Immune System and Atherosclerotic Disease

—— Heterogeneity of Leukocyte Subsets Participating in the Pathogenesis of Atherosclerosis ——

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Atherosclerosis is an inflammatory disease in which a systemic inflammatory reaction is combined with an accumulation of immune cells, such as monocytes/macrophages, dendritic cells (DCs), and numerous lymphocytes, in atherosclerotic plaques. The immune system, comprising innate immunity and adaptive immunity, has been implicated in all stages of atherosclerosis, from initiation through progression and in atherothrombotic complications. It is clear that different subpopulations of leukocytes are involved in the pathogenesis of atherosclerosis and plaque instability. Recent studies have also demonstrated that each heterogeneity of immune-associated cells contributes to the atherogenic and atheroprotective axis. This review highlights recent advances in research and explores the role of the complex heterogeneity of leukocyte subsets, especially monocytes/macrophages (inflammatory monocytes, resident monocytes, M1, and M2), DCs (myeloid DCs, plasmacytoid DCs, pre DCs, conventional DCs, inflammatory DCs), and CD4+ cells (T-helper 1, T-helper 2, regulatory T, and T-helper 17 cells), in the initiation and development of atherosclerotic disease and its complications. (Circ J 2009; 73: 994–1001)

Key Words: Adaptive immunity; Dendritic cells; Innate immunity; Macrophages; Regulatory T cells

Inflammation is an important factor in the initiation and development of atherosclerosis.1–11 The first step preceding the formation of an atherosclerotic lesion is endothelial activation, which is mediated by various inflammatory stimuli and mechanical injury.1–3,12 Secondly, various types of leukocytes adhere to the activated endothelium and migrate into the arterial wall.1–3,12 Subsequently, monocyte-derived macrophages engulfing modified low-density lipoprotein (LDL) and other lipids transform into lipid-laden foam cells to form fatty streaks. The intimal fatty streaks mature into atherosclerotic plaques, which contain not only lipids and debris from dead cells, but also numerous immune cells such as macrophages, dendritic cells (DCs), and T cells.1–3 During the early stages of research, these immune cells were thought to unilaterally induce and promote atherosclerotic processes, however, recent studies have clearly revealed that each heterogeneity of immune-associated cells has both atherogenic and atheroprotective roles.13,14 This review highlights recent research advances and explores the role of the complex heterogeneity of the leukocyte subsets, especially monocytes/macrophages (inflammatory monocytes, resident monocytes, M1, and M2), DCs (myeloid DCs (mDCs), plasmacytoid DCs (pDCs), pre DCs, conventional DCs, inflammatory DCs), and CD4+ cells [T helper (Th) 1, Th2, regulatory T, Th17 cells]), in the initiation and development of atherosclerotic disease and its complications.

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Principals of the Immune System

General Properties of Immune Responses

The principal function of the immune system is to protect individuals against harmful stimuli, including infectious agents and unsafe foreign and/or endogenous substances. This defense system comprises early responses, namely innate immunity, which is not antigen-specific, and late reactions, the so-called adaptive immunity, in which specific antigen receptors are involved (Table).15–17 Innate immunity is composed of neutrophils, natural killer cells, and antigen-presenting cells (APCs), such as macrophages and DCs, utilizing pathogen-associated molecular patterns (PAMP) receptors, including scavenger receptors and Toll-like receptors (TLRs) on the surface of APCs.15–17 Adaptive immune responses must be primed and then followed by clonal expansion of appropriate responder cells, because T and B cells with a fitting receptor for a peptide are rare. The innate immune system begins operating with a few minutes after invasion of a pathogen, whereas adaptive immunity develops over several days.18

