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Trained Immunity and Reactivity of Macrophages and Endothelial Cells

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Abstract

Innate immune cells can develop exacerbated immunological response and long-term inflammatory phenotype following brief exposure to endogenous or exogenous insults, which leads to an altered response towards a second challenge after the return to a non-activated state. This phenomenon is known as trained immunity (TI). TI is not only important for host defense and vaccine response, but also for chronic inflammations such as cardiovascular and metabolic diseases such as atherosclerosis. TI can occur in innate immune cells such as monocytes/macrophages, NK cells, ECs and non-immune cells such as fibroblast. In this brief review, we analyze the significance of TI in endothelial cells (ECs), which are also considered as innate immune cells in addition to macrophages. TI can be induced by a variety of stimuli including LPS, BCG, and oxLDL, which are defined as risk factors for cardiovascular and metabolic diseases. Furthermore, TI in ECs is functional for inflammation effectiveness and transition to chronic inflammation. Rewiring of cellular metabolism of the trained cells takes place during induction of TI, including increased glycolysis, glutaminolysis, increased accumulation of TCA cycle metabolites and acetyl-CoA production, as well as increased mevalonate synthesis. Subsequently, this leads to epigenetic remodeling, resulting in important changes in chromatin architecture that enables increased gene transcription and enhanced pro-inflammatory immune response. However, TI pathways and inflammatory pathways are separated to ensure memory stays when inflammation undergoes resolution. Additionally, reactive oxygen species (ROS) play context-dependent roles in TI. Therefore, TI plays significant roles in endothelial cell and macrophage pathology and chronic inflammation. However, further characterization of TI in ECs and

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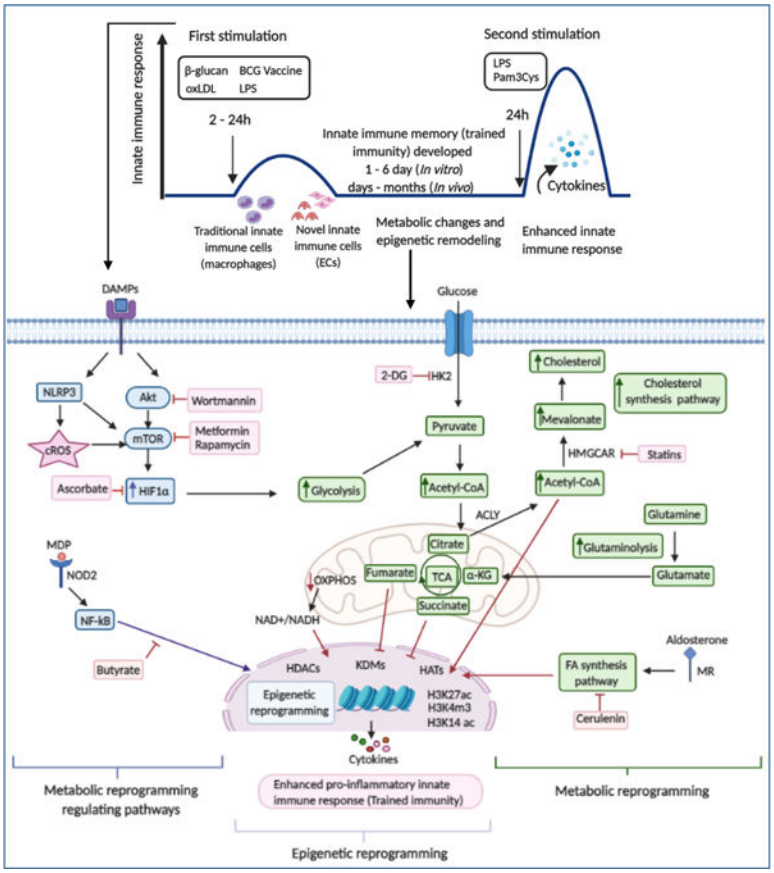
None.

macrophages would provide novel insights into cardiovascular disease pathogenesis and new therapeutic targets.

Graphical Abstract



1. Trained immunity inducers are risk factors for cardiovascular diseases and other chronic metabolic diseases

Cardiovascular disease (CVD) is the leading cause of death worldwide. Sub-endothelial retention of oxidized plasma lipoproteins triggers the recruitment of monocytes, macrophages, T cells, and other immune cells into arteries, suggesting that innate and adaptive immunity contribute to form atherosclerotic plaques[1]. Dogmatic descriptions of the immune system characterize the innate immune system with monocytes, macrophages, dendritic cells, and endothelial cells (ECs) as the rapid, non-specific branch and the adaptive immune system with T cells and B cells as the delayed but specific arm that coordinates the immune response and provides “memory” of the encounter in the form of memory T cells (Tm)[2] for a future exposure to the same pathogen. While this paradigm was mostly accurate, recent discoveries have provided evidence that the innate immune system also has “memory” of past infections/non-infectious stimuli that inform future enhanced immunological responses. Whereas the memory of the adaptive immune system provides

specific defenses against a particular pathogen encoded in the genome of a specialized Tm cell type, innate immune memory in all the innate immune cells provides a more robust but non-specific immune response to a future unrelated immunological threat, which is codified within the host's epigenome[3]. As innate immune memory is a relatively newly described phenomenon in humans, many terms have been used to describe the process[4].

Innate immune memory or trained immunity (TI)[5] can be defined as an initial immune response modifying the immune response to a future exposure of an unrelated pathogen (Figures 1A and 1B). This initial "priming" response can either position the innate immune system for an elevated immune response (trained potentiation with increased pro-inflammatory cytokine generation) or a suppressed immune response (trained tolerance)[5]. These modified immune responses can exist for days to months[6, 7]. TI can be induced by different stimuli including *Bacillus Calmette-Guerin* (BCG), β-glucan, oxidized low-density lipoprotein (oxLDL), Lipopolysaccharides (LPS), and others[8–10] (Table 1A)

TI has not only been identified in myeloid precursors, macrophages and circulating monocytes[11, 12], but also in other innate immune and non-immune cells. For example, TI properties have been demonstrated in vascular smooth muscle cells[13], ECs[14, 15], primed adipose mesenchymal stem cells[16], and bone marrow-derived hematopoietic stem cells (HSC)[17] contributing to both human auto-inflammatory diseases such as hyper-immunoglobulin D (IgD) syndrome (HIDS)[18] and sterile inflammatory diseases such as atherosclerosis[11, 19]. These studies have demonstrated that sterile lipid inflammatory mediators prime myeloid precursor cells in the bone marrow. However, what features of sterile lipid inflammatory mediators have as one of the CVD risk factors identified that are similar to infectious agents in eliciting innate immune responses in the cardiovascular system? As we pointed out recently, the common properties of identified CVD risk factors are to elicit innate immune memory (trained immunity) of the cardiovascular system so that the innate immune responses to the risk factor(s) or other non-specific stimuli can be amplified and long lasting[20].

