



## Seminars in Immunology

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## Long-term activation of the innate immune system in atherosclerosis

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## ABSTRACT

Efforts to reverse the pathologic consequences of vulnerable plaques are often stymied by the complex treatment resistant pro-inflammatory environment within the plaque. This suggests that pro-atherogenic stimuli, such as LDL cholesterol and high fat diets may impart longer lived signals on (innate) immune cells that persist even after reversing the pro-atherogenic stimuli. Recently, a series of studies challenged the traditional immunological paradigm that innate immune cells cannot display memory characteristics. Epigenetic reprogramming in these myeloid cell subsets, after exposure to certain stimuli, has been shown to alter the expression of genes upon re-exposure. This phenomenon has been termed trained innate immunity or innate immune memory. The changed responses of ‘trained’ innate immune cells can confer nonspecific protection against secondary infections, suggesting that innate immune memory has likely evolved as an ancient mechanism to protect against pathogens. However, dysregulated processes of immunological imprinting mediated by trained innate immunity may also be detrimental under certain conditions as the resulting exaggerated immune responses could contribute to autoimmune and inflammatory diseases, such as atherosclerosis. Pro-atherogenic stimuli most likely cause epigenetic modifications that persist for prolonged time periods even after the initial stimulus has been removed. In this review we discuss the concept of trained innate immunity in the context of a hyperlipidemic environment and atherosclerosis. According to this idea the epigenome of myeloid (progenitor) cells is presumably modified for prolonged periods of time, which, in turn, could evoke a condition of continuous immune cell over-activation.

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## 1. Atherosclerosis, a persistent inflammatory disease

Cardiovascular diseases (CVD), including stroke, and myocardial infarction, are the leading cause of mortality worldwide and together are responsible for approximately 17 million deaths per year [1]. The vast majority of cardiovascular events are caused by the rupture or erosion of atherosclerotic plaques in the arterial wall and the subsequent formation of an occluding thrombus. Traditionally, atherosclerosis is regarded as a disease of the developed Western Society, driven by the major classical risk factors dyslipoproteinemia, hypertension, diabetes, obesity, smoking, and a sedentary lifestyle. Interestingly, however, the development of atherosclerosis is not restricted to patients with a Western lifestyle. In a recent study, CT-scanning revealed signs of atherosclerosis in a third of mummies from several ancient populations, even in non-smoking populations that had an almost entirely marine diet [2]. These observations fit the paradigm shift in the early nineties that atherosclerosis is more than simply a vascular cholesterol storage disease, and that chronic low-grade vascular inflammation triggered by the deposited material could contribute to atherogenesis [3]. The inflammatory nature of atherosclerotic plaques was already recognized in the nineteenth century by Virchow [4], who wrote: “In some, particularly violent cases the softening manifests itself even in the arteries not as the consequence of a really fatty process, but as a direct product of inflammation”.

In the last ten years, several novel non-traditional risk factors for atherosclerosis have been identified that are all associated with activation of the immune system. These include chronic inflammatory diseases such as rheumatoid arthritis, gout, psoriatic arthritis, and ankylosing spondylitis, as well as infections with bacteria or viruses, such as *Chlamydia pneumoniae*, *Porphyromonas gingivalis* and *human immunodeficiency virus* (HIV) [5]. In addition, acute cardiovascular events occur more frequently in the weeks following an acute infection, such as pneumonia [6]. Recently, also cardiovascular events itself, such as myocardial infarction [7] or stroke were described to drive subsequent acceleration of atherosclerosis throughout the vascular system [8]. Finally, recent experimental evidence indicates that changes in diet and in dietary composition are able to directly influence lymphoid organs, thus profoundly and continuously influencing immune responses and the development of inflammatory and autoimmune diseases [9].

