 days.

Early atherosclerotic lesions in mouse models are composed almost entirely of myeloid foam cells (Table). The majority of these cells express both macrophage markers and CD11c (Figure 3). Considering that CD11c is expressed by most myeloid cells in the normal intima and in early atherosclerotic lesions, CD11c should be viewed as a myeloid cell marker, analogous to the lungs where alveolar macrophages express CD11c. Intimal expression of CD11c does not necessarily indicate DC origin or differentiation. Additional markers are required to establish this.

The formation of early lesions is critically dependent on monocytes and macrophages. This was demonstrated using osteopetrotic (op/op) mice that are deficient in circulating monocytes, tissue macrophages, and osteoclasts because of a point mutation that disrupts M-CSF. The recruitment of blood monocytes to the arterial intima has been regarded for decades as one of the earliest events in the formation of atherosclerotic lesions. It seems to be most prominent at the periphery of lesions and may contribute to lateral lesion

**Figure 3. Expression of myeloid markers in early atherosclerotic lesions.** Serial frozen sections of the ascending aortic arch from an Ldlr−/− mouse fed a cholesterol-rich diet for 8 weeks were immunostained for CD68, MOMA-2, and CD11c. Nuclei were counter stained with hematoxylin (blue). CD11c expression shows a membrane-staining pattern in the majority of intimal foam cells. CD68 and MOMA-2 are expressed by all foam cells in the intima and occasional cells in the adventitia (A). Aortic lumen (L). Bar, 50 μm.
increased monocyte recruitment. In our experience, macrophage proliferation is essential to the development of atherosclerosis. As early lesions age, monocyte recruitment increases and persists in advanced lesions. Classical (Ly6C\(^{hi}\)) monocytes are preferentially recruited. Reversal of hypercholesterolemia in ApoE\(^{-/-}\) mice by viral transduction of hepatocytes with ApoE resulted in a reduction of intimal lipid within 1 week and reduced monocyte recruitment at 2 weeks. Changes in the rate of monocyte recruitment to atherosclerotic lesions occur 2 weeks after induction or reduction of hypercholesterolemia, which indicates that the regulation of this process is not rapid and suggest that the system behaves like a capacitor that may be dependent on the content of intracellular lipid in intimal myeloid cells.

The molecular regulation of inflammation and intercellular communication during atherogenesis is not fully understood. The textbook explanation is that intimal foam cells produce proinflammatory cytokines that activate luminal endothelium to express leukocyte adhesion molecules and chemokines. However, molecular diffusion in lesions may be complex considering that the flow of water molecules away from the lumen driven by high hydrostatic pressure in blood relative to the interstitial fluid. It is possible that close proximity or direct contact between myeloid cells and endothelium is required. One would expect that the concentration of proinflammatory mediators would be highest in the center of early lesions directly correlating with the abundance and density of myeloid foam cells; however, the most prominent expression of vascular cell adhesion molecule-1 and monocyte recruitment are found primarily at lesion borders.

### Macrophage Proliferation and Dynamics in Atherosclerotic Lesions

As was described above, monocyte-derived macrophages are essential to the development of atherosclerosis. However, our recent data indicate that, in addition to monocyte recruitment, in situ macrophage proliferation also contributes significantly to lesion growth. Evidence of macrophage proliferation in atherosclerotic lesions has previously been observed in relatively advanced lesions in humans and rabbits. Actually, this process begins in early lesions contemporaneously with increased monocyte recruitment. In our experience, macrophage apoptosis detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) is not a prominent feature of early lesions, and increased apoptosis is first detected at the 8-week time point when early lesions begin evolving into advanced (complex) lesions. Macrophage foam cell egress into the blood is considered to be a rare event during atherogenesis, and thus is not likely to have a major impact on macrophage accumulation. The production and secretion of repulsive neuroimmune guidance cues such as netrin-1, ephrin-B, and semaphorins 3A and 3E may contribute to macrophage retention. Hypercholesterolemia also impairs the migration of dermal DCs to regional lymph nodes through generation of platelet-activating factor. Oxidized, but not native LDL, inhibits TLR4-induced peritoneal macrophage efflux into lymphatics and in vitro migration through a process that involves CD36, inactivation of Src homology 2-containing phosphotyrosine phosphatase, sustained activation of focal adhesion kinase, and alteration of cytoskeletal dynamics. On reversal of hypercholesterolemia or treatment with statins, the induction of CCR7 in plaque macrophages may promote plaque regression. Collectively these data suggest that monocyte recruitment and macrophage proliferation, but not macrophage egress, are the key cellular events regulating foam cell lesion progression.

