The official journal of the Japan Atherosclerosis Society and
the Asian Pacific Society of Atherosclerosis and Vascular Diseases

Review

J Atheroscler Thromb, 2022; 29: 297-307. http://doi.org/10.5551/jat.RV17059

Emerging Roles of the Innate Immune System Regulated by DNA Sensors in the Development of Vascular and Metabolic Diseases

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Sterile chronic inflammation causes cardiometabolic disorders; however, the mechanisms are not fully understood. Previous studies have demonstrated the degradation of cells/tissues in the vasculature and metabolic organs in lifestyle-associated diseases, such as diabetes and hyperlipidemia, suggesting the release and/or accumulation of nucleic acids from damaged cells. DNA is indispensable for life; however, DNA fragments, especially those from pathogens, strongly induce inflammation by the activation of DNA sensors. Growing evidence suggests that DNA-sensing mechanisms, which are normally involved in self-defense against pathogens as the innate immune system, are associated with the progression of inflammatory diseases in response to endogenous DNA fragments. There are several types of DNA sensors in our bodies. Toll-like receptor 9 (TLR9)—one of the most studied DNA sensors—recognizes DNA fragments in endosome. In addition, stimulator of interferon genes (STING), which has recently been extensively investigated, recognizes cyclic GMP-AMP (cGAMP) generated from DNA fragments in the cytosol. Both TLR9 and STING are known to play pivotal roles in host defense as the innate immune system. However, recent studies have indicated that the activation of these DNA sensors in immune cells, such as macrophages, promotes inflammation leading to the development of vascular and metabolic diseases associated with lifestyle. In this review, we discuss recent advances in determining the roles of DNA sensors in these disease contexts. Revealing a novel mechanism of sterile chronic inflammation regulated by DNA sensors might facilitate clinical interventions for these health conditions.

Key words: DNA sensor, TLR9, STING, Atherosclerosis, Insulin resistance, Inflammation

Abbreviation: Ang II; angiotensin II, ApoE KO; Apolipoprotein E-deficient, BM; bone marrow, cfDNA; cell-free DNA, cGAMP; cyclic GMP-AMP, cGAS; cGAMP synthase, DAMPs; damage-associated molecular patterns, ER; endoplasmic reticulum, HFD; high-fat diet, HMGB1; high mobility group box protein-1, IFN; interferon, IKK; IκB kinase, IRFs; interferon regulatory factors, LC-MS/MS; liquid chromatograph-mass spectrometry, mtDNA; mitochondrial DNA, MyD88; myeloid differentiation primary response 88, NAFLD; non-alcoholic fatty liver disease, NF-κB; nuclear factor kappa B, PRRs; pattern recognition receptors, RIG-I; retinoic acid inducible gene I, SAVI; STING-associated vasculopathy with onset in infancy, STING; stimulator of interferon genes, STING KO; STING-deficient, TBK; TANK binding kinase, TLRs; Toll-like receptors, TNF-α; tumor necrosis factor-α, WTD; western-type diet

Introduction

The immune system consists of two parts: the innate and the adaptive immune systems. These two systems closely work together but have different roles. The innate immune system uses pattern recognition receptors (PRRs) to recognize nonselfmicrobial products (pathogen-associated molecular patterns (PAMPs)). Following the ligation of PAMPs, PRRs activate various downstream signaling pathways to

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Received: May 13, 2021 Accepted for publication: May 26, 2021

elicit innate immune responses and further activate the adaptive immune system.

Among various PAMPs, nucleic acids, especially those from exogenous organisms, such as bacteria and viruses, are known to strongly activate the innate immune system. A number of sensors for nucleic acids have been identified, implying that sensing exogenous nucleic acids is central for self-defense against infection. Toll-like receptors (TLRs) is the best characterized class of PRRs. Among them, TLR3, TLR7, TLR8, TLR9, and TLR13 recognize endosomal nucleic acid[1, 2]. In addition to endosomal nucleic acid-sensing proteins, cytoplasmic DNA sensors, such as the cyclic GMP-AMP (cGAMP) synthase (cGAS)–cGAMP–stimulator of interferon genes (STING) system, have been reported[3]. There are also RNA sensors in the cytosol, such as retinoic acid-inducible gene I-like receptors[4, 5]. Following the recognition of nucleic acids, these sensors activate multiple pathways that stimulate inflammation, such as interferon (IFN) regulatory factors (IRFs), nuclear factor kappa B (NF-κB), and inflammasome.

