NEW HYPOTHESES IN CLINICAL MEDICINE

Trained Innate Immunity as a Novel Mechanism Linking Infection and the Development of Atherosclerosis

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***Running title*:** Infections, Trained Immunity, and Atherosclerosis

Subject Terms:
Atherosclerosis
Etiology
Inflammation
Mechanisms
Pathophysiology

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In December 2017, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.60 days.

ABSTRACT

Rationale: There is strong epidemiological evidence for an association between acute and chronic infections and the occurrence of atherosclerotic cardiovascular disease (ASCVD). The underlying pathophysiological mechanisms remain unclear. Monocyte-derived macrophages are the most abundant immune cells in atherosclerotic plaques. It has recently been established that monocytes/macrophages can develop a long-lasting pro-inflammatory phenotype after brief stimulation with micro-organisms or microbial products, which has been termed trained immunity.

***Objective*:** To assess whether trained immunity mediates the link between infections and ASCVD.

Methods and Results: Brief exposure of monocytes to various micro-organisms results in the development of macrophages with a persistent pro-inflammatory phenotype: this represents a *de-facto* nonspecific innate immune memory which has been termed trained immunity. This is mediated by epigenetic reprogramming at the level of histone methylation, and a profound rewiring of intracellular metabolism. Although this mechanism offers powerful protection against reinfection, trained macrophages display an atherogenic phenotype in terms of cytokine production and foam cell formation. Trained monocytes are present up to three months following experimental infection in humans. Moreover, a trained immunity phenotype is present in patients with established atherosclerosis.

***Conclusion*:** We propose that trained immunity provides the missing mechanistic link that explains the association between infections and atherosclerosis. Therefore, pharmacological modulation of trained immunity has the potential to prevent infection-related ASCVD in the future.

Keywords:
Infection, trained immunity, innate immune memory, atherosclerosis. immunology, atherogenesis, monocyte.

Nonstandard Abbreviations and Acronyms:

ASCVD	Atherosclerotic cardiovascular disease
BCG	Bacille Calmette-Guérin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBV	Hepatitis B virus
HSPC	Hematopoietic stem and progenitor cell
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IMT	Intima media thickness
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein 1
MMP	Matrix metalloproteinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor

INTRODUCTION

It is often advocated that if we could resist all temptations of modern life (e.g. smoking, fast-food, sedentary lifestyle), clinical manifestations of atherosclerosis could be avoided. However, abundant signs of atherosclerosis are present in ancient mummies from four different geographical regions[1]. Despite their lifestyles being characterized by healthy diets, non-smoking, and abundant physical activity, arterial calcifications occurred at the same locations as in modern day human beings with a similar radiographic appearance on CT-scan. These findings suggest that determinants other than traditional cardiovascular risk factors also play a role in the development of atherosclerosis.

As infectious disease has been a leading cause of morbidity and mortality since ancient times, a connection between the infectious burden and atherosclerosis has been suggested[1]. Indeed, many epidemiological studies in humans and studies in animal models suggest that the infectious burden is associated with later atherosclerotic cardiovascular disease (ASCVD) and that acute infections can trigger the occurrence of cardiovascular events[2, 3]. Although various mechanism have been proposed[2], a unifying pathophysiological explanation of this associations is still lacking. In this *New Hypothesis in Clinical Medicine* paper, we introduce the novel hypothesis that innate immune memory, which is termed *trained immunity*[4], mediates the effects of infections on ASCVD. We first summarize current evidence that infections are linked to ASCVD and then introduce the mechanism of trained innate immunity. We outline preliminary evidence that this mechanism mediates, at least in part, this association, and discuss the potential clinical implications.

Acute and chronic infections are associated with atherosclerosis.