Until recently, the importance of macrophage proliferation relative to monocyte influx was not known. Using innovative experimental approaches including parabiosis—a procedure that allows circulating cells to enter partner tissues—and continuous in vivo delivery of the thymidine analog BrdU via subcutaneously implanted osmotic pumps, we demonstrated that in advanced atherosclerosis (lesions characterized by the presence of lipid-laden macrophages, a necrotic core, and a developing fibrous cap) turnover of macrophages depended mostly on local macrophage proliferation rather than recruitment of circulating monocytes. In contrast, in early lesions (comprised mainly of lipid-laden macrophages) both monocyte recruitment and macrophage proliferation contribute to lesion growth. These data highlight the complexity of chronic inflammation in atherosclerosis, and suggest there is a point during atherogenesis where monocytes stop differentiating and macrophage proliferation takes over (Figure 4). Furthermore, these data suggest that intercellular communication between myeloid cells, endothelium, and stromal cells is distinct in early versus advanced lesions.

Lesion macrophages could accumulate gradually and be long-lived or turnover rapidly. We recently addressed this question through continuous BrdU delivery in osmotic pumps. BrdU incorporates into newly synthesized DNA and reports on a cell’s proliferative history. Strikingly, the data revealed that macrophage turnover in advanced lesions is rapid, and nearly all macrophages stained for BrdU within 4 weeks. Furthermore, despite increases in lesion size over time, the aortic root macrophage burden in established disease did not change significantly, suggesting the processes that contribute to cell loss counterbalance macrophage renewal.

Unlike early lesions where the abundance of intimal macrophages increases over time, in advanced lesions the macrophage burden remains constant or even decreases. Advanced lesions grow primarily as a result of increasing matrix
deposition and acellular regions with necrotic cell debris and dystrophic calcification. Yet, several studies have documented conclusively that monocyte recruitment continues to advanced lesions.105,106,126 This raises an interesting question about the fate of monocytes recruited to advanced lesions in light of our recent data that circulating monocytes contribute minimally to maintaining the macrophage population at this stage of atherosclerosis.63 Possibilities include that intimal monocytes in advanced lesions persist but do not differentiate to macrophages, undergo apoptotic cell death, or migrate though lesions before reentering back into the circulation. Although they do not contribute to the macrophage pool, recruited monocytes may produce soluble mediators that promote matrix deposition and remodeling. Elucidation of molecular mechanisms for the preferential persistence of macrophages over recruited monocytes in advanced lesions will be fruitful area for future investigations.

In vitro studies have shown that macrophages proliferate in response to oxidized LDL (oxLDL).127,128 We showed that proliferation of macrophages in developing atherosclerotic lesions depends on the type 1 scavenger receptor class A (Msr1).63 scavenger receptors promote the uptake of modified lipoproteins, clear apoptotic cells and debris, activate cellular signal transduction, and induce macrophage apoptosis.129 Msr1 is one of the most important receptors in the uptake of oxLDL130 and is expressed on monocytes and macrophages, smooth muscle cells, and endothelial cells. Its role in atherogenesis is multifaceted. In vivo, Msr1 may contribute to lesion complexity by facilitating plaque foam cell formation, inducing macrophage apoptosis, and promoting inflammatory gene expression.130 Macrophages with targeted deletion of Msr1 take up oxLDL poorly in vitro.131 Msr1-mediated internalization of lyso phosphatidylcholine, a phospholipid component of oxLDL, induces peritoneal macrophage proliferation in vitro.132 It remains to be determined whether macrophage proliferation in lesions is a general feature of scavenger receptor activity or supported by Msr1 alone. Additional scavenger receptors of interest include macrophage receptor with collagenous structure, a class A scavenger receptor with considerable homology to Msr1 but whose role in atherosclerosis is not known, and CD36, a class B scavenger receptor that has been implicated in the development of necrotic lesions in advanced atherosclerosis.