Recent studies have demonstrated that PRRs also recognize host-derived molecules called damage-associated molecular patterns (DAMPs). These are generated by prolonged stresses, including infection, physiological stress, and overnutrition, and lead to the induction of inappropriate immune responses and the collapse of homeostasis[6]. Emerging evidence has revealed that nucleic acid sensors participate not only in innate immune systems but also in the pathogenesis of various diseases, such as autoimmune and lifestyle-related diseases. This review briefly summarizes the role of DNA sensors in the pathogenesis of vascular and metabolic diseases that are related to lifestyle-associated diseases.

DNA Damage in Vascular and Metabolic Diseases Related to Lifestyle-Associated Diseases

Sterile chronic inflammation causes atherosclerosis and insulin resistance; however, it remains a major medical challenge to understand the molecular mechanisms of sterile chronic inflammation in the vasculature and metabolic organs. Physiological and pathological processes in unhealthy lifestyles (such as smoking, physical inactivity, and diet), metabolic risk factors (such as hypertension, diabetes, and hyperlipidemia), and other factors (such as genetics, gender, and aging) degrade cells/tissues in the vasculature and metabolic organs and subsequently damage both nuclear and mitochondrial DNA[7-11], resulting in the release and/or accumulation of DNA fragments[12-15]. The underlying mechanism of DNA damage is multifactorial[16]. Numerous studies have demonstrated that higher oxidative stress[17] and lower oxygen pressure[18], caused by physiological and pathological processes associated with lifestyle-associated diseases, induce DNA damage. Ballinger *et al.* reported that mitochondrial DNA (mtDNA) damage not only correlated with the development of atherosclerosis in humans and apolipoprotein E-deficient (ApoE KO) mice but also preceded atherogenesis in young ApoE KO mice[19]. mtDNA damage is also shown to increase vulnerability of atherosclerotic plaques[20]. DNA damage also induces the development of metabolic diseases, such as obesity-induced insulin resistance[15, 21]. Macrophages play a central role in the pathophysiology of cardiometabolic diseases[12, 22]. Our previous studies using immune-electron microscopy demonstrated the accumulation of DNA fragments in macrophages, which infiltrated into atherosclerotic lesions and obese adipose tissues[23, 24].

DNA damage is also of interest because it can potentially be a marker for inflammatory diseases. The existence of extracellular DNA in human plasma, also known as cell-free DNA (cfDNA), has been known since the 1940s[25]. Recent studies have demonstrated the elevation of cfDNA and its association with the pathophysiology and severity of inflammatory diseases[26]. For example, circulating cfDNA is increased in several autoimmune diseases (such as systemic lupus erythematosus[27-31]) and other diseases in which inflammation is involved (such as experimental pulmonary thromboembolism[32] and end-stage renal disease[33]). Furthermore, recent clinical studies have indicated the contribution of elevated plasma cfDNA to the development of cardiometabolic disorders in humans. Borissoff *et al.* demonstrated that plasma double-stranded DNA and nucleosome levels in patients with severe coronary artery disease (diagnosed by coronary computed tomographic angiography) were significantly higher than those of a control group[34]. Our previous study revealed the positive correlation between cfDNA level in the target vessel of patients with acute myocardial infarction and the inflammatory features of plaques in the target lesion (determined by optical coherence tomography)[23]. In addition, we demonstrated that obese subjects had higher plasma levels of cfDNA and that the plasma levels of cfDNA positively correlated with abdominal adipose tissue area and insulin resistance severity[24].