Innate Immunity, TLRs, and Atherosclerosis

Innate immunity is the first line of defense that is rapidly mobilized to detect PAMPS, using PAMP receptors such as scavenger receptors and TLRs.15–17 So far 11 TLRs have been found in mammals and a great number of exogenous and endogenous ligands has been identified.16 Recent studies have shown that TLR-associated signaling consists of 4 adaptor proteins, including myeloid differentiation factor 88 (MyD88), Toll-receptor associated activator of interferon (TRIF), Toll-receptor associated molecule (TRAM), and MyD88-adaptor-like/TIR-associated protein (MAL/TIRAP),
and 3 primary kinases: IL-1R-associated kinase 1 (IRAK1), IRAK-4, and TANK-binding kinase 1. TLRs consist of homodimers or heterodimers, as well as their adaptor proteins. Ligands binding to TLRs initiate signals that involve many kinases [eg, TRAF6 (tumor necrosis factor [TNF]-receptor-associated factor 6), IRAF (interferon [IFN] regulatory factor 3), NF (nuclear factor)-κB, STAT1 (signal transducer and activator of transcription 1 isoform β)]. Consequently, TLRs signals induce the production of numerous cytokines, chemokines, and other inflammatory mediators.

TLRs are expressed by macrophages and ECs in murine and human atherosclerotic lesions. Activation of human ECs through either lipopolysaccharide (LPS) or IFN-γ induces upregulation of TLR2 and TLR4 PAMPs, including LPS, heat-shock protein (HSP), and unmethylated CpG DNA, bind different TLRs. Oxidized LDL (OxLDL), which plays a crucial role in the development of atherosclerosis and pathogenesis of cardiovascular events is reported to be a TLR4 ligand. In addition, other endogenous ligands such as minimally modified LDL, and oxidi- zed 1-palmitoyl-2- arachidonoyl-sn-glycero-3-phosphoryl-choline are also thought to be recognized by TLR4, suggesting that oxidation-related self antigens might be important endogenous ligands that trigger activation of the innate immune system and consequently lead to atherosclerotic disease. A broad spectrum of TLRs, including TLR1, TLR2, TLR4, and TLR5, are found in carotid atherosclerotic plaques. However, TLR4 expression appears not to be limited to macrophages infiltrating atherosclerotic lesions. TLR4 is also detected by vascular smooth muscle cells.

In addition, TLR4 expression on circulating monocytes is increased in patients with stable or unstable angina compared with healthy controls. Therefore, evidence from both animal models and human studies should be considered.

## Subpopulations of Monocytes/Macrophages in Atherosclerosis

### Inflammatory Monocytes and Resident Monocytes

Monocytes and macrophages play a crucial role in athero- genesis. Atherosclerotic lesions contain a large number of lipid-laden macrophages, known as foam cells, which are derived from circulating monocytes. Recent attention has been focused on the heterogeneity of circulating monocytes and lesional macrophages. Human and mouse monocytes can be divided into at least 2 phenotypically distinct subsets: Ly-6C

### Adaptive Immunity and Atherosclerosis

Adaptive immunity mainly consists of T and B cells, which recognize specific epitopes on each pathogen or antigen and generate multiple antigen-specific T-cell recep-
tion of atherosclerotic lesions and neointimal formation induced wire-injury in apoE knockout mice \(^5\). CX3CL1/fractalkine has a chemoattractant function and adhesion properties for monocytes and T cells \(^9\). CX3CL1 and its receptor CX3CR1 are expressed on endothelial cells, coronary smooth muscle cells, and macrophages \(^8\). Deletion of CX3CR1 decreases atherosclerotic lesions in apoE and LDL-receptor knockout mice \(^6\). These recent data suggest that Ly-6C<sup>hi</sup>CCR2<sup>+</sup>CX3CR1<sup>low</sup> monocytes as “inflammatory monocytes” lead to an inflammatory and atherogenic axis.