Although it is now well established that inflammation plays an essential role in the initiation, progression, and destabilization of atherosclerotic plaques, the mechanisms that drive the persistent non-resolving inflammation in the vessel wall remain incompletely understood. Macrophages are the most abundant inflammatory cells present in atherosclerotic plaques. Cells of the adaptive immune system such as T and B lymphocytes are also present, but in lower numbers. Evidence is rapidly accumulating that innate immune cells can adopt a persistent pro-inflammatory phenotype after brief exposure to a variety of stimuli, a phenomenon that has been termed ‘trained innate immunity’ [10].

In this review, we discuss the concept that trained immunity contributes to the development of atherosclerosis, both in the setting of traditional cardiovascular risk factors, and in the setting of non-traditional risk factors, such as acute and chronic infections, and non-infectious chronic inflammatory disorders. In addition, it might well be that elevated dietary fats and consequently an alteration of the gut microbiota, impact immune homeostasis (dysbalance between inflammatory and tolerogenic processes), thus

inducing a long-term epigenetic reprogramming of innate immune cells.

## 2. The innate immune system and atherosclerosis

Within atherosclerotic plaques, macrophages are the most abundant subset of leukocytes. Arterial resident macrophages are derived from embryonic CX3CR1<sup>+</sup> precursors and from bone marrow-derived monocytes that colonize the tissue immediately after birth [11,12]. During atherogenesis, arterial plaque macrophages are sustained by local proliferation, but also by recruitment of Ly6C<sup>high</sup> monocytes [13]. Monocytes and macrophages are critically promoting the initiation, progression, and destabilization of atherosclerotic plaques by several mechanisms [14,15]. In the early stages of atherosclerosis, macrophages contribute to the clearance of reactive lesion oxidized low-density lipoprotein (oxLDL) particles through scavenger receptor A (SR-A) and CD36 mediated uptake. In later stages, macrophages are impaired in their phagocytic functionality due to intracellular accumulation and defective efflux of oxLDL. Foam cell macrophages undergo apoptosis due to cellular stress and inflammatory responses, a process, which ultimately contributes to the formation of the pro-thrombotic necrotic core that characterizes mature atherosclerotic plaques. Damage Associated Molecular Patterns (DAMPs), which accumulate in the atherosclerotic plaques, represent a source of endogenous danger ligands that can further activate macrophages by binding to specific Pattern Recognition Receptors (PRR), such as membrane-bound Toll Like Receptors (TLR), SR, and intracellular NOD-like receptors (NLRs) [16]. Multiple pro-inflammatory triggers could hence cause an inflammatory milieu in the plaque. In addition, even the phase transition of cholesterol can cause inflammation as cholesterol crystals can activate the Nlrp3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome, triggering the secretion of pro-inflammatory IL-1 $\beta$  and IL-18 [17]. Finally, lesion macrophages contribute to the remodeling of atherosclerotic plaques into a rupture-prone unstable phenotype by the production of proteases, such as matrix metalloproteinases [18]. Overall, plaque macrophages display a marked phenotypic heterogeneity [19], which is dictated by the composition of the local DAMPs that are present in the specific environment during different stages of plaque development.

Several studies have shown that hypercholesterolemia considerably increases the number of circulating Ly6C<sup>high</sup> pro-inflammatory monocytes in mice (or the CD14<sup>++</sup>CD16<sup>-</sup>/CD14<sup>++</sup>CD16<sup>+</sup> equivalent in humans), thereby accelerating atherosclerosis [20,21]. Additionally, an acute myocardial infarction, stroke or sepsis can accelerate subsequent atherosclerosis by inducing monocytosis [7,22]. Which factors are driving monocytosis in the context of a hypercholesterolemic environment remains to be fully resolved. Besides, although the adverse contribution of myeloid cells in atherosclerosis is generally accepted, it is still an open question why a strong pro-inflammatory response within the arterial wall fails to be resolved even after reducing potential inflammatory triggers. Recent studies have illuminated the mechanisms underlying the hypercholesterolemia-induced myelopoiesis and a shifting towards the pro-inflammatory Ly6C<sup>high</sup> monocytes [23,24]. Hitherto, the debate has primarily focused on the link between cholesterol accumulation within the plaque macrophage and local ongoing inflammatory processes. However, myeloid priming and