Another important unanswered question is whether macrophage proliferation is mediated directly by intracellular signaling through the Msr1 or other scavenger receptors or indirectly through uptake of lipids into the cell. Indeed, genetic deficiencies in the reverse cholesterol transport machinery of hematopoietic stem and progenitor cells results in increased plasma membrane lipids and upregulation of the common β chain of the IL-3/granulocyte/macrophage-CSF receptor.133 Notably, infusion of high-density lipoprotein and liver X receptor agonists in this setting restores reverse cholesterol transport and reduces hypercholesterolemia-induced hematopoietic stem and progenitor cell proliferative responses.134

Macrophage proliferation may also depend on different mechanisms at different stages of disease. Indeed, granulocyte/macrophage-CSF, a growth factor that regulates early intimal foam cell proliferation in nascent lesions,61 does not seem to orchestrate macrophage proliferation in established disease because neither neutralizing antibodies to the growth factor nor genetic manipulation targeting its receptor affected macrophage proliferation.63 Future studies will be required to determine mechanisms of macrophage proliferation at different stages of disease and the contribution of signaling that promotes cell survival, including CXCL1 and its receptor CXCR1.81

It is clear that both local and systemic factors influence myeloid cell accumulation in atherosclerosis, although the interplay between these is poorly understood. Systemic risk factors for atherosclerosis, such as hyperlipidemia, hypertension, hyperglycemia, smoking, etc, are well established. As was mentioned above, hypercholesterolemia promotes the accumulation of lipid in the arterial intima, and this together with local hemodynamics promotes a local inflammatory response. This local inflammatory response may, in turn, release systemic mediators, but it is also likely that hypercholesterolemia directly affects myeloid cells in the bone marrow, spleen, and blood. There is a strong evidence for systemic myeloid responses to hypercholesterolemia. In mice, hypercholesterolemia induces increased levels of circulating classical (Ly6C[hi]) monocytes,105,106 and elevated blood monocyte counts are an established risk factor for human atherosclerosis.135–137

High levels of hypercholesterolemia in mice induce the formation of foamy monocytes, with abundant cytoplasmic lipid droplets and a high side scatter pattern when analyzed by flow cytometry. Monocyte lipid uptake is dependent on CD36 and is associated with upregulated expression of CD11c, chemokine receptors, and activation of CD49d (α4) integrin, which mediates adhesion to vascular cell adhesion molecule-1.138–141 Monocytes with cytoplasmic lipid droplets can also be found in humans after a high-fat high-cholesterol meal, but the extent of intracellular lipid is much lower relative to mice.139,140,142,143 The systemic effects of hyperlipidemia on circulating monocytes promote monocyte recruitment to atherosclerotic lesions. Other systemic effects, such as production of cytokines and growth factors, may influence macrophage proliferation, survival and polarization in lesions.

**Functions of DCs in Atherosclerosis**

Many studies have shown that adaptive immunity can profoundly affect the progression of atherosclerosis in mouse models by modulating the accumulation of myeloid cells in lesions.144 As was mentioned above, DCs are key regulators of adaptive immunity, and thus, it is not unexpected that they influence atherogenesis. DCs may also modulate lipoprotein metabolism,145 although the molecular mechanisms remain unknown.

Pathogenic stimuli rapidly induce DC maturation that includes upregulation of cell surface costimulatory molecules CD80/86/7-1 and CD86/B7-2 as well as antigen presentation machinery (MHC-II), required for stimulation of T cells. Combined deficiency of CD80 and CD86 in Ldlr−/− mice resulted in reduced early and to some extent advanced atherosclerotic lesion formation (8 and 20 weeks of 1.25% CRD), a reduction in MHC-II+ cells in early lesions and a modest decrease in lesion CD3+ cells.146 T cells isolated from the spleen and lymph nodes of CD80/CD86-deficient mice after 8 weeks
of diet showed reduced production of IFNγ in response to stimulation by heat shock protein 60, which suggested that priming of a Th1 response to a self-antigen was compromised in the absence of key costimulatory molecules. Similarly, deficiency of the invariant chain (CD74), a molecule that functions as a chaperone and participates in the trafficking and transport of peptide-loaded MHC-II to the cell surface, resulted in reduced atherosclerosis and activated CD25+ T cells in lesions of Ldlr−/− mice at 12 to 26 weeks of CRD.149 Cd74−/−Ldlr−/− mice had elevated IgM and IgG3 and decreased IgG1, IgG2b, and IgG2c antibodies directed to malondialdehyde-modified LDL, and after immunization with malondialdehyde-modified LDL had lower levels of all anti–malondialdehyde-modified LDL immunoglobulins. Collectively, these studies suggest that antigen presentation can influence atherogenesis by modulating both cellular and humoral immunity, including B-1 cells that produce natural IgM antibodies to oxidized phospholipids found in oxLDL.148 Because both DCs and macrophages can express MHC-II, CD80, and CD86, the relative contribution of these cell types remains unknown. Furthermore, it is not clear where antigen-presenting cells encounter oxLDL and whether pathogenically relevant antigen presentation occurs in lymphoid tissues or lesions. Dynamic interactions between antigen-presenting CD11c+ cells and T cells in the artery wall can lead to local T-cell activation and proinflammatory cytokine production, which promotes macrophage uptake of modified LDL.149