In contrast, Ly-6C<sup>low</sup>CCR2<sup>+</sup>CX3CR1<sup>hi</sup> monocytes have a patrolling function in steady-state healthy tissues depending on CX3CR1 \(^6\). Once an early inflammatory response, such as acute infection, has occurred these “resident monocytes” rapidly invade injured tissues and differentiate into macrophages \(^6\). Ly-6C<sup>low</sup>CCR2<sup>+</sup>CX3CR1<sup>hi</sup> monocytes in apoE knockout mice fed a high-fat diet acquire a high-fat diet expressing the DC-associated marker CD11c \(^6\). In contrast, Ly-6C<sup>hi</sup>CCR2<sup>+</sup>CX3CR1<sup>low</sup> monocytes enter atherosclerotic plaques partially dependent on CCR5 instead of CX3CR1. In contrast Ly-6C<sup>hi</sup>CCR2<sup>+</sup>CX3CR1<sup>low</sup> monocytes require not only CX3CR1 but also CCR2 and CCR5 for trafficking to plaques. Therefore, CCR2, CX3CR1, and CCR5 are all associated with the atherosclerotic process \(^9\). Indeed, recent studies utilizing multiple knockout mice, which were CCR2/CX3CL1/apoE or CCL2/CX3CR1/CXCR5/apoE deleted, demonstrated a dramatic reduction in atherosclerosis \(^5,6\).

Classically Activated Macrophages and Alternatively Activated Macrophages

Macrophage subsets also participate in inflammatory processes. LPS, IL-1<beta>, and Th1 cytokines, such as IFN-\gamma, induce a “classically” activating profile (M1), in contrast to Th2 cytokines, such as IL-4 and IL-13, which lead to an “alternatively” activating profile (M2) (Figure 1) \(^5,53,55,56,66\). Activated M1 macrophages, which produce proinflammatory cytokines such as TNF-\alpha, IL-6, and IL-12, can switch to alternative M2 macrophages, which dampen the inflammatory state by producing anti-inflammatory properties, including IL-10, IL-1 receptor antagonist, and transforming growth factor (TGF)-\beta \(^5,6\). A recent report demonstrated that both M1 and M2 macrophages are present in human atherosclerotic lesions, and that M2 marker expression positively correlated with that of peroxisome proliferators-activated receptor (PPAR)-\gamma \(^6\). In addition, PPAR-\gamma activation primes primary human monocytes for M2 differentiation \(^7\). These data suggest that PPAR-\gamma may be a critical player in the atherosclerotic process by regulating macrophage subclasses \(^5,6\).

Macrophage infiltration into adipose tissue is linked to insulin resistance in diet-induced obesity \(^70,71\), suggesting that insulin resistance induced by obesity and atherosclerosis have a common underpinning in macrophage-mediated inflammation. Macrophages isolated from adipose tissue in lean mice express several markers of alternative activation, characterized as “alternative” M2 phenotypes such as Ym1, arginase 1, and IL-10. In contrast, macrophages isolated from adipose tissue of diet-induced obese mice exhibit a proinflammatory phenotype characterized by a “classical activated” M1 phenotype, including TNF-\alpha and inducible nitric oxide synthase (iNOS) \(^2\).