In epidemiological studies, a large number of different micro-organisms have been linked with an increased risk for ASCVD[2, 5]. This association is predominantly based on serological positivity against specific pathogens, particularly *Chlamydophilia pneumoniae*, *Helicobacter pylori*, as well as viral pathogens, including human immunodeficiency virus, and cytomegalovirus. The *infectious burden concept* postulates that the cumulative infectious burden, rather than exposure to specific pathogens, is associated with the future development of ASCVD[6]. Also, chronic infections with specific micro-organisms have been linked to ASCVD, including CMV[7] and HIV[8].

In addition, there is increasing epidemiological evidence that acute infections such as pneumonia or influenza are temporally associated with a increased incidence of acute cardiovascular events, with a four-fold increased risk in the first 30 days following admission for community acquired pneumonia[9] . Interestingly, ASCVD risk remains elevated for 10 years following hospitalization with pneumonia[9].

A key concept is that although clinically manifest in adulthood, the development of atherosclerosis begins in childhood when the infection burden is greatest[10]. Recent evidence suggests that childhood infections may also accelerate the process of atherosclerosis. In a population-wide data linkage study, hospitalization with infection in childhood was associated with ASCVD events in adulthood[11]. Childhood infections are also associated with sub-clinical markers of atherosclerosis. In the prospective *Cardiovascular Risk in Young Finns Study*, infection-related hospitalization in early childhood was associated with impaired endothelial function at a mean age of 22 years[12]. Perinatal exposure to the maternal microbiome may be associated with changes to arterial structure; in the prospective *Barwon Infant Study*, maternal pet ownership/livestock exposure or colonization with group B streptococcus in pregnancy, both of which are associated with differences in the maternal enteric microbiome, were associated with differences in aortic intima-media thickness (IMT) at six weeks of age. The associations were only observed in vaginally born infants, but not in those born by cesarean section, implicating early microbial exposure

from the maternal microbiome as a possible determinant of the infant intermediate vascular phenotype[13]. The long-term implications of increased aortic IMT in infancy are unknown and longitudinal studies are ongoing.

Several hypotheses have been put forward regarding the mechanisms that link infections to atherogenesis[2], including direct effects of micro-organisms in the atherosclerotic plaque with local activation of endothelial cells and macrophages. Regardless of the presence of pathogens within atherosclerotic plaques, infections also induce a systemic inflammatory response and activation of the immune system. Molecular mimicry between microbial and host factors has also been suggested as causal mechanism. An important potential confounder in the association between infections and atherosclerosis could be the impact of antibiotics on the gut microbiome, since this could also regulate the development of atherosclerosis[14]. Furthermore, underlying genetic predisposition could play a role in both susceptibility to infections and atherogenesis.

Many trials have sought to prevent infection-related ASCVD with antibiotics, but the majority fail to show any effect[2]. This could be explained by an incomplete eradication of pathogens, but also by pathogenic (immunological) processes that are set in motion by the triggering micro-organism that continue unabated after removal of the pathogen. Understanding these pathogenic immunological mechanisms is a key knowledge gap. Here we propose a role for innate immune memory, also termed *trained immunity*, as a driving mechanism of the association between infection, atherosclerosis and ASCVD.

The innate immune system can build immunological memory: trained immunity.

Monocytes and monocyte-derived macrophages are key players in atherosclerosis development[15]. Following activation of endothelial cells by turbulent local laminar blood flow and by systemic triggers such as smoking and dyslipoproteinemia, monocytes bind to endothelial cells and enter the intimal space. Macrophages contribute to plaque formation and progression by the production of cytokines and chemokines, and foam cell formation, and they can trigger plaque destabilization and rupture by expression of proteases, such as matrix metalloproteinases (MMPs) [15].