reprogramming under hypercholesterolemic conditions can occur already within the bone marrow, due to an overload of cholesterol and defective efflux pathways in hematopoietic stem and myeloid precursor cells. Animal studies have shown that cholesterol efflux pathways in hematopoietic stem cells (HSC) are mediated by apolipoprotein E (ApoE), ATP-binding cassette transporter A1 (ABCA1) and ABCG1 and these processes are controlling HSC expansion in the bone marrow. Transplantation of ABCA1<sup>-/-</sup> ABCG1<sup>-/-</sup> bone marrow into *Ldlr*<sup>-/-</sup> mice fed a Western type diet, as well as studies in *ApoE*<sup>-/-</sup> mice fed a Western type diet, induced dramatic leukocytosis (monocytosis and neutrophilia), which could be reversed by overexpression of the transgenic human Apo-AI [25–27]. The proliferative response in HSC was favored by increased cholesterol storage into the plasma membrane (due to impaired efflux), which resulted in increased expression of the proliferative cytokine receptors to IL-3, IL-5 and GM-CSF. Recent evidence suggests that Western type diet feeding also induces epigenetic modifications within HSC and myeloid progenitor subsets, potentially provoking increased myelopoiesis [28,29]. Van Kampen et al. [29] revealed in competitive bone marrow transplantation experiments using *Ldlr*<sup>-/-</sup> recipients fed a chow diet that donor bone marrow from Western type diet fed animals markedly increased myeloid progenitor proliferation compared to donor bone marrow from chow fed animals. Bone marrow from Western type diet fed mice showed hypomethylation in CpG-regions in the genes encoding PU.1 and IRF8, key regulators of monocyte proliferation and macrophage differentiation. Consistently, blood leukocyte numbers were significantly increased as a result of increased circulating monocytes, along with the presence of a more activated splenic macrophage phenotype and increased plaque sizes. In agreement with these data, Seijkens and colleagues [28] demonstrated in a competitive bone marrow transplantation study (transfer of normocholesterolemic bone marrow cells into either chow or high fat diet fed *Ldlr*<sup>-/-</sup> recipients) that a hypercholesterolemic BM microenvironment induced loss of HSC quiescence, characterized by increased expression of cyclin B1, C1 and D1, and skewed HSC development towards myeloid lineages, especially towards granulocytes and Ly6C<sup>high</sup> monocytes. HFD priming of bone marrow HSC remained even after transfer into normocholesterolemic mice and was associated with reduced H3K9/14 acetylation at the promoter of the retinoblastoma (RB) gene (involved in the control of excessive cell growth). In summary, these studies shed new light on the causality between hypercholesterolemia, altered epigenetic patterning in HSC and myeloid progenitors, and increased susceptibility to atherosclerosis.

Indeed, the concept of epigenetic regulation is being increasingly acknowledged as an important contributor in the pathogenesis of atherosclerosis and other metabolic diseases. Research within the last decade has provided an essential link between pro-atherogenic factors (amongst others diet, disturbed blood flow patterns, dyslipoproteinemia, hyperglycemia and microbiome) and reprogramming of the epigenome (altered DNA methylation and histone modifications) in various cell types such as leukocytes, vascular endothelial cells (EC) and vascular smooth muscle cells (vSMC) [30,31].