DCs express a repertoire of TLRs that sense diverse maturation signals and 2 key adaptor molecules, MyD88 and TRIF, integrate intracellular signaling downstream of TLRs. Germline deficiency of TLR4150 or TLR9151 in Ldlr−/− mice was inhibited; however, one could expect that this phenotype was not recapitulated by transplantation of Ldlr−/− mice into Ldlr−/− Apoe−/− mice resulted in reduced atherosclerosis in Ldlr−/− mice but not the healthy artery wall, restricted Treg expansion through secretion of CCL17.158 CCL17+ DCs are mature (CD40+, CD80+, and CD86+) DCs that express CD11c, MHC-II, CD11b, and CD205 cell surface markers159; however, further studies such as antigen-presentation capability and allogeneic T-cell stimulatory capacity (eg, in a mixed leukocyte reaction) are required to determine if CCL17+ DCs represent a distinct DC subset. Collectively, it is clear that cDCs play a critical role in Treg homeostasis, although CCL17+ DCs seem to have an opposing role. Tregs diminish myeloid cell accumulation in lesions likely through several different mechanisms. Treg abundance within lesions seems to be important, but these cells may also function in lymphoid tissues to modulate systemic factors that, in turn, influence myeloid cell biology in lesions.

pDCs can also modulate atherogenesis. Several studies adopted a pDC depletion approach using antibodies targeting PDCA1/BST2, but reached disparate conclusions. Depletion of pDCs in Ldlr−/− mice exacerbated the development of lesions in the aortic root and in a carotid artery cuff model, whereas reduced atherosclerosis was observed in Apoe−/− mice.161,162 The depleting antibody, the dose and the interval of administration may have accounted for different effects on lesions in these studies. It is also important to remember that expression of PDCA1 is not restricted to pDCs and influences myeloid cell biology in lesions.

Recently, E2-2/TCF4, a transcription factor critical for pDC development, was deleted using CD11c-Cre and atherosclerosis was studied following bone marrow transplantation into Ldlr−/− mice.163 In TCF4-depleted chimeras, the neutral lipid content and T cells were reduced in early (8 weeks of CRD) lesions located in the aortic root in spite of elevated total plasma cholesterol levels. A compensatory increase in cDCs and B220+ CD11c+ MHC-II cells was found in lymphoid organs, consistent with previous studies, but Treg levels were comparable in the spleen. The authors attributed the reduced atherogenesis to defective pDC-driven MHC-II expression were decreased in these atherosclerotic lesions, whereas CCL2 expression, monocyte recruitment, and myeloid cells were increased.155 This study suggested that the atheroprotective effect of Tregs dominates over effector T cells in atherogenesis and highlights the requirement of MyD88 signaling in DCs in the induction of Tregs.