Subpopulations of DCs in Atherosclerosis

mDCs, pDCs, and Atherosclerotic Diseases

DCs are highly efficient APCs that are central to the induction and regulation of adaptive immune responses \(^73,74\). Compared with other APCs, such as macrophages, DCs have specialized capacities for homing to the T-cell zones of lymphoid organs to interact with T lymphocytes \(^73,74\). DCs are present at the adventitia–media border in normal human arteries \(^7\). Another group reported that tissue-resident and immature-phenotype DCs (CD1<sup>+</sup>S<sup>+</sup>-100<sup>+</sup>CDCD83<sup>-</sup>CDCD86<sup>-</sup>) accumulated most densely in the arterial intima of healthy young individuals \(^7\). Human lymphocyte antigen (HLA)-DR<sup>+</sup>CD1<sup>+</sup>S<sup>+</sup>-100<sup>+</sup>DCs have been observed in atherosclerotic lesions \(^7\). Increased numbers of DCs are primarily observed in the intimal neovascularization area, media and adventitia of vessels with inflamed atherosclerotic lesions \(^7\). These arterial DCs express TLR1, TLR2, TLR3, TLR4,
Until recently, 2 types of DCs have been investigated in regard to atherosclerotic diseases: (1) mDCs, which have a dendritic form, express CD1c, CD11c, and CD33 and secrete IL-12, and (2) pDCs, which have a plasma-cell-like appearance, express CD123 (IL-3 receptor α-chain), and produce type I IFN (Figure 2).\textsuperscript{73,74} Circulating mDC precursors, but not pDC precursors, decrease significantly in patients with coronary artery disease (CAD), including stable angina, unstable angina, and acute myocardial infarction, possibly because of increased recruitment into atherosclerotic lesions.\textsuperscript{79} In contrast, other studies have shown a significant reduction in the circulating number of pDCs and a significant increase in the number of mDCs in patients with CAD compared with controls.\textsuperscript{20,81} This discrepancy may be explained by differences in mDC and pDC surface markers. A high frequency of mDCs with high levels of HLA-DR expression and T-cell contacts are observed in advanced atherosclerotic lesions.\textsuperscript{82} Moreover, the emergence of mDCs in carotid atheroma is associated with vulnerable plaque and acute cerebral events, suggesting that mDCs may participate in plaque destabilization.\textsuperscript{83} The expression of the activating marker, CD86, and the capacity for T cell proliferation in circulating monocyte-derived DCs are significantly higher in patients with unstable angina than in control subjects.\textsuperscript{83} The expression of TLR4, phosphor-Erk1/2, and phosphor-JNK in circulating monocyte-derived DCs are much higher in patients with ACS than in either patients with stable angina or controls.\textsuperscript{84} Monocyte-derived DCs activated by human C-reactive protein induce IFN-γ production and T-cell proliferation. These results were obtained in a case-controlled fashion, so how DCs interfere with the atherosclerotic process has not yet been investigated. We recently reported that LPS administration induced activation of vessel wall DCs in a novel bioengineered human artery model characterized by CD83 upregulation and production of CCL19 and CCL21.\textsuperscript{86} In addition, autologous CD4+ cells were induced to produce IFN-γ and infiltrate the model artery. These findings suggest that TLR4 ligation by LPS is sufficient to induce breaking of T-cell tolerance and initiate vascular inflammation.\textsuperscript{86} Dyslipidemia activates DCs and inhibits their migration from tissues into lymph nodes.\textsuperscript{88} In contrast, migration of activated DCs from atherosclerotic lesions to secondary lymphatic tissues may be an important process for interaction with other immune cells. Indeed, the lymph nodes and spleen contain OxLDL-reactive T cells\textsuperscript{87} DCs primed by modified LDL, such as OxLDL, induce upregulation of co-stimulatory molecules and proliferation of T cells; however, a high-dose stimulation of OxLDL promotes apoptosis of DCs\textsuperscript{88} In concordance with this result, severe dyslipidemia inhibits TLR-induced production of proinflammatory cytokines and co-stimulatory molecule expression of DCs, resulting in modulation of Th1-biased responses to Th2-dominated responses\textsuperscript{89} A recent study revealed that DCs maintain antigen processing, presentation capability, and CD4+ T-cell stimulation, both within the plaque microenvironment and in secondary lymphoid tissues under dyslipidemic conditions.\textsuperscript{80} That study demonstrated that DCs are gatekeepers for adaptive immunity in lipiddenized circumstances\textsuperscript{80} pDCs recognize oligodeoxynucleotides containing particular CpG motifs via TLR7 and TLR9, and secrete IFN-α (Figure 2).\textsuperscript{73,74} In atherosclerotic carotid arteries, especially unstable plaques, pDCs as well as mDCs are localized to the shoulder lesion with IFN-α production, which correlates with plaque instability.\textsuperscript{91} In addition, IFN-α enhances TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis of coronary vascular smooth muscle cells.\textsuperscript{91} Coexistence of mDCs and pDCs synergizes the inflammatory effects of the TLR4 and TLR9 ligands, including amplified production of TNF-α, IL-12, and IL-23, and (2) pDCs, which have a plasma cell-like appearance, express CD123 (IL-3 receptor α-chain), and produce IFN-α. Recently, DC subtypes and precursors have been divided into (1) pre-DCs, (2) conventional DCs, which are further subdivided into migratory DCs and lymphoid-tissue-resident DCs, and (3) inflammatory DCs, which are not present in the steady state but appear as a consequence of inflammation. DCs, dendritic cells; TLR, Toll-like receptor; TNF, tumor necrosis factor; BDCA, blood dendritic cell antigen; IFN, interferon.