Until recently, it was generally assumed that, in contrast to cells of the adaptive immune system, monocytes and macrophages do not have capacity for immunological memory, mounting an identical naïve response each time they are stimulated. However, recent studies have demonstrated that the innate immune system can adopt a long-term activated phenotype by previous encounters with various microbiological products (Figure 1). This non-specific immunological memory has been described as *trained innate immunity*[4]. In isolated human monocytes, brief exposure to *Bacille Calmette-Guérin* (BCG), *Candida albicans* or its cell wall component β-glucan leads to a long-lasting proinflammatory phenotype characterized by increased production of pro-inflammatory cytokines upon restimulation with Toll-like receptor (TLR) agonists 6 days after the initial exposure[16, 17]. In addition, a low concentration of lipopolysaccharide (LPS) also induces a trained immune phenotype, in contrast to a high dose of LPS, which induces immune tolerance[18]. Interestingly trained immunity can also be induced in vitro by brief exposure of human primary monocytes to endogenous pro-atherogenic substances, such as oxidized low-density lipoprotein (LDL) and lipoprotein (a)[19].

Trained immunity is mediated by metabolic rewiring and epigenetic reprogramming and occurs at the level of bone marrow progenitors.

In a recent series of in vitro experiments, we reported that trained immunity is caused by epigenetic reprogramming at the level of histone methylation and acetylation[20]. The trained macrophage phenotype, by brief exposure to β-glucan, BCG or oxLDL is characterized by an enrichment of the activating histone

modifications H3K4me3 and H3K4me1, and the trained phenotype is prevented by co-administration of pharmacological inhibitors of histone methyltransferases[20, 21].

The epigenetic reprogramming of trained monocytes is driven, at least in part, by a profound rewiring of intracellular metabolic pathways (reviewed in[22]). First, a switch from oxidative phosphorylation to increased aerobic glycolysis is essential for the development of the trained phenotype[17]. Furthermore, increased glutaminolysis and subsequent accumulation of fumarate occurs, which can impact on histone methylation by inhibition of the histone demethylase KDM5[23]. Finally, β-glucan-induced trained immunity critically depends on the intracellular accumulation of mevalonate and the subsequent activation of the insulin-like growth factor1 (IGF1)-receptor[24].

Interestingly, although augmented cytokine production appears to be a general hallmark of the trained phenotype, the accompanying changes in the intracellular metabolism might differ depending on the training stimulus. For example, β-glucan-induced trained monocytes are characterized by a shift from oxidative phosphorylation to aerobic glycolysis (ie the Warburg effect)[17], whereas BCG-induced trained cells show an pan-activation of these metabolic pathways[25].

Notably, the observation that trained circulating monocytes are present several months after BCG vaccination strongly suggest functional reprogramming of bone marrow progenitors[16]. Indeed, in mice, the administration of β-glucan induces long-term transcriptional and metabolic changes of hematopoietic stem and progenitor cells (HSPCs) resulting in their expansion and bias towards myelopoiesis which results in a more favorable response to a secondary LPS challenge and protection from chemotherapy-induced myelosuppression[26]. This long-term reprogramming is associated with increased surface expression of CD131, the common β-subunit of the interleukin-3/granulocyte-macrophage colony-stimulating factor (IL-3/GM-CSF) receptor. Interestingly, this similar mechanism is responsible for the myeloid expansion and increased inflammation in the setting of hypercholesterolemia in atherosclerosis-prone mice[27], suggesting a potential role for trained immunity in the context of traditional cardiovascular risk factors. Indeed, a comparable reprogramming of HSPCs occurs in response to a Western type diet in atherosclerosis-prone *Ldlr-/-* mice. Christ *et al* have reported that a Western type diet for four weeks induces systemic inflammation in *Ldlr*-/- mice and a profound transcriptional and epigenetic reprogramming of circulating monocytes and bone marrow progenitors. Importantly, the innate immune reprogramming persisted even after switching to a chow diet for four weeks[28].

Trained immunity protects against reinfections but may accelerate atherosclerosis formation.

