Atherosclerosis is considered a chronic inflammatory disease and an intervention targeting the inflammatory process could be a new therapeutic strategy for preventing atherosclerotic cardiovascular diseases (CVD). We hypothesized that the intestine, which is considered the biggest immune organ in the human body, could be a therapeutic target for preventing CVD. We demonstrated that oral administration of anti-CD3 antibody or an active form of vitamin D3 reduced atherosclerosis in mice via induction of regulatory T cells and tolerogenic dendritic cells in the gut-associated lymphoid tissues. Similar to regulatory immune responses achieved by oral tolerance, our method had systemic effects that ultimately contributed towards atherosclerosis reduction. Recently, we have been interested in the gut microbiota, which have been reported as highly associated with intestinal immunity and systemic metabolic disorders, including obesity and diabetes. Notably, the guts of obese individuals are predominantly colonized by *Firmicutes* over *Bacteroidetes*. The association between atherosclerosis and microbiota has been attracting increased attention, and gut microbiota have been shown to participate in the metabolism of a proatherogenic compound called trimethylamine-N-oxide (TMAO) and aggravate CVD. Our investigation of the relationship between susceptibility to CVD and the gut microbiota revealed a characteristic flora type. Here, we discuss the evidence for the relationship between the gut microbiota and cardiometabolic diseases, and consider the gut microbiota as new potential therapeutic targets for treating CVD. (Circ J 2015; 79: 1882–1890)

**Key Words:** Atherosclerosis; Gut microbiota; Intestinal immunity; Regulatory T cell; Tolerogenic dendritic cell
Intestinal Immunity and Gut Flora in Atherogenesis

Proinflammatory and Antiinflammatory Immune Responses in Atherogenesis

Atherosclerosis is considered a chronic inflammatory disease involving many types of immune cells. Several immune responses are critical in the initiation and progression of atherosclerosis (Figure 1). The first step preceding the development of various “omics” technologies, including genomics, proteomics, and metabolomics, have enriched our understanding of the ecological system of commensal bacteria in the intestine. Now, this understanding has prompted large-scale projects to comprehend microbe-host interactions, such as the United States’ Human Microbiome Project (HMP) and the European Metagenomics of the Human Intestinal Tract (MetaHIT) Consortium.

Recent studies have demonstrated that gut microbe-derived factors may actually lead to many chronic diseases, such as ulcerative colitis and Crohn’s disease. In addition, massive data from both animal models and human studies are expanding our understanding of the associations between the functions of the gut microbiota and metabolic diseases: obesity, MetS, and CVD. In this review, we will describe how specific gut microbial changes could affect host metabolism, and how these findings lead to novel therapeutic targets for CVD as well as MetS.
Atherosclerotic lesion formation is endothelial dysfunction and LDL-cholesterol deposition in the arterial wall, which are mediated by coronary risk factors such as dyslipidemia, hypertension, DM, and smoking. Secondly, the accumulated LDL is oxidized and the resultant formation of oxidized LDL (OxLDL) has been suggested to be the critical event in deleterious inflammation in the vascular wall. Thirdly, monocytes and various other types of leukocytes adhere to the activated endothelium, migrate into the arterial wall via upregulation of adhesion molecules, and produce proinflammatory cytokines or chemokines. Subsequently, monocyte-derived macrophages take up OxLDL via scavenger receptors, leading to the formation of lipid-laden foam cells. Following these steps, the initial fatty streaks are developed, which contain lipids and numerous immune cells such as macrophages, dendritic cells (DCs), and T lymphocytes. However, not only proinflammatory cells but also anti-inflammatory immune cells [regulatory T cells (Tregs) and tolerogenic DCs] are involved in the atherosclerotic lesions. As the atherosclerotic process progresses, the lesion becomes more complex, consisting of migrated smooth muscle cells (SMCs), apoptotic cells, and extracellular matrix such as collagen and proteoglycans. Finally, such indolent advanced atherosclerotic plaques may suddenly rupture and induce life-threatening coronary thrombosis presenting as an acute coronary syndrome. The notable features of unstable rupture-prone plaque include infiltration of many inflammatory cells, large lipid core, and thin fibrous cap (Figure 1). Although not yet successful, therapeutic interventions targeting the atherosclerotic inflammatory process described may lead to establishment of definitive treatments for atherosclerotic CVD.