Pre-DCs, Conventional DCs, and Inflammatory DCs

All DCs are capable of antigen uptake, processing and presentation to naive T cells; however, the DC subtypes differ in localization, immunological function, and inflammatory reaction for their generation.\textsuperscript{79} Recently, DC subtypes and precursors have been divided into (1) pre-DCs, which are capable of developing into DCs without dendritic form (eg, some monocytes and pDCs), (2) conventional DCs, which are further subdivided into migratory DCs (eg, Langhans cells and dermal DCs), and lymphoid-tissue-resident DCs, which are unable to migrate into lymph nodes, and (3) inflammatory DCs, which are not present in the steady state but appear as a consequence of inflammation (eg, TNF- or iNOS-producing DCs).\textsuperscript{73,74} Deletion of CX3CR1 decreases the accumulation of DCs and the atherosclerotic burden in the aorta of apoE deficient mice\textsuperscript{93} which suggests that inflammatory DCs might predominantly differentiate from Ly6C\textsuperscript{low} monocytes and promote atherosclerotic lesions.\textsuperscript{14}
The phenotype of vascular DCs remains incompletely defined; a portion of them are supposed to be inflammatory DCs, but both Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{low} monocytes are capable of differentiating into DCs in vivo.\textsuperscript{94} A recent study demonstrated that the number of mDC precursors, pDC precursors, and total DC precursors are significantly lower in patients who required revascularization than in controls.\textsuperscript{95} Even after adjusting for confounding factors, the decreased number of these precursors was an independent predictor of subsequent therapeutic procedure.\textsuperscript{95} However, further investigations are required to clarify the development of DC precursors into each subpopulation and classification of DCs subgroups regarding atherosclerotic disease.

**Subpopulations of CD4\textsuperscript{+} Cells in Atherosclerosis**

**Th1 and Th2**

Adaptive immunity, which includes cell-mediated immunity and humoral immunity, mainly consists of T and B lymphocytes. Mosmann et al demonstrated that 2 types of CD4\textsuperscript{+} T cells, the so-called Th1 and Th2, can be distinguished by their cytokine profiles.\textsuperscript{96} The Th1 subset, which drives cell-mediated immunity, is reliant on IFN-\(\gamma\) and IL-12, whereas the Th2 subset, which drives humoral immunity, depends on IL-4 and IL-5 (Figure 3).\textsuperscript{3,14,96,97} Th1 function includes the activation of macrophages, neutrophils, and CD8\textsuperscript{+} cytotoxic T lymphocytes to remove damaged tissue and eliminate phagocytosed microbes. In contrast, the function of Th2 is to stimulate antibody production on B cells and plasma cells to neutralize microbes and their toxins. It has already been recognized that multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and graft-vs-host disease are Th1-predominant diseases. In contrast, allergic diseases and systemic lupus erythematosus are supposed to be Th2-biased diseases, suggesting that overactivation of either pattern, the so-called Th1/Th2 imbalance, will cause a specific disease.\textsuperscript{3,13,96,97} Th1 cytokine-producing cells, including those that produce IFN-\(\gamma\), IL-12, but few Th2 cytokine-producing cells, such as those that produce IL-4, have been identified in human atherosclerotic plaques.\textsuperscript{98,99} Several clinical studies have demonstrated that Th1-type cytokines dominate not only in atherosclerosis but also in ACS\textsuperscript{4,100,101}, suggesting that atherosclerotic disease, including ACS, is a Th1-dominant disease. This evidence is supported by mice models that lack IFN-\(\gamma\), IL-12, or T-box expressed in T cells (T-bet), which is a critical transcription factor for Th1 induction.\textsuperscript{104} My group and others have shown that early statin treatment modulates the Th1/Th2 imbalance in patients with ACS\textsuperscript{50,51}. The mechanism by which statins regulate this balance is thought to be as follows. The signal-transducer-and-activator-of-transcription (STAT) protein family, T-bet, and GATA binding protein 3 regulate Th1 and Th2 cell differentiation. IL-12 and IL-4 bind to their receptors and activate STAT4 and STAT6 to promote Th1 and Th2 differentiation, respectively.\textsuperscript{105} Indeed, STAT4 transcriptional levels in CD4\textsuperscript{+} T lymphocytes are upregulated in patients with stable angina and ACS\textsuperscript{101}. Atorvastatin promotes Th2 bias in a Th1-shifted model, experimental autoimmune encephalomyelitis (EAE), by inhibiting STAT6 phosphorylation.\textsuperscript{105} Another explanation is a decrease in isoprenoid production. Isoprenoids, such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate, regulate Th1 differentiation.\textsuperscript{106} Atorvastatin treatment induces a Th2 bias accompanied by a decrease in signals between both Ras and extracellular signaling-regulated kinase, and between RhoA and p38 at the T-cell membrane in EAE mice.\textsuperscript{106}
Regulatory T Cells and Th17