DNA damage is increasingly recognized as a causal factor in the initiation and progression of vascular and metabolic diseases[15, 16]. Therefore,

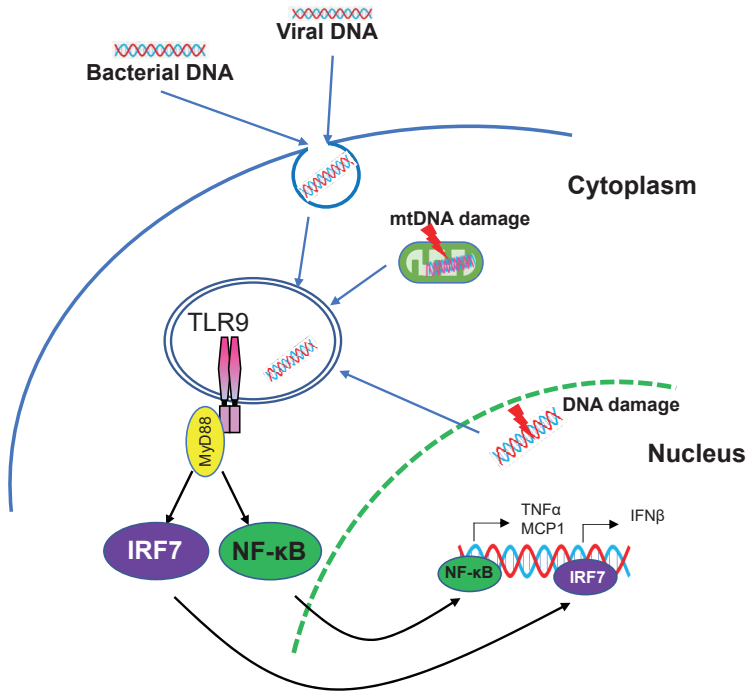

Fig. 1. TLR9 signaling

TLR9 recognizes DNA fragments that contain unmethylated CpG DNA in endosomes and promotes inflammatory responses. Following ligation with the DNA fragments, it produces type I IFN and inflammatory cytokines through IRF7 pathway and NF–κB pathway, respectively.

investigating the role of DNA fragments derived from host cells and the mechanisms by which DNA fragments promote vascular and metabolic inflammation have become research topics of great interest.

Mechanism of the Activation of DNA Sensors

TLR9 is one of the most studied DNA sensors for nucleic acids. It recognizes DNA fragments that contain unmethylated CpG DNA and plays a role in innate immunity[35, 36]. TLR9 localizes in the endoplasmic reticulum (ER) in multiple types of immune cells, including macrophages, B-cells, dendritic cells, and plasma cells. After the uptake of the ligands by phagocytosis, TLR9 immediately redistributes from the ER to CpG DNA-containing structures such as endosome. After binding with ligands, TLR9 accelerates inflammatory responses by the production of type I IFN through the myeloid differentiation primary response 88 (MyD88)–IRF7 pathway or inflammatory cytokines through the MyD88–NF–κB pathway **(Fig. 1)**[37-40].

STING has been investigated as a major cytosolic DNA sensor that recognizes bacterial and viral DNA as well as endogenous DNA, regulating innate immune response[41-44]. It is a transmembrane protein that locates in the surface of the ER. Once cGAS recognizes DNA fragments present in the cytoplasm, cGAS synthesizes cGAMP from ATP and GTP. This is an intracellular second messenger that serves as a ligand, binding to STING and subsequently activating the IRF3 and NF-κB pathways via TANK binding kinase 1 and IκB kinase, respectively. This leads to the production of type 1 IFN and cytokines **(Fig. 2)**[29, 41, 45-48].

The Role of TLR9 in Vascular Diseases

In addition to its contribution to self-defense by recognizing exogenous DNA fragments, recent studies have suggested that TLR9 has causal roles in vascular inflammation and atherogenesis. Our previous study demonstrated that the genetic deletion of TLR9 attenuates atherogenesis in angiotensin II (Ang II)-infused ApoE KO mice fed a western-type diet (WTD)[23]. The pharmacological blockade of TLR9 by iODN2088—one of the inhibitory oligodeoxynucleotides of TLR9—also attenuated atherogenesis in Ang II-infused ApoE KO mice, compared with control oligodeoxynucleotide. The genetic deletion of TLR9 and the administration of TLR9 inhibitor decreased macrophage and lipid accumulation and inflammatory