2. Trained immunity in endothelial cells is functional for inflammation effectiveness and transition to chronic inflammation

We previously argued that ECs are conditional innate immune cells[14, 21]. ECs function to regulate vascular permeability, metabolite and waste exchange, and immune cell extravasation into tissue[22–25]. ECs also express many of the same pattern recognition receptors and cytokines as other profession immune cells such as macrophages[24, 26]. Importantly, ECs have an immune response modulation (promotion or suppression) functions on both the innate and adaptive immune systems by expressing T cell co-stimulation/immune checkpoint receptors[27–29]. Proper EC function is so critical that EC dysfunction is the first step towards the inflammatory cascade that leads to the development of CVD[22]. EC detection of hyperlipidemia, hyperglycemia, and hyperhomocysteinemia metabolic danger-associated molecular patterns (DAMPs) leads to the expression of cytokines and adhesion molecules that initiate the recruitment of monocytes and T cells into major arteries leading to atherosclerosis[30–37]. However, two important questions remain: *1)*

whether DAMPs-sensing capacities are functionally linked to TI in ECs? And *2)* whether pro-atherogenic stimuli make ECs undergo metabolic and epigenetic reprogramming, which leads to TI?

TI has also been characterized in ECs. One such study illustrates that how innate immune potentiation in EC uses the same atherogenic metabolic DAMP[38], oxLDL, that elicits innate immune training, similar to that in monocytes, oxLDL-mediated immunologic memory in ECs[15]. In this study, they demonstrate that oxLDL promotes an increased innate immune cell phenotype (increased cytokine production, increased adhesion molecule expression), switch from oxidative phosphorylation (OXPHOS) to glycolysis via metabolic reprogramming and epigenetic modifications downstream protein kinase B (Akt)-mammalian target of rapamycin (mTOR) signaling. Other studies have identified oxLDL-mTOR signaling in monocytes, and reported that shear stress triggers hypoxia-inducible factor 1-α (HIF1-α) in ECs[9, 39–41]. Another study characterizes the effects of bacterial endotoxin LPS induced-immune tolerance in human umbilical vein ECs (HUVECs) and the potential use of monophosphoryl lipid A (MPLA), a compound similar in structure to LPS, to induce trained tolerance as a potential therapeutics to suppress innate immune inflammation[42–44]. These results suggest that ECs function as conditional innate immune cells; and that they are the gatekeepers and make decision on trained immunity or trained tolerance that control the progression towards atherosclerosis.

EC are highly heterogeneous despite arising from a common progenitor[45]. This heterogeneity is reflected by distinctive patterns of inflammation-induced changes in leukocyte recruitment and protein expression between different ECs[46, 47]. Certain vascular beds including arteries and veins in heart, kidney and lung are more susceptible to the development of inflammation[48]. For example, EC in certain location in the arterial tree as the aortic arch and carotid arteries are particularly susceptible to atherosclerosis (atherosclerotic prone regions) whereas others such as the distal internal carotid and upper extremity arteries, are usually spared[41, 49]. Indeed, ECs lining those arteries in the atherosclerotic prone regions characterized by hyper-inflammatory state and probably upregulation of the TI pathways. Furthermore, microvascular ECs inflammation and immune response contributes to hypertension[50–52], presumably via TI pathways. Additionally, a growing body of evidence suggests a role for consideration of ECs activation and inflammation as a major contributor to the pathophysiology of venous thromboembolism[53, 54]. Therefore, the key event in the initiation of venous thromboembolism formation is most likely vein wall inflammation and studies showed that probable association between venous thromboembolism and several other markers of inflammation such as IL-6, MCP1, IL-8, and tumor necrosis factor-alpha exists[55–57]. Moreover, microvascular ECs inflammation, hypertension, and thromboembolism have been shown to be associated with COVID-19[58, 59] presumably also via TI pathways. Why do ECs need to have TI function? ECs have TI function to achieve the following purposes: *1)* making EC sensing of DAMPs more effective; *2)* guiding trans-EC migration of monocytes, T cells and other immune cells to the atherogenesis-prone regions of major arteries such aortic sinus in TI-enhanced manners; *3)* differentiating CVD risk factors (inducing TI pathways) from non-inflammatory stimuli (not inducing TI pathways), for example, all the 70,926 compounds identified in the foods (https://foodb.ca/compounds) that have potential

to get into human blood circulation[20]; and *4)* making inflammation triggered by CVD risk factors become chronic. This conclusion opens exciting new possibilities in targeting EC trained immunity for the treatment of not only CVD, but also for the treatment of other metabolic inflammatory diseases.

3. Reprogramming of bioenergetic metabolic pathways in trained immunity generates compounds for epigenetic memory

The five classical signs of inflammation are heat, pain, redness, swelling and loss of function, in which ECs play essential roles. The processes have to be highly effective in secreting cytokines, recruiting innate immune cells, phagocytosis, cell locomotion, and killing of pathogens or cells, all of which require high energy via bioenergetic metabolic reprogramming[4] selected from 2,847 metabolic pathways (https://metacyc.org/)[14]. To provide the energy needed to function properly, the metabolic processes of the innate immune cells are regulated precisely. Trained immunity is intrinsically linked to changes in cellular bioenergetic metabolism. These metabolic pathways include the glycolytic pathway, tricarboxylic acid cycle (TCA) cycle, acetyl-coenzyme A (acetyl-CoA) generation, and mevalonate pathway, which are closely intertwined, because of shared fuel inputs and the dependence on the products of other pathways to serve as precursors (Figure 1C)[18, 60].