In the context of recurrent infections, trained immunity provides robust protection against reinfection and improves mortality in animal models of sepsis[16, 29]. Administration of a low dose of *Candida albicans* to mice confers protection against a subsequent exposure to a lethal dose of *Candida*[29]. Similarly, vaccination with BCG profoundly lowers mortality when the mice were exposed two weeks later with a lethal dose of *Candida*[16]. These protective effects were retained in mice models with disrupted adaptive immunity. Based on the phenotype of trained macrophages, and the pivotal role of macrophages in atherogenesis, we recently proposed that the persistent state of heightened innate immune cell activation in trained immunity, albeit beneficial in the context of recurrent infections, contributes to progression of atherosclerosis development and to acute destabilization of existing atherosclerotic plaques[30].

In vitro*,* extensive phenotyping of trained macrophages indeed reveals an atherogenic phenotype, characterized by increased production of pro-atherogenic cytokines and chemokines, including tumor necrosis factor-α (TNF α), interleukin (IL) 6 (IL6), monocyte chemotactic protein 1 (MCP1), and MMP2 and 9, and increased foam cell formation[21]. The effect of training on other relevant functions of monocytes, such as vascular patrolling, endothelial adhesion and transmigration, apoptosis, and efferocytosis is currently unknown. Moreover, based on the micro-organism-specific immunometabolic profile of trained

monocytes, the effect of these cells on the vascular wall might depend on the specific micro-organism involved.

Moreover, in vivo, in *ApoE*-/- mice, the administration of an ultra low dose of LPS for 4 weeks substantially aggravated atherosclerosis development, which was associated with a pro-inflammatory reprogramming of circulating monocytes. Adaptive transfer of these monocytes to non LPS-treated animals also accelerated atherosclerosis formation[31].

We hypothesized that BCG vaccination would accelerate atherosclerosis by trained immunity, but in a recent study, this was associated with a reduced atherosclerotic plaque area in ApoE*3-Leiden.CETP mice. However, this study was confounded by a profound reduction in cholesterol levels due to ongoing disseminated chronic infection by BCG[32]. In previous studies in which plasma cholesterol levels were monitored and kept unchanged, BCG vaccination did aggravate atherosclerosis formation[33]. Further animal studies that better mimic the timing, dose and administration of BCG vaccination are warranted. A large Danish case-cohort study found that BCG vaccination was associated with a reduced incidence of cardiovascular diseases[34]. Although apparently in contradiction with the concept of trained immunity, this could well be explained by a reduction in subsequent infections by BCG, which, by themselves, could have induced trained immunity to increase ASCVD.

Trained immunity occurs in humans in vivo.

Recent studies have been able to translate the concept of trained immunity to the human in vivo situation. Three months after vaccination of healthy subjects with BCG, circulating monocytes display an increased cytokine production capacity in response to stimulation with various microorganisms ex vivo[16]. *In utero* exposure to hepatitis B virus (HBV) in HBV infected mothers triggers a state of trained immunity in the newborns, which is characterized by an increased production of pro-inflammatory cytokines when cord blood mononuclear cells are exposed to various unrelated bacteria ex vivo[35].

A similar trained immune phenotype has also been observed in circulating monocytes from patients with risk factors for atherosclerosis or established atherosclerosis. Circulating monocytes from patients with isolated elevated plasma levels of lipoprotein (a) (an independent risk factor for ASCVD) showed an enhanced ex vivo cytokine production capacity and an increased endothelial cell adhesion and migration, which is consistent with a trained immune phenotype[19]. Similarly, cytokine production capacity was increased in patients with severe established coronary atherosclerosis[36]. This was associated with an upregulation of glycolytic enzymes and a reprogramming at the level of histone methylation which is also consistent with a trained immune phenotype.

Future clinical implication.

Multiple levels of experimental evidence have now supported the concept that exposure of the innate immune system to a variety of micro-organisms triggers a prolonged state of hyperactivation, which has been termed trained immunity. Trained monocytes and macrophages display a profound pro-atherogenic phenotype, with increased production of pro-atherogenic cytokines/chemokines and increased foam cell formation. This is mediated by a metabolic rewiring and epigenetic reprogramming at the level of histone methylation. These processes occur not only in circulating monocytes, but also in bone marrow progenitors cells, which ensures a prolonged state of innate immune cell hyperactivation.