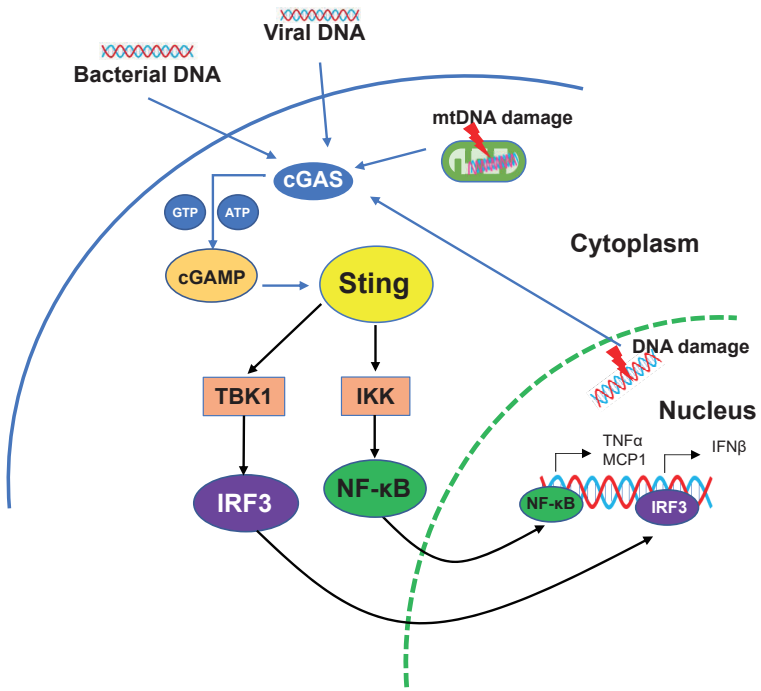

Fig. 2. STING signaling

STING recognizes cGAMP generated from DNA fragments in the cytoplasm by the action of cGAS. After binding with cGAMP, STING produces type 1 IFN and inflammatory cytokines through the activation of IRF3 and NF–κB pathways, respectively.

molecule expression in the aorta. On the other hand, the restoration of TLR9 in bone marrow (BM) in TLR9-deficient ApoE KO mice promoted atherogenesis. Similarly, we have reported that the activation of TLR9 accelerated the hyperplasia of the neointima after mechanical vascular injury in mouse femoral arteries—another type of vascular lesion—which was blocked by the administration of an anti-high mobility group box protein-1 (HMGB1) antibody[49]. In these studies, TLR9 deletion did not affect blood pressure and plasma lipid profiles. These findings indicate the proatherogenic roles of TLR9 in immune cells derived from BM (such as macrophages), at least in part. In *in vitro* studies using macrophages, we and others have shown that the activation of TLR9 by its agonists, or DNA fragments, accelerated the expression of proinflammatory molecules and the formation of foam cell[23, 49, 50]. Other studies have consistently reported the role of TLR9 in atherogenesis. Ma *et al.* showed that the inhibition of TLR9 by IRS869—an inhibitory oligodeoxynucleotide of TLR9—reduced plaque burden and attenuated proinflammatory activation of macrophages[51]. Krogmann *et al.* also demonstrated that intravenous administration of a TLR9 agonist to ApoE KO mice promoted the development of atherosclerotic lesions, partially through the impairment of re-endothelialization after acute vascular injury[52]. However, interestingly, Koulis *et al.* have reported incongruous findings. They demonstrated that the genetic deletion of TLR9 in high-fat diet (HFD)-fed ApoE KO mice enhanced the development of atherosclerotic lesion, along with increase in the accumulation of macrophages, dendritic cells, and CD4+ T cells in plaques, suggesting the antiatherogenic roles of TLR9 [53]. In line with the result of their genetic deletion experiments, they also showed a reduction of atherosclerotic lesion development in HFD-fed ApoE KO mice by the administration of CpG-ODN1668—a TLR9 agonist. Of note, they also found that TLR9 deletion elevated plasma lipid levels by an undetermined mechanism. Several studies have suggested a link between metabolic parameters and the innate immune system[54, 55], which might have affected their results. Along with the effect of TLR9 on lipid levels, a previous study mentioned conflicting roles of TLR9 activation according to the concentration of its ligand[56]. Therefore, differences in study design (such as mouse model, food, duration of study, and types of agonist or antagonist) might cause differences in ligand levels, resulting in the

discrepancies observed in previous studies. Thus, both the pro- and antiatherosclerotic roles of TLR9 have been described. Further experiments are needed to determine the effect of TLR9 in atherosclerotic diseases.