Glycolysis

Glycolysis is a major metabolic pathway and plays an important role in biosynthetic pathways. In this pathway, glucose is converted to pyruvate in the cytoplasm and enters into the TCA cycle or fermented into lactate. The “Warburg Effect,” first identified in highly metabolically active cancer cells, has been identified as a key metabolic transition to glycolysis, which is seen in cells undergoing biosynthesis of pro-inflammatory molecules[10, 61, 62]. Additionally, the transition from OXPHOS to aerobic glycolysis seen in the Warburg Effect[63] is also an important metabolic change in TI. This cellular metabolic switch has been demonstrated in β-glucan- and BCG-induced, innate immune training in monocytes[10, 64, 65]. *In vitro* studies reported that β-glucan-trained human monocytes have dramatically reduced oxygen consumption and increased glucose consumption and lactate production[66]. Additionally, *in vivo* studies reported that isolated peripheral blood mononuclear cells trained with BCG induce-TI associated with upregulation of the key enzymes involved in glycolysis pathways such as hexokinase 2 (HK2) and phosphofructokinase platelet (PFKP)[60]. The molecular mechanism underlying β-glucan and BCG induced-TI have been outlined in monocyte *in vitro* studies. β-glucan and BCG training promote a metabolic transition to glycolysis via the pathway of mammalian target of rapamycin (mTOR) activation of hypoxia-inducible factor 1α (HIF1α)[66, 67]. Pharmacological inhibition of Akt-mTOR-HIF1α pathway or glycolysis pathway using wortmannin, rapamycin, and 2-deoxyglucose (2-DG) (Table 1B), as well as gene knockout of HIF1α in myeloid cells reverses the pro-inflammatory phenotype in trained innate cells[66].

Pro-atherogenic lipids, such as oxLDL and lysophosphatidylcholine (LPC), have been shown to drive TI phenotypes in innate immune cells as well as ECs[9, 20, 68–71]. Training of monocytes with oxLDL induces TI and increases glycolysis, however, inhibition of

glycolysis with 2-DG and inhibition of the inducible PFK-2/FBPase isozyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) prevent development of TI[72]. Genetic variation in PFKFB3 and PFKP genes is associated with the increased tumor necrosis factor-α (TNF-α) and inteleukin-6 (IL-6) production *ex vivo* upon training with oxLDL[73]. Human aortic endothelial cells (HAECs) primed with oxLDL exhibit increased glycolysis and lactate production[15]. Similarly, the TI phenotype in oxLDL-primed HAECs is reversed upon treatment with mTOR inhibitors (Torin1), glycolysis inhibitors 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) and HIF1α inhibitors (KC7F2)[15].

Tricarboxylic acid cycle (TCA) for citrate-Acetyl-CoA generation and fumarate accumulation

Another important metabolic event in trained immune cells is the TCA cycle's anabolic repurposing towards synthesizing cholesterol from acetyl-CoA and citrate. The TCA cycle in mitochondria represents a critical pathway of cellular energy metabolism resulting in the oxidation of different substrates, including pyruvate generated from glycolysis in the cytosol[18].

In addition to the transition from OXPHOS to glycolysis, β-glucan trained macrophages have increased levels of glycolysis-related metabolites (including Acetyl-CoA). The TCA cycle-related metabolites such as citrate, succinate, malate, and fumarate are increased in trained monocytes due to the partial shutdown of the TCA cycle[66]. Citrate can be generated from glycolysis via pyruvate or generated from glutamine (glutaminolysis) and converted into α-ketoglutarate to enter the TCA cycle[74, 75]. Then citrate is transported from the mitochondria into the cytoplasm by the citrate carrier (CIC), is converted into acetyl-CoA by adenosine triphosphate (ATP)-citrate lyase (ACLY)[76] and used as a precursor in mevalonate/cholesterol biosynthesis pathway and lipogenesis pathway. Moreover, ACLY-catalyzed acetyl-CoA is a key determinant of cellular protein acetylation and used as an acetyl donor for histone acetylation, regulating the expression of several genes[77]. Importantly, acetyl-CoA generated from both glycolysis and glutamine can induce histone acetylation of genes of glycolytic enzymes, including; hexokinase 2 (HK2), phosphofructokinase (PFK), and lactate dehydrogenase (LDH) resulting in increased glycolysis[78]. Additionally, ACLY acts as a key enzyme in macrophage inflammatory response. Different inflammatory stimuli including LPS, interferon-γ (IFN-γ), and TNF-α induce ACLY expression in immune cells[79].

Metabolomics and transcriptomic studies of β-glucan trained immune cells showed that these trained cells increase accumulation of the TCA cycle metabolite fumarate[80]. Several lines of evidence showed that succinate and fumarate accumulation is associated with stabilization of HIF-1α leading to increased glycolysis and IL-1β production[81, 82]. Furthermore, fumarate accumulation leads to epigenetic reprogramming by inhibiting lysine-specific histone demethylase 5 (KDM5)[80]. Furthermore, increased glutamine metabolism (glutaminolysis) is one of the important metabolic changes in macrophages trained with BCG and β-glucan to produce more citrate for Acetyl-CoA generation in the cytosol[60, 80]. Notably, the glutamine pathway inhibition by the glutaminase inhibitor, Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) inhibits β-glucan and BCG induced TI *in vitro* and *in vivo*[60, 80].

Mevalonate synthesis pathway

The cholesterol biosynthesis is an effector molecule involved in inflammatory responses[83]. Acetyl-CoA generated from the TCA cycle in the cytoplasm can enter the cholesterol biosynthesis pathway and generate cholesterol using the enzyme, 3-hydroxy-3-methylglutaryl CoA reductase (HMGCAR), which is the rate-limiting step in the cholesterol biosynthesis pathway. Cholesterol plays a critical role in the inflammatory function of different immune cells. Lipid rafts in the cellular membrane are important for inflammatory signal transduction pathways[83]. Furthermore, cholesterol accumulation in monocytes/macrophages play a key role in the acceleration of atherosclerosis[84]. Increased cholesterol synthesis is an important hallmark of β-glucan trained monocytes. In fact, this is accomplished not by cholesterol biosynthesis but rather by an accumulation of the upstream metabolite mevalonate[18]. As highlighted earlier, Akt-mTOR-HIF1α drives metabolic reprogramming through the mevalonate pathway, which is considered the underlying mechanism that induces TI. In addition, β-glucan trained macrophages show an upregulation of cholesterol and fatty acid synthesis pathways. Of note, increased cholesterol synthesis is critical for the β-glucan-induced training of mature myeloid cells and the hematopoietic stem and progenitor cells (HSPCs)[12].