Given the importance of feedback regulatory loop between DCs and Tregs, several other studies investigated the role of DCs in Treg homeostasis in atherosclerosis. Fli3-dependent DCs were important in maintaining Treg homeostasis, and lack of these DCs resulted in more atherosclerosis in Ldlr−/− mice fed a cholesterol-enriched diet for 12 weeks.80 In line with this, Treg depletion by anti-CD25 antibody156 and Foxp3-DTR157 also promoted atherosclerosis. Increased plasma cholesterol and very low–density lipoprotein levels and decreased clearance of very low–density lipoprotein and chylomicron remnants in Treg-depleted Foxp3-DTR mice contributed to this effect.157 The molecular basis for hepatic lipoprotein metabolism modulation by Tregs has not been established. In contrast to the above studies, CCL17-expressing DCs, which are found in advanced atherosclerotic lesions of Apoe−/− mice but not the healthy artery wall, restricted Treg expansion through secretion of CCL17.158 Dynamic interactions between antigen-presenting CD11c+ cells and T cells in the artery wall can lead to local T-cell activation and proinflammatory cytokine production, which promotes macrophage uptake of modified LDL.149

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antigen presentation to T cells based on both in vitro and in vivo experiments, and not to altered IFN-α production. Given the expansion of cDCs in TCF4-depleted chimeras, the known atheroprotective functions of most cDCs, and the important role of Tregs in atherosclerosis, further studies may be necessary to fully appreciate the observed atherosclerosis phenotype in E2-2/TCF4-deficient chimeric mice.

Although published studies on the role of pDCs in atherosclerosis are informative, spatio-temporal information about aortic pDCs during different stages of atherosclerosis is lacking and it is still not known whether aortic pDCs produce type I IFNs in lesions. Given the potential for nonspecific cell depletion by anti-PDCA1 antibodies and compensatory responses to constitutive pDC depletion in E2-2/TCF4-deficient mice, it will be necessary to use more specific and inducible pDC depletion approaches. Lymphoid tissue pDCs can be deleted efficiently in BDCA2-DTR mice. Once pDC accumulation in aortic atherosclerotic lesions is evaluated, it remains to be determined if DT will effectively delete these cells in BDCA2-DTR mice and what impact this will have on lesion formation.

**DC Therapeutics for Atherosclerosis**

To harness DCs as a therapeutic vaccine, bone marrow–derived DCs (cultured with granulocyte/macrophage-CSF) were loaded with oxLDL and adoptively transferred to Ldlr−/− mice. These studies resulted in a significant reduction in atherosclerotic lesion development, reduced oxLDL–specific T cells, increased oxLDL–specific IgG levels, and induced Tregs. In contrast, DCs loaded with malondialdehyde-modified LDL aggravated atherosclerosis in Apoe−/− mice. Similarly, increased lesion development was found when Apoe−/− mice expressing the β-galactosidase under the transcriptional regulation of the SM22α promoter received bone marrow–derived DCs loaded with β-galactosidase–specific peptides.

The above DC immunization approaches have several limitations. Bone marrow–derived DCs cultured with granulocyte/macrophage-CSF constitute a heterogeneous population of DCs and macrophages. Even purification of DCs using approaches such as microbeads coated with antibodies to CD11c fails to achieve a uniform population. The maturation status of DCs was not evaluated. It is well established that steady-state immature DCs promote T-cell tolerance through deletion of reactive T cells and induction of Tregs, whereas mature DCs (after exposure to TLR ligands) induce Th1 polarization. It would be interesting to assess effects of adaptively transferred cultured DCs loaded with modified LDL (eg, oxLDL) and exposed to a maturation stimulus (eg, a TLR agonist) in an animal model of atherosclerosis.

Given the limitations of immunization with cultured DCs loaded with an atherosclerosis-related protein/peptide, a new approach was pioneered by Drs Steinman and Nussenzweig to directly target antigens to DCs in vivo through the antibodies against endocytic surface receptors that are highly expressed in DC subsets (Figure 5). This DC targeting antibody approach can be used in all species and does not require lengthy in vitro manipulation steps, which can be problematic when translated to patients. Targeting receptors include CD205/DEC-205, manIgG1, mouse IgG1 isotype heavy chain constant region; Vκ light chain variable region; VH, heavy chain variable region; and Vκ, light chain variable region. Linker is a 12 to 14 amino acid peptide for flexible fusion of proteins or peptides to the targeting antibody.
systemic factors that include modulation of acquired immunity by DCs. Mouse models provide a means for elucidating molecular insights into how cellular processes such as monocyte recruitment and macrophage proliferation are regulated at different stages of lesion formation, what are the functions of newly recruited monocytes versus established proliferating macrophage populations, what molecules mediate intercellular communication in lesions, and how these are modulated by various DC subsets residing in lesions and in lymphoid tissues. Intimal myeloid cells do not only influence the progression of atherosclerotic lesions but also ultimately may contribute to lesion destabilization. With respect to the latter, it will be important to discern the role of newly recruited monocytes versus established proliferating macrophages because understanding the relationship between these may yield novel therapeutic strategies. Modeling of thrombotic complications will be difficult with mouse models. Furthermore, in contrast to mouse models, a persistent and extreme hypercholesterolemia is not a usual feature in the majority of patients with atherosclerosis; therefore, disease progression and the interplay between monocyte recruitment and macrophage proliferation may be more complex and influenced by multiple systemic risk factors. Nevertheless, we feel that gaining a thorough understanding of myeloid cell biology in atherogenesis using the diverse approaches that are available in association with mouse models is an important endeavor that will ultimately affect human health.