The Role of TLR9 in Metabolic Diseases

In a similar way to atherogenic vascular diseases, metabolic abnormalities, such as insulin resistance and hepatic steatosis, share sterile chronic inflammation in metabolic organs as a fundamental aspect of their pathology. The release of various endogenous ligands (including saturated fatty acids, heat-shock protein, and self-derived DNA) has been demonstrated[24, 57-59] and therefore, the role of PRRs has been extensively studied.

We previously reported the causal role of TLR9 in adipose tissue inflammation and subsequent insulin resistance in obese mice[24, 58]. To investigate the role of TLR9, we genetically deleted TLR9 in C57BL/6 mice, because we found an elevation of TLR9 expression in adipose tissue in obese C57BL/6 mice fed a HFD. The genetic deletion of TLR9 decreased the accumulation of macrophages in adipose tissue and inhibited the development of obesity-induced adipose tissue inflammation and insulin resistance. Similarly, the administration of iODN2088—an inhibitory oligonucleotide for TLR9—to HFD-fed wild-type mice attenuated inflammation in adipose tissue and improved insulin resistance, suggesting that the pharmacological blockade of TLR9 is a potential therapeutic strategy for obesity-induced insulin resistance. On the other hand, BM-specific TLR9 expression worsened insulin resistance under HFD feeding, compared with that in mice lacking TLR9. Here, DNA fragments released from obese adipocytes stimulated the proinflammatory activation of macrophages through TLR9, at least partially. These results suggest a link between TLR9 and obesity-induced insulin resistance and the potential of cfDNA–TLR9 signaling as a therapeutic target. In these studies, we found an elevation of plasma levels of cfDNA—a potential TLR9 agonist—in obese mice. Other TLR9 ligands might also contribute to TLR9 activation in obese subjects. Guzmán–Ruiz *et al.* showed that the elevated levels of HMGB1 in plasma and visceral adipose tissue correlate with the markers of adipose tissue inflammation[60]. However, another study showed that TLR9 deficiency promoted insulin resistance in HFD-fed mice, suggesting the anti-inflammatory roles of TLR9 in macrophage activation[61]. As described above, several differences in study design, including diet and duration of feeding, might account for these discrepancies. Further studies are required to establish the role of TLR9 in the pathogenesis of obesity-induced insulin resistance.

Recently, the role of TLR9 in the development of nonalcoholic fatty liver disease (NAFLD) has been reported[62, 63]. NAFLD is characterized by excessive fat accumulation in the liver without other clear reasons (such as alcohol use) and is observed with high prevalence in obesity and/or type 2 diabetes. Metabolic stresses related to fat accumulation in the liver are suggested to cause cellular damage and accelerate cell/tissue degradation, which results in the inappropriate release or accumulation of self-derived DNA, leading to the activation of TLR9 and the subsequent development of NAFLD.

The prevalence of obesity and related disorders is increasing because of changes in our lifestyles. This may direct more attention to the role of TLR9 and increase the importance of this system in our understanding of the development of metabolic diseases.

The Role of STING in Vascular Diseases

Over the past decade, STING has been investigated as a major cytosolic DNA sensor that recognizes bacterial and viral DNA, as well as endogenous DNA, and regulates innate immune response[41-44]. The role of STING in various inflammatory diseases has been reported.

Recently, Luo *et al.* reported the contribution of STING to the development of aortic aneurysm and dissection[64]. They induced aortic aneurysm formation with a combination of HFD-feeding and Ang II infusion in wild-type mice and STING-deficient (STING KO) mice. STING KO mice demonstrated a significant reduction in aortic diameter, dissection, and rupture, which suggested a causal role of STING signaling in this vascular disease. DNA damage and subsequent smooth muscle cell apoptosis, as well as proinflammatory macrophage activation induced by DNA fragments released from smooth muscle cells, is suggested as the underlying mechanism. They also showed that STING is a potential therapeutic target for this disease context by showing the inhibitory effects of a STING inhibitor—C-176. These results suggested the contribution of STING to the development of vascular inflammation.