The functions of the mevalonate synthesis pathway in TI can be summarized: *first*, human monocytes exposed to mevalonate for 24 hours increase cytokine production and induce TI associated with increased aerobic glycolysis and enrichment of histone 3 lysine 4 tri-methylation (H3K4me3) on the promoters of several cytokine genes[18]. *Second*, inhibition of the mevalonate pathway by using statins (HMGCAR inhibitor) significantly reduces TI induced by oxLDL and β-glucan *in vitro* and *in vivo*[18, 85] presumably by a negative feedback to decrease Acetyl-CoA generation for histone acetylation. *Third*, pharmacological inhibition studies demonstrated that mevalonate enhances TI via activation of the insulin-like growth factor 1 receptor (IGF1-R) and subsequent stimulation of mTOR signaling and glycolysis pathway by a positive feedback loop[18].

Multiple levels of experimental evidence showed the *in vitro* pharmacological inhibitors of mevalonate pathway, glycolysis, glutaminolysis, and pharmacological blockers of histone methyltransferases could ameliorate induction of TI. Furthermore, *in vivo* studies using mouse models have confirmed these results[80]. These results would allow the development of novel pharmacological strategies to inhibit TI. However, future extensive studies and further elucidation of the mechanism of TI in endothelial cells will provide an exciting novel prospect for the development of pharmacological strategies to reduce atherosclerotic cardiovascular diseases and other metabolic diseases.

4. Epigenetic reprogramming is formed for enhancement of cytokine responses when encountering subsequent specific or non-specific challenges

The epigenetic reprogramming of trained immune cells is driven, at least in part, by a rewiring of intracellular metabolic pathways[86]. A switch from OXPHOS to increased glycolysis is key for the development of the trained phenotype[69]. In addition, increased

glutaminolysis and fumarate accumulation can inhibit histone demethylase KDM5 and affects histone methylation[80]. β-glucan induced TI depends on the intracellular accumulation of mevalonate and the subsequent activation of the IGF-1 receptor[18]. Therefore, epigenetic modification plays a critical role in mediating TI. During the TI process, the first stimulations, such as BCG, oxLDL or LPS, rewrite the epigenetic modifications of inflammatory cytokines' enhancers or promotors in innate immune cells. After the epigenetic modification, second stimuli induce high cytokine production, activate intracellular signaling molecules, and enhance inflammatory response[87]. These epigenetic modification in TI includes histone methylation and acetylation[88, 89]. However, the epigenetic modifications induced by different stimuli are variable in mechanisms and cell types of TI.

BCG vaccination promotes the binding of H3K4me3 at the promoters of inflammatory genes encoding TNFα, IL6, and Toll-like receptor 4 (TLR4)[90]. Besides H3K4me3, BCG significantly augments IL-1β production via histone 3 lysine 27 acetylation (H3K27ac) enrichment and protects against virus infection in monocytes[91]. In addition to enrichment of histone modification, studies also found that BCG leads to decreased H3K9me3, a repressor mark, and enhances TI response[60]. Furthermore, β-glucan priming increases cytokine production by enrichment of H3K4me3 but not H3K27me3 in monocytes[92] via an increased levels of long non-coding RNAs in macrophage[93]. The top 500 genes, induced by H3K4me3 in β-glucan treatment, are related to the production of cytokines and chemokines in atherosclerosis progression[9]. To clearly study the relationship of H3K4me3 with TI, studies found that inhibition of histone demethylase KDM5 by fumarate accumulation[80] significantly increases the levels of H3K4me3, suggesting that the TCA cycle product fumarate plays an important role in β-glucan mediated TI[9]. In addition to H3K4me3, β-glucan also increases H3K4me1 and H3K27ac in monocyte-to-macrophage differentiation and TI[67].

oxLDL, an established risk factor for CVD and chronic kidney disease, is also critical for TI induction. oxLDL priming significantly increases the enrichment of H3K4me3 at the promoters or enhancers of pro-atherogenic cytokines and chemokines[9] as well as H3K27ac at the promoters of IL-6 and IL-8 via mTOR-HIF1α signaling in ECs[15]. The oxLDL induced TI is abrogated by non-specific histone methyltransferase inhibitor. Besides this, inhibition of histone methylation by pan-methyltransferase inhibitor 5'-methylthioadenosine (MTA) blocks the phenotype of TI induced by β-glucan[94] and BCG[92]. LPC is another risk factor and DAMP for atherosclerosis[36] has been reported to induce TI enzymes via H3K14ac in HAECs[20].

Additionally, aldosterone, the main mineralocorticoid hormone, induces the gene expressions related to fatty acid metabolism and pro-inflammatory cytokines production via H3K4me3 enrichment in macrophage[95]. Super-low dose LPS has been shown to induce TI *in vivo* and *in vitro*. LPS promotes enhancer activities in H3K4me1 and H3K27ac and TI in HSCs[96] and macrophage[97]. Studies reported that the epigenetic changes could maintain 12 weeks, which are mediated by CCAAT/enhancer binding protein (C/EBP) transcription factor family in LPS-induced TI[96]. In addition to active epigenetic reprograming, LPS also induces TI by inhibiting histone 3 lysine 9 di-methylation (H3K9me2), a suppressive histone

modification, via stress-response transcription factor ATF7 phosphorylation-mediated mechanism[98]. Human cytomegalovirus (HCMV) also induces TI via DNA hyper-methylation at promoters of altered cytokines in natural killer (NK) cells[99].

Based on these publications, H3K4me3, H3K27ac, H3K14ac, and H3K4m1 have been reported to participate in most stimuli-induced TI in different cell types including ECs. In addition, future work is needed to identify other stimuli involved in TI and epigenetic reprogramming in ECs.