Acknowledgments

We acknowledge the assistance of Angela Li and Tae Jin Yun in preparation of figures and Magar Ghazarian for performing immunostaining.

Sources of Funding

Our research programs are supported by grants from the Canadian Institutes of Health Research (CIHR): MOP-84446 (M.I. Cybulsky), MOP-89740 (M.I. Cybulsky), MOP-106522 (M.I. Cybulsky), MOP-125933 (C. Cheong), HIG-133050 (C. Cheong), and MOP-133390 (C.S. Robbins), the National Research Foundation of Korea (GRN-2013S1A2A2035348 to C. Cheong), and the Peter Munk Cardiac Centre at University Health Network. M.I. Cybulsky is the recipient of a Canada Research Chair. C. Cheong holds a Chercheur-Boursier 2013S1A2A2035348 to C. Cheong), and the Peter Munk Cardiac Centre at University Health Network. M.I. Cybulsky is the recipient of a Canada Research Chair in Aortic Disease Research and a CIHR New Investigator Award, and C. S. Robbins holds the Peter Munk Chair in Aortic Disease Research and a CIHR New Investigator Award, and of a Canada Research Chair; C. Cheong holds a Chercheur-Boursier 2013S1A2A2035348 to C. Cheong), and the Peter Munk Cardiac Centre at University Health Network.

Disclosures

None.

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Macrophages and Dendritic Cells: Partners in Atherogenesis
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Circ Res. 2016;118:637-652
doi: 10.1161/CIRCRESAHA.115.306542

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/118/4/637

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/02/22/CIRCRESAHA.115.306542.DC1
Dendritic cell origin and classification

DCs are subdivided into classical (cDCs) and plasmacytoid (pDCs). Murine cDCs were discovered in lymphoid tissues and defined based on functional and morphological features that distinguish them from monocytes and macrophages. These include: (a) nonadherent cells with probing morphology (continuously forming and retracting processes), (b) preferential localization to T cell areas thereby facilitating clonal section of antigen-specific T cells, (c) strong stimulators of the mixed leukocyte reaction that measures the capacity for stimulating allogeneic T cells, (d) superior antigen presentation to CD4 and CD8 T cells relative to other myeloid cells in the context of major histocompatibility complex (MHC) class II (MHC-II) and MHC-I, (e) lower phagocytic activity compared with monocytes and macrophages, and (f) loss of monocyte and macrophage markers.1-5 pDCs were identified subsequently. Their morphology is distinct from cDCs, and upon stimulation by foreign nucleic acids they have the unique capacity to produce abundant type I interferon (IFN) and prime T cells against viral antigens.4,5 In mice, both cDCs and pDCs reside in lymphoid and most nonlymphoid tissues, but only pDCs are detected in the blood.6

DC subsets are based on their origins, yet within tissue DC populations are thought to acquire distinct phenotypes based on local environmental cues. Both cDCs and pDCs are derived from bone marrow precursors, although the mapping of DC developmental pathway is still being debated. Previously, a common macrophage-DC-restricted precursor (MDP) was proposed7-9; however, the existence of this precursor was recently challenged10, and additional studies will be necessary to resolve this controversy. Nonetheless, has been firmly established that a common DC progenitor (CDP) can generate cDCs and pDCs in lymphoid organs, but lacks the potential to generate monocytes and macrophages11,12 (Figure 1). Precursors of cDCs (pre-cDCs) form in the bone marrow and disseminate to lymphoid tissues.8,13,14 In addition to cDCs derived from CDPs and pre-cDCs, DCs can originate from blood monocytes (Mo-DCs).2,15-18 Mo-DCs are as capable as cDCs at antigen presentation, including cross-presentation of proteins and live gram-negative bacteria.2 Mo-DCs can be distinguished from CDP-derived cDCs by cell surface markers (see below). Although DCs and macrophages constitute the bulk of tissue myeloid cells, it should be noted that DC developmental pathways have been studied mostly in lymphoid tissues, and additional work will be required to map DC development in other tissues, including the aorta. For example, pre-cDCs have not been identified in the normal or diseased aorta.