Intestine as a Therapeutic Target for Preventing Atherosclerosis

The mucosal and intestinal immune system discriminates between potentially harmful foreign antigens and harmless ones. The gut acts to tolerate harmless antigens, but remains able to eliminate harmful pathogens. To accommodate the exposure to harmless antigens, including food components and commensal gut bacteria, the gut has evolved an anti-inflammatory environment (Figure 2). Recent research revealed that tolerogenic DCs in the gut present food antigens to T cells as tolerogens and induce antigen-specific immune suppression. It is now accepted that oral tolerance involves either anergy/apoptosis of CD4+ effector T cells or induction of Tregs, which
Intestinal Immunity and Gut Flora in Atherogenesis appear to come in several different forms. Originally described as CD4+/CD25+ cells, the naturally occurring Tregs (nTreg) generated in the thymus express the transcription factor Foxp3 and are involved in maintaining systemic homeostasis and preventing autoimmunity. Immunosuppressive mediators of Tregs include inhibitory molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and the immunoregulatory cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). IL-10-producing Tr1 cells were firstly described for splenocytes cultured and matured in vitro in the presence of IL-10. Tr1 cells, which do not express Foxp3, have been demonstrated in Peycer’s patches of mice fed a low dose of β-lactoglobulin and produce large amounts of IL-10. Th3 cells are TGF-β-producing latency-associated peptide (LAP)+CD4+ T cells originally isolated from mesenteric lymph nodes of orally tolerant mice. TGF-β induces expression of Foxp3 in naïve CD4+ T cells, and Th3 cells can influence the Treg development of neighboring cells. In particular, retinoic acid promotes the conversion of naïve CD4+ T cells into Foxp3+ peripherally inducible Tregs (pTreg) with the help of TGF-β in the gut, suggesting significant roles for intestinal DCs that produce retinoic acid (Figure 2). Oral tolerance is locally induced in an antigen-specific manner, but its effects are not constrained to the local immune response within the gut. The anti-inflammatory immune responses could be seen in other tissues or organs, including distal non-lymphoid organs such as skin. Taken together, we hypothesized that modulation of intestinal immunity or induction of oral tolerance affects the systemic immune responses and might prevent atherosclerosis. Recent studies have shown that oral anti-CD3 antibody is biologically active and induces TGF-β-producing CD4+ LAP+ Tregs, namely Th3, that suppress experimental autoimmune encephalitis, autoimmune DM in a TGF-β-dependent fashion. Autoimmune diseases are shown to be suppressed by only low doses of oral anti-CD3 antibody in association with.
an increase in LAP⁺ Tregs, but not by high doses, although there is no evidence of antigen specificity with oral anti-CD3 antibody. As observed in mucosal tolerance induction, only low doses of oral anti-CD3 antibody administration may result in the induction of Th3 by delivering a weak signal to T cells, although further elucidation of the cellular and molecular mechanisms underlying induction of various Tregs is needed. We applied this method for the treatment of atherosclerosis in apolipoprotein E-deficient (apoE⁻/⁻) mice and demonstrated that oral anti-CD3 antibody treatment induced Th3 and Foxp3⁺ Tregs, which suppressed pathogenic immune processes pivotal for atherogenesis through a TGF-β-dependent mechanism and consequently inhibited atherosclerotic plaque formation (Figure 3). Furthermore, we examined the effect of oral anti-CD3 antibody treatment on the phenotypes of DCs in the mesenteric lymph nodes in mice and confirmed that CD80 and CD86 expressions in DCs were reduced in anti-CD3 antibody-treated mice compared with controls.

Active vitamin D₃ (calcitriol) has been shown to induce immature DCs and Tregs. We tried to examine the effects of orally administrated calcitriol on atherosclerosis in animal models and first demonstrated that it decreases atherosclerosis in apoE⁻/⁻ mice by promoting induction of tolerogenic DCs and Foxp3⁺ Tregs. A cell-based therapy strategy using tolerogenic DCs revealed that apolipoprotein B100 (apoB100)-pulsed tolerogenic DCs inhibit the proliferative and proinflammatory T-cell response to apoB100, promoted Treg induction, and reduced atherosclerotic lesion formation. Oral administration of Hsp60 or Hsp65 might also induce Tregs in the gastrointestinal tract and affect atherogenesis (Figure 3). Although further studies are needed to clarify the precise role of the several types of vascular DCs in atherogenesis, effective methods to induce atheroprotective DCs could be novel therapies for prevention of atherosclerosis.

**Figure 4.** Human gut commensal microbiota and their classification. The most dominant Gram-positive phylum in the human gut flora is Firmicutes and the most dominant Gram-negative phylum is Bacteroidetes. The 4 major phyla of Firmicutes, Bacteroides, Actinobacteria, and Proteobacteria account for more than 98% of all human gut microbiota. The total number of bacteria in the human intestine is more than one hundred trillion. Those bacteria are classified into several hundreds of species.