Although it has been clearly established that micro-organisms can induce trained immunity in humans in vivo and that monocytes from patients with established atherosclerosis are characterized by a trained immunity phenotype, it is now key to investigate whether this mechanism indeed drives the increased ASCVD risk associated with infections. Support of this should come from studies in mice directly

showing that deficiency of trained immunity pathways prevents infection-associated atherosclerosis development.

Of note, in vitro, pharmacological inhibitors of glycolysis, glutaminolysis, and the mevalonate pathway, as well as pharmacological blockers of histone methyltransferases, prevent trained immunity. These effects have been confirmed in mouse models in vivo in which pharmacological inhibition of glutaminolysis and of the mevalonate synthesis pathway ameliorates the induction of trained immunity by intraperitoneal administration of β-glucan[23]. This knowledge would allow the development of novel pharmacological approaches to reduce ASCVD risk in high-risk individuals that encounter acute infections, such as pneumonia, and also the possibility of reducing the potential deleterious effects of childhood infections on later ASCVD risk. Hence, confirmation that trained immunity links infections to the development of ASCVD and further elucidation of the mechanism of trained immunity will offer exciting novel possibilities for the development of pharmacological strategies to prevent ASCVD.

SOURCES OF FUNDING
This study was supported by a European Union's Horizon 2020 research and innovation grant (REPROGRAM; No 66837) and a CVON grant (IN-CONTROL) to NPR, LAB, and MGN. MGN was supported by an ERC Consolidator Grant (#310372) and a Spinoza Grant of the Netherlands Organization for Scientific Research.

DISCLOSURES
None.

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FIGURE LEGEND

Figure 1. Schematic representation of the development of trained immunity in human monocytes. Brief exposure of monocytes to various micro-organisms of micro-organism derived stimuli (right upper corner) or to endogenous pro-atherogenic stimuli (oxLDL and Lp(a)) triggers an intracellular signaling cascade that involves activation of glycolysis and mevalonate synthesis. Subsequently, intermediate metabolites of these pathways regulate gene transcription by modulating histone methylation and acetylation. The trained immune phenotype is characterized by an increased production of pro-atherogenoc cytokines, chemokines, and proteases, and increased foam cell formation.

NOVELTY AND SIGNIFICANCE

What Is Known?