Epidermal Langerhans cells, which express Langerin (CD207), constitute a subset of cDCs. Interestingly, these cells are maintained by local proliferation19 and are radio-resistant in bone marrow chimera experiments where they remain as host-derived cells for at least 18 months.20 In contrast, Langerin+ dermal DCs are rapidly replaced by donor cells in bone marrow chimera experiments, implying differential origin of dermal and epidermal Langerin+ DCs.21 Epidermal Langerhans cells are derived predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived cells,22 but in inflamed skin they are derived from blood monocytes.23

Cell surface markers are commonly used for identification of DCs. Mouse DCs including dermal and epidermal Langerin+ DCs express CD11c (alpha-X integrin) and high levels of MHC-II, but lack T, B, natural killer cell, granulocyte and erythroid markers. Other markers include chemokine receptors, and c-type lectin receptors. Mouse pDCs express CD4, Siglec-H, mPDCA1/BST2/CD317, B220/CD45RA, and low levels of CD11c and MHC-II (Figure 1). CDP-derived cDCs express CD8α in lymphoid tissue and CD103 (alpha-E integrin) in nonlymphoid tissues, and may express CD11b (alpha-M integrin), but do not express macrophage markers such as F4/80 and CD206 (mannose receptor). It is presumed that lymphoid CD8α+CD103+ cDCs are equivalent to nonlymphoid CD103+CD8α+ cDCs.24 Mo-DCs express macrophage markers CD11b, F4/80, CD14, CD206 and CD209a/DC-SIGN, but do not express CD103 or
CD8α. It is important to note that lineage tracing approaches were not used to establish the above paradigm, and it is possible that in some tissues local environmental cues may dominate over origin (CDP versus monocyte) in establishing the local cDC phenotype.

Surface markers are insufficient for identification of distinct myeloid cell subsets across tissues. Several markers previously considered to be DC-specific are actually expressed by other cell types. For example, some macrophages and T cells can also express CD11c. Thus, when using the CD11c promoter to regulate the expression of Cre or DTR, it is important to remember that other cell types may be targeted in addition to DCs. The pDC-specific markers mPDCA1/BST2/CD317 and Siglec-H can be expressed by many cell types including inflamed macrophages. CD11b is not a reliable marker for CD8α DC, and CD11b+ DCs may be a mixed population of DCs and macrophages. Given the promiscuous expression of CD11c and higher autofluorescence of macrophages, it is prudent to use CD64 and MerTK in combination with CD11b and F4/80 to identify macrophages and to exclude these cells from analysis of DC populations. It should also be noted that many markers that are used successfully to identify DC subsets in lymphoid organs are difficult to use in tissues such as the aorta that require a combination of collagenases for cell isolation. In this case, intracellular staining can be employed to visualize some markers. For example, CD4 and CD206/MR expression can be rescued by intracellular staining. Thus, in addition to cell surface markers, multiple parameters including intracellular staining and transcription factors (see below) should be used to identify DC subset equivalents to lymphoid counterparts in the aorta. Recently, a classification of DC subsets mainly in the bone marrow and lymphoid tissues was proposed based on surface markers, growth factor requirements, and subset-specific transcription factors.