5. Reactive oxygen species (ROS) play context-dependent roles in trained immunity

Cellular metabolic pathways and signaling molecules are significantly correlated to epigenetic rewriting in TI. We recently proposed that ROS systems are a new integrated network for sensing homeostasis and alarming stresses in organelle metabolic process[100]. Previous studies reported that ROS production can be regulated by circular RNAs and associated with different cardiovascular metabolic inflammation[101]. In addition, ROS generated in metabolic processes are reported to participate in the process of TI and the metabolic reprogramming during immune responses leads to excessive ROS production[102]. We previously reported that ROS mediate LPC-induced TI enzyme transcription in HAECs[20, 100] and that IL-35 can downregulate three ROS promoters and upregulate one ROS attenuator[103]. In addition, ROS generation is increased by BCG and oxLDL priming in monocytes[8]. Studies further reported that oxLDL-induced ROS production via mTOR signaling plays critical role in promoting TI[71]. To further study ROS production's function in TI *in vivo*, mice are first trained with BCG followed by a second stimulation with a type of ROS hypochlorous (HOCL). After ROS stimulation, the productions of cytokines and chemokines, such as C-C chemokine receptor type 2 (CCR2), C-X-C receptor type 4 (CXCR4), lymphocyte antigen 6 (Ly6C) and C-C motif chemokine ligand 2 (CCL2) are significantly increased[104]. However, other publications reported that β-glucan decreases ROS production and increases mTOR signaling in trained monocytes[8]. Additionally, studies reported that mitochondrial ROS (mtROS) production inhibits the transcriptional factors and gene expressions, but not epigenetic remodeling in TI[105]. Therefore, further studies are needed for the detailed role and mechanisms for ROS production and TI in ECs.

ROS are the upstream direct activator of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3)-caspase-1 inflammasome, which is an important mediator of TI. The nucleotide-binding site domain in NLRP3 is highly sensitive to ROS[100]. After tightly binding with, the NLRP3 is activated and promotes the progression of TI. In addition, oxLDL and extracellular ATP promote the activation of NLRP3 inflammasome and induction of TI via H3K4me1, H3K4me3 and H3K27ac enrichment[9]. Thus, the ROS-NLRP3 inflammasome pathway may be the potential regulating mechanism of TI. However, the other detailed mechanisms have been less reported than the ROS-NLRP3 inflammasome pathway, and further studies are needed. Taken together, ROS play context-dependent roles in TI.

6. Trained immunity pathways and inflammatory pathways are separated to ensure memory stays when inflammation undergoes resolution

Adaptive immunological memories are carried out by special functional cell populations, memory T^2 and memory B lymphocytes, which are separated from effector T cells/B cells[89]. Thus, this mechanism ensures that the robust immune responses to dominant pathogens are carried out by effector cells. In contrast, immune memories are carried out by a small number of memory cells with high antigen specificities. However, an important question remains whether this important mechanism exists in innate immune cells. We previously found out that innate immune memory pathway and effector pathway are separated in LPC-trained ECs; and that LPC-induced H3K14ac binds more TI gene genomic regions (78.95%) than that of effector genes (43.12%)[20]. Another publication reported that epigenetic reprogramming-modulated innate immune memory is separated from the transcriptional factors-regulated innate immune effector pathways in HAECs. Anti-inflammatory cytokines, such as IL-35 and IL-10, inhibit the effector pathways in LPC-induced TI in ECs, whereas having no inhibition affects the expression of memory pathway genes[105]. Further studies are needed to determine the detailed mechanisms of separation of memory and effector pathways in TI.

7. Trained immunity plays significant roles in endothelial pathology and chronic inflammation

Infections

Immune memory is a defining feature of the acquired immune system. However, reprogramming of innate immunity can also result in enhanced responsiveness to subsequent triggers, which forms the basis on which chronic inflammatory diseases develop[4]. ECs have been documented the existence of non-specific characteristics of innate immunity when exposed to exogenous or endogenous stimuli[14]. Dengue viruses cause two severe diseases that alter vascular fluid barrier functions, dengue hemorrhagic fever and dengue shock syndrome[106]. ECs are critical targets of dengue virus infection that can contribute to viremia, elicit immune-enhancing responses via high-level induction and secretion of activating and recruiting cytokines by immune cells[107]. The immune memory of prior infection of the dengue virus could provide protection for 2–3 months. When short-term cross-protection wanes, patients with secondary infections (not limited to dengue virus) are at higher risk of severe disease than patients without prior infection[108], suggesting the innate immune training on ECs may have harmful effects on how the immune system responds to subsequent same or other pathogen invasions[109]. Based on these findings, several trials are currently underway to determine whether BCG can help prevent the Coronavirus Disease 2019 (COVID-19)[110].

Association between infections and cardiovascular diseases

Epidemiological evidence indicates that TI links infection and CVD[111, 112]. Impaired anti-coagulant function and expression of the endothelium genes upon infection with influenza viruses or other respiratory viruses have been linked to a risk of myocardial infarction

beyond the short-term post-infection period[113]. Prior instances of bacteremia and sepsis also substantially increase the 5-year risk of cardiovascular events. Endothelial dysfunction and pro-coagulant changes in the blood provide a biological basis for the association[114, 115]. The strength and time-related patterns of the association between infections and increased risk of cardiovascular events suggest a causal relationship, that the associations are stronger and last longer when the infections are severe[111]. Indeed, rather than exposure to specific pathogens, the accumulated non-specific infectious burdens, including infections of *Chlamydophila pneumoniae, Helicobacter pylori,* cytomegalovirus and human immunodeficiency virus (HIV), are associated with future development of atherosclerosis[116–118]. A longitudinal population-based study found that severe early life infections are associated with sub-clinical atherosclerosis in adulthood[119, 120]. Regardless of the induction of a systemic inflammatory response and activation of the immune system, exposure to pathogens correlates with local activation of ECs and the appearance of pro-coagulant activity, leading to endothelial dysfunction. In addition to the atherogenic phenotype of trained macrophages, enhanced adhesive markers and cytokine production capacity in trained ECs also indicate that the TI protects against re-infections but may accelerate the development of chronic inflammation such as atherosclerosis[112].

Other than microbial products, endogenous sterile stimuli could trigger TI in multiple clinical scenarios. In the atherosclerosis-prone mice model, Western-type diet- induced systemic inflammations contribute to the enhanced responses to subsequent LPS stimulation[68]. The transcriptional and epigenetic reprogramming of circulating monocytes and their bone marrow myeloid progenitor cells devote to the TI phenotype, which persists even after the mice had been switched to a normal chow diet, regardless of circulating cholesterol levels and systemic inflammatory markers returning to normal. We previously demonstrated that ECs also underwent transcriptional and epigenetic alterations upon LPC stimulation *in vitro* and Western diet feeding *in vivo*[121]. The changes of a group of enzyme genes lasted even when the endothelial activation markers are obstructed by the administration with anti-inflammation cytokines, suggesting endogenous sterile stimuli induced immune memory could also be built-in ECs[105].