For exploring direct evidence of the contribution of STING signaling to atherogenesis, we tried to find evidence of DNA damage in atherosclerotic lesions in WTD-fed ApoE KO mice—a commonly used hypercholesterolemia-induced atherosclerotic model—because DNA fragments released by DNA damage are

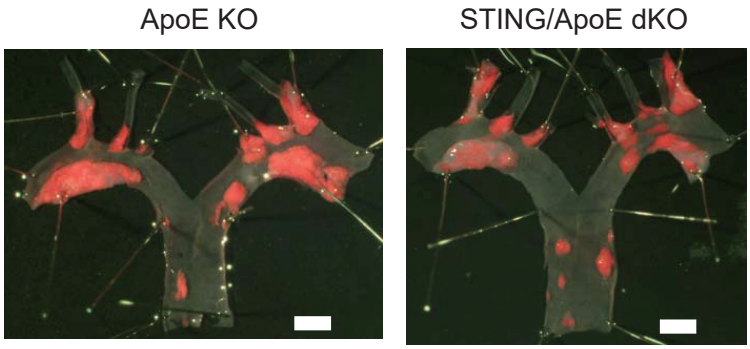

Fig. 3. Genetic deletion of STING suppressed development of atherosclerosis

Representative figures of Sudan IV staining of aortic arch. The genetic deletion of STING in ApoE KO mice on a WTD for 20 weeks decreased the development of atherosclerosis. Bar: 1 mm.

potential agonists for STING[65]. The results of western blotting and immune-electron microscopic analysis in this mouse model demonstrated the expression of DNA damage markers (such as γH2AX) and the accumulation of DNA fragments in macrophages that accumulated in atherosclerotic lesions. Furthermore, using high-sensitive liquid chromatograph–mass spectrometry (LC–MS/MS), we succeeded in showing the existence of cGAMP—a direct agonist for STING—in the atherosclerotic aorta of WTD-fed ApoE KO mice. To examine the role of STING in atherogenesis, we genetically deleted STING in ApoE KO mice fed a WTD. The genetic deletion of STING attenuated the development of atherosclerotic lesions **(Fig. 3)** along with the reduction of lipid deposition, accumulation of macrophage, and expression of inflammatory molecules. Similarly, the pharmacological blockade of STING using C-176—a specific STING inhibitor—significantly reduced atherosclerotic lesion development in the aorta and lipid deposition in atherosclerotic lesions, compared with controls. On the other hand, BM-specific expression of STING promoted atherogenesis in ApoE KO mice. These findings indicate that STING contributes to the development of vascular inflammation and atherogenesis. Furthermore, Lui *et al.* showed that the genetic deletion of IRF3—a downstream component of STING signaling—reduced the development of atherosclerotic lesion and the vulnerability of plaque in ApoE KO mice[66]. Likewise, daily injection of IFN-β—a downstream molecule of STING signaling—accelerated atherosclerotic plaque and the accumulation of macrophage in ApoE KO and low-density lipoprotein receptor-deficient mice[67]. The results of our study also showed a reduction of IFN-β in the aorta of ApoE KO mice lacking STING[65]. We further demonstrated that cGAMP or mtDNA promoted the expression of IFN-β and other cytokines, such as TNF-α, in both mouse and human macrophages[65]. These studies suggest that the activation of STING signaling promotes vascular inflammation and atherogenesis, at least in mouse models, and that STING signaling can be a potential therapeutic target for vascular inflammation.