CD4+CD25+ regulatory T (Treg) cells expressing the forkhead/winged helix transcription factor (Foxp3) have recently been described as a special subset of T cells that tightly control the effector function of activated T cells through a contact-dependent mechanism or production of anti-inflammatory cytokines such as IL-10 and TGF-β (Figure 3). The development of CD4+CD25+Foxp3+ Treg cells is induced by “tolerogenic” APCs in the thymus, as well as by adaptive antigen-triggered or IL-10 and TGF-β-producing cells. These cells migrate into peripheral tissues to regulate the immune response. Treg cells are absent in normal human arteries; however, Treg cells that express Foxp3 or GITR are observed in the intima during all stages of developing human atherosclerotic plaques. In patients with ACS, the number of circulating Treg cells is reduced and their functions hampered, including susceptibility of apoptosis induced by OxLDL and decreased ability to suppress helper T-cell proliferation. In animal experiments, a decreased number of Treg and their compromised function were observed in apoE-deficient mice compared with controls. In addition, OxLDL induced a more robust depletion of splenic Treg than of helper T-cells. Depletion of peripheral Treg using an anti-CD25 antibody increases the size of atherosclerotic lesions and vulnerability in apoE-deficient mice. In contrast, adaptive transfer of Treg reduces atherosclerotic formation and increases plaque stabilization in apoE knockout mice. A recent study showed that statin therapy increases the number of circulating Treg cells in vivo, as well as the number of Foxp3+ T-cells in peripheral blood, suggesting that conversion of Th cells to Treg might be a mechanism by which statins modulate atherogenesis. Although Th17 cells have an essential role in protecting against various pathogens, the reduced Treg cells in vivo, as well as the number of Foxp3+expressing cells in vitro, suggesting that conversion of Th cells to Treg might be a mechanism by which statins modulate atherogenesis. Th17 cells are another CD4+ T-cell lineage that express retinoic acid-related orphan receptor γ (RORγt), which plays an important role in adaptive immunity. Although Th17 cells have an essential role in protecting against various pathogens, Th17 cells reacting to self-antigens leads to the development of autoimmune and allergic reactions by producing IL-17. Significant increases in the number of peripheral Th17 cells, Th17-related cytokines (IL-17, IL-6 and IL-23), and transcription factor (RORγt) levels are observed in patients with ACS. Interestingly, obvious decreases in Treg numbers, Treg-related cytokines (IL-10 and TGF-β), and transcription factor (Foxp3) levels are also demonstrated when stable angina patients and controls are compared. These findings suggest that a Th17/Treg imbalance may play an important role in plaque destabilization and the onset of ACS.

Conclusions

There is growing evidence that atherosclerotic disease is an immune disease. Leukocyte subsets, including monocytes/macrophages, DCs, and lymphocytes, obviously regulate and participate in the initiation of atherosclerosis and atherothrombotic complications. However, the entire pathophysiology of atherosclerosis, even in terms of immunopathophysiology, is unclear. The balance and dynamic equilibrium of other leukocyte subsets must be involved in the process. Further detailed basic and clinical studies will be required in this regard.

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