Ischemia/reperfusion injury

Organ transplantation has been investigated as another clinical scenario, in which endogenous sterile stimuli could trigger TI in myeloid cell[122]. Meanwhile, hypoxia/ischemia as a stimulus for TI has been hypothesized[123]. Endothelial dysfunction plays a significant role in ischemia/reperfusion (I/R) injury in multiple organs[124–126], however, it could be prevented by ischemic conditioning with a protective intervention based on limited intermittent periods of ischemia and reperfusion[127]. The molecular mechanisms and signal transduction reprogramming including less ROS production, reduced neutrophils recruitment, and diminished inflammatory reactions during the pre-conditioning process indicate the TI characteristics in ECs[128, 129]. In the context of endothelial training in I/R injury, low-dose LPS pre-treatment could also downregulate the expression of endothelial-cell adhesion receptors, hence alleviate neutrophil invasion into the tissues[130].

Recently, we have reported that ischemic pre- and post-conditioning induce upregulation of the canonical and non-canonical inflammasome regulators and TI regulators[131]. In addition, ischemic pre-conditioning has been demonstrated to alter the brain's epigenetic profile from ischemic intolerance to ischemic tolerance[132]. Meanwhile, the miRNA transcriptome changes have also been observed to be involved in the epigenetic reprogramming in the brain ischemic tolerance[133, 134]. As the induction of miR-15a expression has been experimentally verified to contribute to ischemic endothelial cell damage functionally, the TI phenotype in ECs during the ischemic pre-conditioning may provide neuroprotective roles in later ischemia injuries[135].

Smoking

Cigarette smoking (CS)-induced EC damage and endothelial dysfunction seem to be dose-related and contribute to vascular injury, atherogenesis, chronic obstructive pulmonary disease (COPD) and increased CVD risk[136–138] presumably via increasing TI. Smoking cessation leads to prolonged improvements in endothelial function[139, 140] presumably by inhibiting TI. During this process, epigenetic alterations have been demonstrated to play important roles in the specificity and duration of gene transcription[141, 142]. Indeed, the modification of histone deacetylase 6 (HDAC6) has been evidenced to mediate the disruption of lung endothelial barrier integrity after CS exposure, enhancing the susceptibility to following acute lung injury[143]. Besides, protein arginine methyltransferase 6 (PRMT6) has been identified in mediating CS-induced apoptosis and inflammation in HUVECs; and modulating this methyltransferase has been demonstrated to be associated with HIV-1 infectivity[144] and lung tumor progression[145]. Taken together, those TI findings may explain the enhancement of CS-induced endothelial impairment to the development of AIDS and tumorigenesis[146].

Conclusion

The complexity of the immune system remains at the heart of both infectious and sterile inflammatory diseases. In the case of cardiovascular diseases, the inflammatory response starts with endothelial cell activation[147, 148], which coordinates the subsequent leukocyte infiltration. In this context, endothelial cells function as conditional innate immune cells. Recent studies have shown that innate immune memory (trained immunity) is a feature of monocytes, macrophages, NK cells and endothelial cells[149, 150]. Taken together, these studies support our argument that endothelial cells, in their capacity as conditional innate immune cells with TI function, are the gatekeepers that control the initiation and progression of cardiovascular disease, including atherosclerosis.

Endothelial cells are primed for a potentiated innate immune response common driver of atherosclerosis. Recent studies have demonstrated how the canonical atherogenic DAMP, oxLDL, elicits innate immune training in endothelial cells via *1)* increased cytokine and adhesion molecule expression production, *2)* a metabolic switch from OXPHOS to glycolysis, *3)* epigenetic modifications of pro-inflammatory genes, and *4)* ATK-mTOR-HIF1α signaling. Furthermore, changes in shear stress at atheroprone sites in the vasculature

have shown key features of trained immunity including *1)* upregulation of glycolysis, *2)* histone modifications, and *3)* HIF1α signaling[41].

These conclusions open exciting new possibilities in targeting EC trained immunity for the treatment of not only cardiovascular disease but also for the treatment of other metabolic and inflammatory diseases. The regulation of trained innate immunity may provide new insights and therapeutic targets in various disease contexts[151]. Promoting trained immunity is beneficial for preventing diseases, for example, using the BCG vaccine for children after birth can reduce child morbidity and mortality[152], and can be used as immunotherapies in the treatment of various cancers such as lymphomas, leukemia[153], melanomas[154], and bladder cancer[155, 156]. On the other hand, potentiating TI could lead to persistent non-resolving vascular inflammation and chronic inflammatory diseases such as atherosclerosis. TI inducers such as oxLDL, LPS, and a Western-type diet induce intense inflammatory responses and epigenetic reprogramming, leading to exacerbate atherosclerotic cardiovascular diseases[3, 60, 68]. Therefore, promoting the reverse of trained immune potentiation, a phenomenon known as trained immune tolerance has potential therapeutic uses. Endothelial cell tolerance to LPS challenge induced by MPLA characterizes the effects of LPS induced-immune tolerance in HUVEC and the potential use of MPLA, a compound similar in structure to LPS, to induce trained tolerance as a potential therapeutic to suppress innate immune inflammation[44]. Further studies are required to elucidate the molecular underpinnings driving endothelial cell TI and their roles in human inflammation and disease.

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Nonstandard Abbreviations and Acronyms

CVD	Cardiovascular disease
ECs	Endothelial cells
TI	Trained immunity
OxLDL	Oxidized low-density lipoprotein
BCG	Bacillus Calmette-Guerin
LPS	Lipopolysaccharides
DAMPs	Danger-associated molecular patterns

LPC	Lysophosphatidylcholine
OXPHOS	Oxidative phosphorylation
mTOR	Mammalian target of rapamycin
HIF1-α	Hypoxia-inducible factor 1-α
HUVECs	Human umbilical vein ECs
HAECs	Human aortic endothelial cells
TCA	Tricarboxylic acid cycle
Acetyl-CoA	Acetyl-coenzyme A
ACLY	Adenosine triphosphate (ATP)-citrate lyase
H3K4me3	Histone 3 lysine 4 tri-methylation
H3K27ac	Histone 3 lysine 27 acetylation
ROS	Reactive oxygen species

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Highlights

- Innate immune cells can develop exacerbated immunological response after exposure to endogenous or exogenous insults, which phenomenon as trained immunity (TI).
- TI can occur in innate immune cells such as monocytes/macrophages, NK cells, and ECs.
- TI inducers are risk factors for CVD and other metabolic diseases
- TI characterized by metabolic reprogramming and epigenetic modification.
- Reactive oxygen species (ROS) play context-dependent roles in TI.