There is some evidence supporting the role of STING in human vascular diseases. In a recent study, we revealed the expression of STING in atherosclerotic lesions in the carotid artery (which had been obtained by carotid endarterectomy) at the RNA and protein levels. Furthermore, we detected the STING ligand cGAMP using high-sensitivity LC–MS/MS. STING and cGAMP expression was significantly higher in atherosclerotic lesions, compared with those of macroscopically normal carotid arteries obtained from a tissue bank[65]. Furthermore, several studies have demonstrated that the gain-of-function mutation in STING is associated with vasculopathy observed in STING-associated vasculopathy with onset in infancy (SAVI)—a rare autoinflammatory disease[68-70]. The transcription of IFN-β in peripheral blood mononuclear cells in patients was increased compared with that of donors who did not have mutations, with or without cGAMP simulation[69]. A recent study by Hamann *et al.* demonstrated the protective effect of single-nucleotide polymorphism R293Q on STING in obesity-associated cardiovascular disease[71]. Although evidence regarding the cGAS–cGAMP–STING axis related to cardiovascular diseases is still limited, these studies suggest that STING signaling contributes to the pathogenesis of vascular inflammation in humans, at least partially.

The Role of STING in Metabolic Diseases

The expression of upstream/downstream molecules in STING signaling is upregulated in obese mouse models[72-74]. For example, HFD-induced mtDNA release in adipose tissue activated the cGAS–cGAMP–STING pathway, leading to increased chronic sterile inflammatory response and the development of insulin resistance[74, 75]. STING signaling was also activated in pancreatic β-cells of db/db mice, suggesting the involvement of this signaling pathway in the pathophysiology of lipotoxic injury of pancreatic β-cells in type 2 diabetes[76]. Similar to TLR9, the role of STING in the pathogenesis of NAFLD has also been reported[77-79]. Liver injury caused by excessive fat accumulation generates mtDNA dysfunction and mtDNA damage[80] that functions as a STING agonist to produce type I IFN[77, 78], which plays an important role in oxidative stress and inflammation in the development of hepatic diseases[81]. The deletion of STING in wild-type mice attenuated the development of nonalcoholic steatohepatitis and insulin resistance[77, 78]. The promotion of TNF-α and Interleukin-6 expression in Kupffer cells by mtDNA released from hepatocytes (which were inhibited by a NF-κB inhibitor) is suggested to be an underlying mechanism[78]. Furthermore, some clinical studies have suggested that the release of mtDNA, the correlation between STING expression in macrophages, and the development of liver fibrosis and inflammation are disease mechanisms in patients with NAFLD[79, 82]. These findings suggest a role of STING in the pathophysiology of metabolic diseases, such as insulin resistance and hepatic diseases. Further studies are required to elucidate the role of STING signaling in the pathogenesis of metabolic diseases.

Future Prospective

The recognition of foreign DNA is one of the most fundamental functions of the innate immune system, which is a first line of self-defense[40]. Thus, DNA-sensing mechanisms generally have a protective role, although this system also promotes inflammation against endogenous DNA fragments inappropriately in certain disease contexts. The initiating stimuli and machinery that causes the unwanted activation of inflammation to host-derived DNA by the systems that ordinarily handle the immune response remain unidentified. However, it is clear that lifestyle-related risk factors, such as obesity, diabetes, and hyperlipidemia, promote a destructive tissue environment that locally and/or systemically increases DAMP levels, for example, due to greater oxidative stress. Therefore, controlling these risk factors by clinical intervention and promoting a healthy lifestyle is essential to regulate inflammation through DNA-sensing mechanisms. Several animal studies suggest that pharmacological inhibitors for TLR9 and STING attenuate the development of atherosclerosis, insulin resistance, and NAFLD. Again, these DNA-sensing mechanisms are indispensable for life, as a self-defense system against pathogens. Therefore, we need to be careful when targeting them. However, exploring safer methods that can control these signaling pathways, especially locally and/or at a certain time phase of the disease process, would provide new therapeutic strategies for vascular and metabolic diseases caused by lifestyle-associated diseases. Regardless of accumulating evidence, there is still limited knowledge about the role of DNA-sensing receptors in the development of cardiometabolic disorders and their activating mechanisms. Further studies are required to establish therapeutic strategies—against atherosclerotic vascular and metabolic diseases—that target DNA sensors.