While traditional flow cytometry forms the foundations for defining myeloid cell populations, it cannot elucidate the full complexity of the myeloid system. To overcome this, a recent advance was to combine flow cytometry and mass spectrometry. This technology, known as CyTOF, enables determination of more than 30 different cell surface or intracellular markers per cell due to minimum spectral overlap between transition element isotopes conjugated to antibodies. Interestingly, this approach identified more than 28 clusters of myeloid cells across eight tissues. Analysis of the DC transcriptome by transcription profiling has also contributed to defining the ontogeny and subtypes of myeloid cells across the entire mouse hematopoietic lineage. Recent studies through the Immunological Genome (ImmGen) Project have defined a core-DC gene signature compared with macrophages. The above approaches may help to identify aortic DC subsets. The only problem is the small number of aortic myeloid cells, especially cDCs and pDCs. Recent advance in single cell trancriptome-analysis using RNAseq may overcome this limitation.

Bone marrow culture systems have been developed to obtain large numbers of DCs for in vitro studies. This approach also contributed to studies of DC immunizing properties. Unfortunately, bone marrow culture does not fully recapitulate the properties of tissue-resident cDCs in vivo. Moreover, DCs derived from bone marrow cultured with granulocyte/macrophage-colony stimulating factor (GM-CSF or CSF2) were recently shown to be a heterogeneous population consisting of DCs and macrophages. Bone marrow cells cultured with FMS-like tyrosine kinase 3 ligand (Flt3L) also generate multiple subsets of DCs, including pDCs (CD11c+/B220+) and two types of cDCs (CD24+ and CD11b+CD172a+). These cDC subsets are considered equivalent to lymphoid CD8α+ and CD8α- DCs, respectively. However, in vitro-derived cDCs do not express cell surface CD8α, CD103, CD205/DEC-205 and DCIR2/33D1, suggesting the acquisition of cell surface markers is subject to tissue or environment context or appropriate activation signals.

Mature cDCs derived from pre-DCs or monocytes, but not pDCs, monocytes and macrophages, express the transcription factor Zbtb46/BTBD4. Zbtb46 is not required for early cDC
development, but Zbtb46-deficiency resulted in the alteration of the proportion of CD8α+ and CD8α− DCs in the spleen and a small increase in DCs that migrated from the skin to draining lymph nodes. Zbtb46 is a useful tool for visualization and deletion of cDC in vivo. Coupled to a fluorescent reporter transgenic mice with the Zbtb46 promoter enable cDCs visualization, or their selective depletion when coupled to the diphtheria toxin receptor (DTR). Zbtb46 expression is also a useful tool to determine if a population of immune cells has cDC features. For example, monocytes infected with Listeria monocytogenes produce TNF and iNOS and have been called TNF/iNOS-producing DCs (TIP-DCs). However, TIP-DCs do not express Zbtb46, which suggests that they are more closely related to activated monocytes than to DCs. Other transcription factors are also required for cDC development and differentiation. For example, BATF3-deficient mice lack splenic CD8α and nonlymphoid CD103+ DCs. Interferon regulatory factor 8 (IRF8)-deficient mice lack CD8α−CD103+ DCs, but also have reduced pDCs. ID2-deficient mice lack CD8α−CD103+ DCs. In contrast, IRF4 and several additional transcriptional factors such as RelB, RBP-J, and IRF2 are required for CD8α−CD11b+ DC differentiation.

Transcription factors E2-2 and IRF8 are critical for differentiation of pDCs. Interestingly, loss of E2-2 expression in mature pDCs resulted in their spontaneous conversion to cDCs. It is important to take this into consideration when using E2-2 deficient mice to dissect pDC functions in pathophysiological settings. While IRF8 is critical for normal development of CD8α+ DCs and pDCs, BXH-2 mice that harbor a point mutation in IRF8 (R294C) do not exhibit an impairment of pDC development. This suggests that different transcription factors interact with IRF8 and influence CD8α+ DC or pDC development.

DC identification and subtyping is still evolving. It is prudent to rely on a combination of approaches, including surface markers, transcription factor expression and transcriptional profiling to define tissue-resident DC subsets. Approaches such as parabiosis can distinguish self-renewing DC populations versus blood-derived replenishment both in steady-state and inflammatory conditions. Sophisticated lineage tracing tools are lacking for DCs, and hopefully will be developed in the near future. The exception is genetic tracing of Clec9a/DNGR-1 expression, a receptor for dead cells that is expressed by CD11b+ CDPs and pre-cDCs, but not MDPs. This lineage tracing approach revealed that some CD11b+ DC populations in nonlymphoid tissues are in fact derived from CDP and pre-cDC precursors, and is potentially applicable to DC populations in arteries.
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