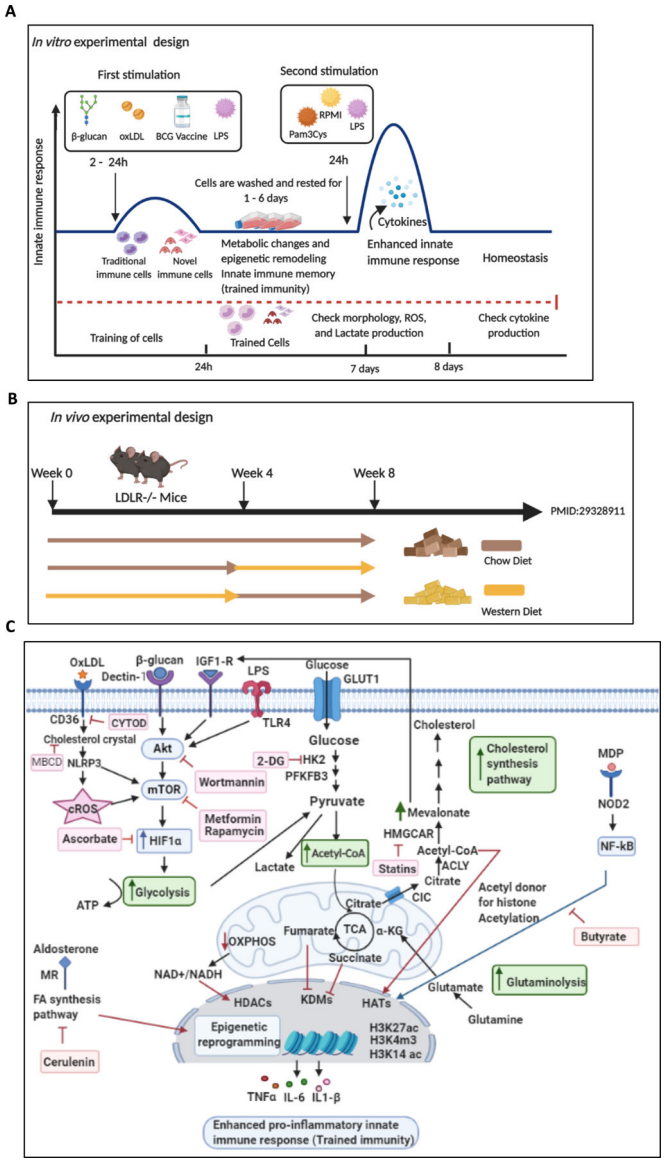

Figure 1.

Schematic overview of trained immunity experimental design *in vitro*, *in vivo*, and the complex metabolic pathways involved in trained immunity. *A. In vitro* induction of trained immunity: Traditional and novel immune cells are trained by β-glucan, oxLDL, LPS, and BCG for 24 hours. Cells are washed twice with PBS and rested in culture media for 24 hours, 3 days, or 6 days, after which the cells are restimulated with LPS or Pam3Cys for 24 hours. Then cytokine productions are measured. *B. In vivo* induction of trained immunity by a Western diet priming and LPS restimulation. Low-density lipoprotein receptor deficient (LDLR−/−) mice are fed with Western diet for 4 weeks, then, normal chow diet for another 4 weeks. Six hours before sacrifice, mice are injected intravenously with LPS or PBS. *C.* Overview Figure Showing the Metabolic and epigenetic reprogramming in trained immunity. Trained immunity inducers bind to their receptors in the cell membrane or intracellular receptors, leading to enhanced signaling of the Akt–mTOR–HIF-1α pathway,

modifications in metabolic pathways (increased glycolysis, increased acetyl-CoA generation, increased mevalonate synthesis), and epigenetic reprogramming. This results in increased pro-inflammatory cytokine production and enhanced innate immune response (trained immunity). Abbreviation: oxLDL: oxidized low-density lipoprotein, BCG: *Bacillus Calmette–Guérin,* LPS: Lipopolysaccharide, cROS: Cytosolic reactive oxygen species, LDLR−/− mice: Low-density lipoprotein receptor knockout mice, Akt: Serine/threonine protein kinase, mTOR: Mammalian target of rapamycin, HIF1: Hypoxia-inducible factor 1α, 2-DG: 2-deoxyglucose, GLUT1: Glucose transporter 1, HK2: Hexokinase 2, PFKFB3: Phosphofractokinase-2/FBPase isozyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, Acetyl CoA: Acetyl Coenzyme A, α-KG: α-ketoglutarate, OXPHOS: Oxidative phosphorylation, CIC: Citrate carrier, ACLY: Adenosine triphosphate (ATP)-citrate lyase, HMGCAR: 3-hydroxy-3-methylglutaryl CoA reductase, H3K27ac: Histone 3 lysine 27 acetylation, H3K4m3: Histone 3 lysine 4 tri-methylation, H3K14ac: Histone 3 lysine 14 acetylation, TNFα: Tumor necrosis factor α, IL-6: Interleukin-6, IL1-β: Interleukin 1-β, NOD2: Nucleotide-binding oligomerization domain-containing protein 2, MDP: Muramyl dipeptide, NF-kB: nuclear factor (NF)-κB, FA: Fatty acid, MR: Mineralocorticoid receptor, HDACs: Histone deacetylase, HATs: histone acetyl transferases, KDM: Histone lysine demethylase. Figure created with BioRender (https://app.biorender.com/user/signin).

Table 1. A.

A schematic summary of training programs induced in different cell types.