Conclusion

Chronic sterile inflammation caused by lifestyle-associated diseases is a central feature of the pathogenesis of atherosclerosis and insulin resistance, in which various cellular and molecular mechanisms are involved. Accumulating evidence indicates that DNA-sensing machinery participates in the pathogenesis of chronic sterile inflammatory diseases. This review has focused on the role of DNA sensors, such as TLR9 and STING, which normally contribute to self-defense against pathogens, in the proinflammatory activation of immune cells and the pathogenesis of vascular and metabolic diseases **(Fig. 4)**. This immune system is indispensable for survival; however, it also causes unwanted inflammation in certain conditions, leading to the development of various inflammatory diseases. Recent changes in lifestyle have dramatically increased the prevalence and incidence of metabolic disorders associated with obesity and nutritional excess. This change may have induced a shift from what are normally favorable physiological processes to become pathological events. In summary, DNA sensors, such as TLR9 and STING, contribute to the pathogenesis of vascular and metabolic diseases. These pathways might be potential therapeutic targets and possible biomarkers for this health threat. Further studies of these inflammatory mechanisms are required to define possible clinical applications.

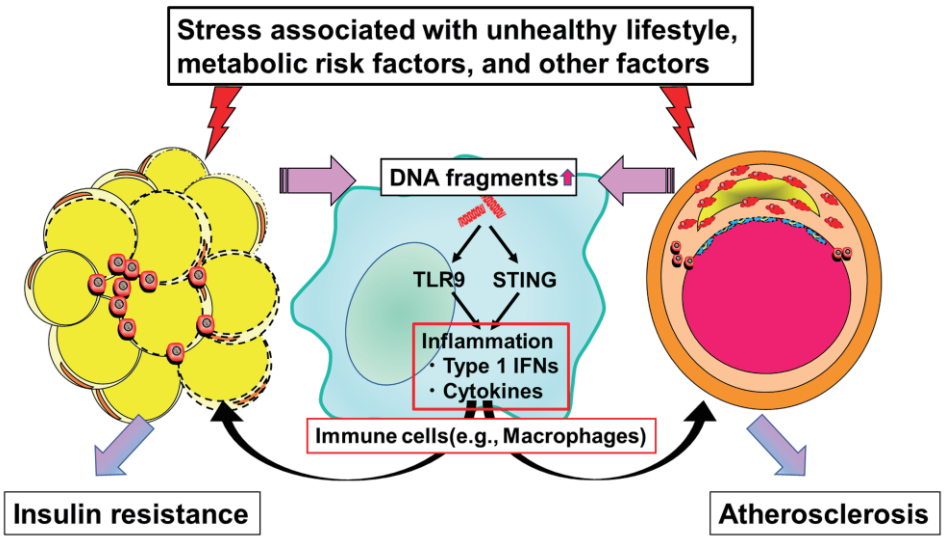

Fig. 4. Role of DNA sensors in development of vascular and metabolic diseases

DNA fragments released from damaged cells/tissues activate the innate immune systems, such as TLR9 and STING, promoting inflammation in vascular and metabolic organs, which contribute to the pathogenesis of cardiometabolic disorders.

Acknowledgements

All authors are grateful to E. Uematsu and S. Okamoto (Tokushima University) for their expert technical assistance.

Sources of Funding

This work was partially supported by JSPS Kakenhi Grants (Number 19K08584 to D.F., Number 19H03654 to M.S.), Bristol-Myers Squibb Research Grants (D.F.), The Uehara Memorial Foundation (D.F.), Takeda Science Foundation (M.S.), and the Vehicle Racing Commemorative Foundation (M.S.). M.S. also received clinical research funding from Bayer Yakuhin, Daiichi Sankyo Co. Ltd., Boehringer Ingelheim Japan, and Suntory Wellness Ltd. and scholarship grants from Takeda Pharmaceutical Co. Ltd., Mitsubishi Tanabe Pharma Co., and Astellas Pharma Inc. The funders had no role in the study design, data collection and analysis, or preparation of the manuscript.

Disclosures

D.F. received lecture fee from Bayer Yakuhin. M.S. received lecture fee from Takeda Pharmaceutical Co. Ltd., Mitsubishi Tanabe Pharma Co., Bayer Yakuhin, Boehringer Ingelheim Japan, Astellas Pharma Inc., Amgen-Astellas BioPharma, Bristol-Myers Squibb, Daiichi Sankyo Co. Ltd., MSD, K.K.

The other authors declare no conflicts of interest.

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