**Gut Microbiota and Their Regulatory Effects on the Intestinal Immunity**

Colonization of the gastrointestinal tract begins after birth, and continues throughout life. The profile of the predominant

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phyllum in the gut changes during childhood and youth, and almost stabilizes by adulthood. Most bacterial species in the adult human and mouse gut belong to the phyla Firmicutes and Bacteroidetes, with less abundant bacterial phyla such as Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (Figure 4). The major 4 phyla of Firmicutes, Bacteroides, Actinobacteria, and Proteobacteria occupy more than 98% of all human gut microbiota. A lot of gut bacteria cannot be cultivated in vitro, making their categorization and identification difficult. Although there is no clear definition of a normal “healthy” gut microbiome in humans, recent metagenomics has allowed 3 major clusters of gut bacteria named “enterotypes” to be distinguished in humans, based on the predominant bacterial genera in fecal specimens: type I is characterized by high levels of Bacteroides, type II has few Bacteroides but Prevotella are common, and type III has high levels of Ruminococcus (Figure 4). The composition of the gut microbiota is remarkably diverse and dynamic over short periods of time because dietary exposure significantly affects the microbial community, but its composition appears to remain remarkably stable over time within individuals and their family members.

It has recently been demonstrated that specific bacterial species are associated with differentiation of specific subsets of T cells in the intestine. Both human and mouse Clostridium clusters IV and XIVa, a spore-forming component of indigenous intestinal microbiota, have been implicated in the induction of Foxp3+ Tregs in the colon of mice (Figure 3). Furthermore, butyrate, a short-chain fatty acid (SCFA) produced by commensal bacteria, promotes Foxp3+ Treg induction. Given this background, it can be speculated that the propagation or sterilization of some specific bacterial species, which will result in augmented generation of Tregs or reduced differentiation of pathogenic T cells, may prevent inflammatory diseases, including atherosclerosis. Further studies are needed to prove this hypothesis and may contribute to the development of novel strategies for preventing atherosclerosis through modulation of intestinal immunity.

Gut Microbial Alternations Associated With Obesity and T2DM

Recent studies in both mice and humans show that obesity is also associated with changes in the gut microbiota compared with lean subjects. It has been indicated that the gut microbiota may function as an environmental factor that contributes to obesity and T2DM. GF mice that consume 27% more food than conventional mice have significantly less body fat, suggesting the role of the microbiota in metabolism and energy balance. In humans, a study including twins concordant for obesity and T2DM proposed that the gut microbiota affects host lipid metabolism. A detailed mechanism for how commensal bacteria may contribute to host lipid and cholesterol metabolism could potentially be explained by microbial regulation of bile acid synthesis and metabolism, but further work is required.

A sequencing study comparing the gut microbiome from patients who had symptomatic and asymptomatic atherosclerotic plaques in the carotid artery and from healthy controls showed that the microbiome was more proinflammatory in the people with atherosclerotic plaques. The shotgun sequencing of the gut metagenome demonstrated that the genus Collinsella was increased in patients with symptomatic atherosclerosis, whereas Roseburia and Eubacterium were enriched in healthy controls. The study also demonstrated that patients with symptomatic atherosclerosis were underrepresented in enterotype I, while overrepresented in enterotype III.

Our research group also has been investigating the association between the fecal microbiota and coronary artery disease (CAD). We will report the results in the near future.

CVD and the Chemical TMAO Connection

Recently, Hazen et al have splendidly reported that gut microbial-
microbiota using broad-spectrum antibiotics cancelled the proatherosclerotic effect of dietary choline, which was associated with the reduction of plasma TMAO levels in antibiotic-treated mice. The study also investigated the relationship between fasting plasma levels of TMAO and incident major adverse cardiovascular events (death, myocardial infarction, or stroke) during 3 years of follow-up in 4,007 patients undergoing elective diagnostic cardiac catheterization. Increased plasma TMAO levels were associated with an increased risk of a major adverse cardiovascular event (Figure 5). Even after adjustment for traditional risk factors, an elevated TMAO level could predict an increased risk of major adverse cardiovascular events. Collectively, the findings suggest that pathways that are dependent on the gut microbiota may contribute to the pathophysiology of atherosclerotic CAD and suggest potential therapeutic targets.