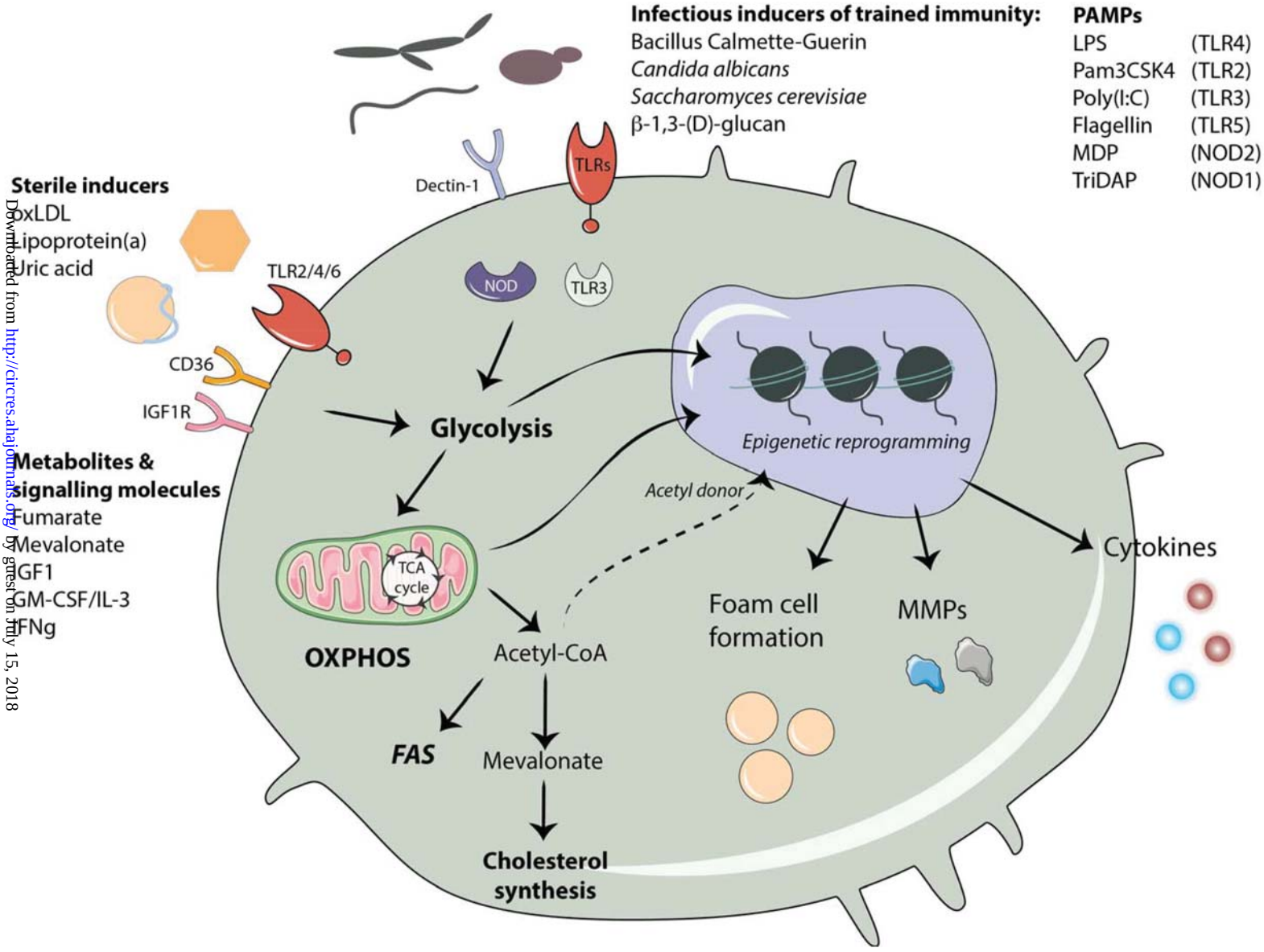
- Infections are associated with an increased risk for atherosclerotic cardiovascular disease (ASCVD).
- Monocytes/macrophages regulate atherosclerotic plaque formation.
- Monocytes/macrophages can build a nonspecific immunological memory after stimulation with micro-organisms, which is termed *trained immunity.*

What New Information Does This Article Contribute?

- Trained monocytes/macrophages display a pro-atherogenic phenotype.
- Trained immunity may be responsible for infection-related ASCVD.
- This offers novel pharmacological targets to prevent ASCVD.

There is a strong epidemiological link between some forms of infections and the occurrence of ASCVD. However, the underlying mechanism is unknown and antibiotic treatment does not limit ASCVD development. Monocyte-derived macrophages are major regulators atherosclerosis. We have recently reported that brief exposure to micro-organisms can induce a persistent nonspecific immunological memory in monocytes/macrophages, named *trained immunity*. This is mediated by changes in the intracellular metabolism and epigenetic landscape and occurs at the level of bone marrow progenitors. Trained monocytes/macrophages display a pro-atherogenic phenotype and we now hypothesize that trained immunity is a key mechanism that links infections to ASCVD and suggest future studies necessary to confirm this hypothesis. This could provide exciting novel pharmacological targets that can be used to prevent infection-related ASCVD.

FIGURE 1



Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. published online January 24, 2018;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571