Stimulation	Receptor	Cell type	Training immunity signaling	Metabolic remodeling	Epigenetic remodeling	PMID
β-glucan	Dectin-1	Monocytes BMPCs	Akt-mTOR-HIF1α IL-1 GM-CSF/CD131	Glycolysis Glutaminolysis Mevalonate synthesis	H3K4me1 H3K4me3 H3K27ac	25258083 29328908 27866838
BCG	NOD2	Monocytes BMPCs	Akt-mTOR, IFN-r, IL-32	Glycolysis Glutaminolysis Mevalonate synthesis	H3K9me3 H3K4me3 H3K27ac	27926861 29328912 29324233
OxLDL	TLR	Monocytes	mTOR dependent ROS	Glycolysis Mevalonate synthesis	H3K4me3	27733422 30723479
LPS	TLR4	Monocytes	IRAK-M, Tollip,JNK- miR24, ATF7	Glucose and cholesterol metabolism	H3K4me1 H3K4me3 H3K9me2 H2K27me	27824038 26322480
Aldosterone	Mineralocorticoid	Monocytes	Fatty acid synthesis pathway	Fatty acid synthesis	H3K4me3	31119285
HMGB1	TLR RAGE	Splenocytes	IRAK-M			24089009 26970440
LPC	GPCR	Endothelial cells	ROS	Glycolysis Acetyl-CoA generation Mevalonate synthesis	H3K14ac	31153039
Fungal Chitin	TLR	Monocytes	-	-	Histone methylation	26887946
WD	-	BMPCs	NLRP3 IL-1	-		29328911
CMV		NK cells	SYK PLZF	-	Methylation	25786175 25786176
Uric acid	-	Monocytes	IL-1β Akt	-	Histone methylation	28484006 31853991

Abbreviation: HIF1α: Hypoxia-inducible factor 1-alpha, BMPCs: Bone marrow progenitor cells, GM-CSF: Granulocyte-macrophage colony-stimulating factor, CMV: Cytomegalo-virus, BCG: Bacillus Calmette-Guerin, oxLDL: Oxidized low-density lipoprotein, LPS: Lipopolysaccharides, GPCR: G-protein coupled receptor, NOD2: Nucleotide-binding oligomerization domain-containing protein 2, IFN-γ: Interferon gamma, TLR: Toll-like receptor, IRAK-M: IL-1 Receptor-Associated Kinase M, HMGB1: High mobility group box 1, RAGE: receptor for advanced glycation end-products, LPC: Lysophosphatidylcholine, WD: Western diet, H3K27ac: Histone 3 lysine 27 acetylation, H3K4m3: Histone 3 lysine 4 tri-methylation, H3K14ac: Histone 3 lysine 14 acetylation, H3K9m2: Histone 3 lysine 9 dimethylation, Akt: Serine/threonine protein kinase, mTOR: Mammalian target of rapamycin, NLRP3: NLR family pyrin domain containing 3.

Table 1B.

Trained-immunity-regulating pathway inhibitors.

1st stimulus	2nd stimulus	Inhibitor	Function	Effect on trained immunity	PMID
I- Inhibition of signaling pathways					
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	Wortmannin	Akt inhibitor	Inhibit trained immunity	25258083
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	Rapamycin	mTOR inhibitor	Inhibit trained immunity	25258083
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	AICAR	mTOR inhibitor	Inhibits trained immunity	25258083
β-glucan (10 μg/ml for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	Metformin	AMPK inhibition	Inhibit trained immunity	25258083
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	Ascorbate	HIF1α inhibitor	Inhibit trained immunity	25258083
LPS (200 ng/ml) for 6 h	OxLDL (50 μg/mL) for 24 h	Z-VAD-fmk	NLRP3 activation inhibitor	Inhibits IL-1β secretion	23812099
LPS (200 ng/ml) for 6h	OxLDL (50 μg/mL) for 24 h	MβCD	Cholesterol crystal formation inhibitor	inhibits the cholesterol crystals and IL-1β secretion	23812099
II- Inhibition of metabolic pathways					
β-glucan (10 μg/ml) for 24 h	LPS (10ng/ml) Pam3Cys (10 g/ml)	2-DG	Inhibits HK2 (glycolytic rate limiting step)	Inhibit trained immunity	27926861 25258083
OxLDL (10 μg/mL) for 24 h	LPS (10 ng/ml)	3PO	Inhibit glycolytic enzyme PFKFB3	Inhibit trained immunity	32350546
β-glucan (5 μg/ml), oxLDL (10 μg/ml), (R)-mevalonic acid (100–1000 μM), BCG (5 μg/ml)	LPS (10 ng/ml) Pam3Cys (10 μg/ml)	fluvastatin	HMGCAR inhibitor	Inhibit trained immunity	29328908
β-glucan (5 μg/ml), oxLDL (10 μg/ml), (R)-mevalonic acid (100–1000 μM), BCG (5 μg/ml)	LPS (10 ng/ml) Pam3Cys (10 μg/ml)	6-fluoromeval-onate	Mevalonate-pyrophosphate decarboxylase inhibitor	Enhance trained immunity	29328908
LPS (200 ng/ml) for 6 h	OxLDL (50 μg/mL) for 24 h	CYTOD	CD36 inhibitor	inhibit formation of cholesterol crystals and IL-1β secretion	23812099
Aldosterone for 24 h	LPS (10 ng/ml) Pam3Cys (10 μg/ml)	Cerulenin	FAS inhibitor	Inhibit trained immunity	31119285 32241223
III- Inhibition of epigenetic reprogramming					
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	MTA	Non-selective Methyltransferase inhibitor	Inhibit trained immunity	25258083 29328908 30380404
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	ITF (ITF2357)	HDACi	Inhibit trained immunity	25258083
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	Cyproheptadine	Set7 methyltransferase inhibitor	Inhibit glycolysis	25258083
β-glucan (10 μg/ml) for 24 h	LPS (10 ng/ml) Pam3Cys (10 g/ml)	Resveratrol	HDAC/sirtuins activator	Inhibit trained immunity	25258083 30380404
-	-	UMLILO	Inhibit H3K4me3 epigenetic priming	Inhibit trained immunity	30733945

Abbreviation: 2-DG: 2-deoxyglucose, HK2: Hexokinase2, PFKFB3: Phosphofractokinase-2/FBPase isozyme 6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3, HMGCAR: 3-hydroxy-3-methylglutaryl CoA reductase, IL1-β: Interleukin 1-β, HDACi: Histone deacetylase inhibitor, AICAR: Adenosine monophosphate–activated protein kinase (AMPK) activator, Z-VAD-fmk: Benzyloxycarbonyl-Val-Ala-Asp-

fluoromethyl ketone, MβCD: Methyl-β-cyclodextrin, 3PO: 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one, CYTOD: Cytochalasin D, FAS: Fatty acid synthetase, MTA: 5′-Methylthioadenosine, UMLILO: Upstream Master LncRNA of the inflammatory chemokine Locus.