It was also clarified that microbial processing of L-carnitine, which is abundant in red meat and contains a TMA structure similar to that of choline, elevates plasma TMAO concentrations and enhances atherosclerosis in a microbial-derived metabolites, trimethylamine (TMA) and TMA N-oxide (TMAO), are proatherogenic in both mice and humans. First, they used a metabolomics approach to generate unbiased small-molecule metabolomic profiles in plasma that predict for CVD. Three metabolites of the dietary lipid phosphatidylcholine (choline, TMAO and betaine) were identified and these metabolites were associated with atherosclerosis risk in humans and promote atherosclerosis in mice (Figure 5). Oral feeding of choline, rather than parenteral delivery, was necessary to generate the metabolite TMAO, suggesting that a necessary phase in this biochemical pathway was performed within the intestine. Generation of TMAO and its precursor TMA were shown to be dependent on the gut microbiota in both humans and mice. That is, they found that dietary choline is metabolized by intestinal bacteria to TMA, which is subsequently absorbed into the host and metabolized to TMAO in the liver by the FMO (hepatic flavin monooxygenase) family of enzymes. Moreover, dietary supplementation of apoE−/− mice with choline promoted upregulation of multiple macrophage receptors and enhanced atherosclerosis. Deletion of gut microbiota using broad-spectrum antibiotics cancelled the proatherosclerotic effect of dietary choline, which was associated with the reduction of plasma TMAO levels in antibiotic-treated mice. The study also investigated the relationship between fasting plasma levels of TMAO and incident major adverse cardiovascular events (death, myocardial infarction, or stroke) during 3 years of follow-up in 4,007 patients undergoing elective diagnostic cardiac catheterization. Increased plasma TMAO levels were associated with an increased risk of a major adverse cardiovascular event (Figure 5). Even after adjustment for traditional risk factors, an elevated TMAO level could predict an increased risk of major adverse cardiovascular events. Collectively, the findings suggest that pathways that are dependent on the gut microbiota may contribute to the pathophysiology of atherosclerotic CAD and suggest potential therapeutic targets.

It was also clarified that microbial processing of L-carnitine, which is abundant in red meat and contains a TMA structure similar to that of choline, elevates plasma TMAO concentrations and enhances atherosclerosis in a microbial-
dependent manner.\textsuperscript{45} It was also recently found that 3-butyrobetaine is the major proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMA and TMAO.\textsuperscript{46} One of the proatherosclerotic mechanisms of TMAO might be increasing macrophage foam cell formation and suppressing reverse cholesterol transport (RCT) in vivo. Bacterial taxa belonging to the families Clostridiaceae and Peptostreptococcaceae were positively associated with TMAO production in humans, suggesting that L-carnitine-metabolizing bacteria may belong to these families. However, the molecular mechanisms through which gut microbial formation of TMAO leads to inhibition of RCT are not entirely clear. Further, this study may in part explain why excessive red meat consumption has been associated with increased CVD and mortality risks.

**Future Perspectives**

As imbalance in the gut microbiota, termed dysbiosis, significantly contributes to various human diseases, the fecal microbiota are regarded as important players in the development of metabolic disorders and subsequent CVD. As currently available evidence is mainly based on animal and human cohort studies, additional work in the future is earnestly desired to clarify how altered gut microbiota can be used in both the diagnostic and therapeutic setting. For example, FMT from a healthy donor by duodenal tubing, colonoscopy, or enema has been shown as effective in patients with pseudomembranous enteritis infected by *Clostridium difficile*.\textsuperscript{47} Transplanting microbiota from lean donors to recipients with MetS led to an improvement in insulin sensitivity, with increased levels of butyrate-producing bacteria.\textsuperscript{48} There will be many obstacles in the development of commercial products based on FMT, for example donor selection, quality assurance, risk of pathogen contamination, and patient acceptance. FMT from a healthy donor to CVD patient might enable us to identify causally involved intestinal microbes in CVD and might be a new therapeutic strategy for CVD, as in the case of treating *Clostridium difficile* infection.

**Concluding Remarks**

Intestinal immunity has been attracting much attention as a novel therapeutic target for atherosclerosis. Since the discovery of Tregs and DCs, knowledge about the biology and pathophysiology of these regulatory immune cells has accumulated in the fields of atherosclerosis and autoimmune diseases. It is now clear that several types of Tregs and tolerogenic DCs are essential for the regulation of pathogenic T-cell immune responses in atherogenesis. Gut-associated immune tolerance induction by oral administration of drugs or therapeutic agents possessing immunoregulatory activities is simple, easy, and a hopeful way of regulating inflammatory diseases, though the detailed mechanisms of how intestinal immunity affects systemic immunity remain to be clarified.

In association with intestinal immunity in atherogenesis, we have been interested in the gut bacteria that might be involved in the pathogenesis of atherosclerotic CVD and the associated gut flora types. Simple classification of the gut microbiota associated with CVD, especially CAD, must be the first step. Sequencing the microbial genes by a metagenome-wide association study is the second step. However, this may be insufficient, because the presence of DNA alone neither necessarily translates into protein synthesis nor relates to function. Further studies are needed for understanding of the functional level of some specific microbial pathways and their products that contribute to maintaining our physiological homeostasis and that contribute to disease processes. We hope the novel therapeutic strategies that intervene in the gut microbiota for the prevention of atherosclerotic CVD will be developed in the near future and contribute to patients’ wellbeing.

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