### 3. Trained innate immunity

Although cells of the innate immune system are traditionally considered incapable of building an immunological memory, plants and invertebrates that lack an adaptive immune system are protected against reinfection with pathogens, suggesting that the response of the innate immune system can be modified by previous encounters [10]. Trained innate immune responses were confirmed in human monocytes *ex vivo* by showing that mono-

cytes can adopt a long-term pro-inflammatory phenotype after brief exposure to various micro-organisms or microbial products, including the bacille Calmette-Guérin (BCG) vaccine, *Candida albicans*, or its cell wall component  $\beta$ -glucan [32,33]. This phenomenon could also be shown in humans *in vivo* by demonstrating that BCG vaccination in healthy human subjects induced an increased production of pro-inflammatory cytokines when isolated monocytes were exposed *ex vivo* to various unrelated microbial metabolites [32]. This enhanced pro-inflammatory phenotype of the monocyte could be detected even three months after the first vaccination suggesting that trained innate immune responses can persist for relatively long periods. The powerful immunologic effects of this phenomenon are illustrated by the findings in *scid* mice, which lack T and B lymphocytes, where BCG vaccination provided robust protection against a subsequent lethal dose of *C. albicans*. In humans, BCG vaccination prior to influenza vaccination results in a more pronounced increase and accelerated induction of functional antibody responses against an influenza vaccine [34].

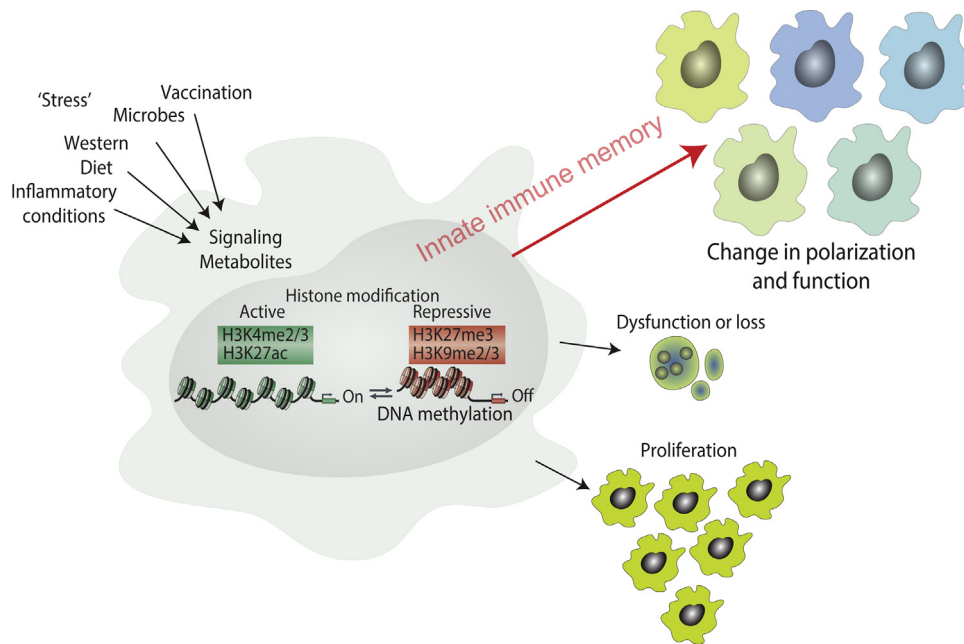
Importantly, trained innate immunity cannot only be induced by microbial products, but most likely also by metabolites that are relevant in the development of metabolic diseases and its complications. In the context of atherosclerosis, innate immune training might occur in a high fat and high cholesterol environment occurring within the plaque and potentially elsewhere, such as in the gut, liver or bone marrow. We postulate that long-term epigenetically reprogrammed myeloid precursor cells that are characterized by a hyper-inflammatory phenotype may contribute to a sustained disease progression [35] (Fig. 1).

#### 3.1. Human *in vitro* studies

In an *in vitro* model in human isolated monocytes, we found that trained innate immunity can be induced by modified LDL (oxLDL as well as acetylated LDL), but not by native LDL [36]. Twenty-four hour exposure of human monocytes to low concentrations of oxLDL resulted in an increased production of pro-atherogenic cytokines and chemokines (including IL-6, TNF $\alpha$ , IL-8, and MCP-1) upon re-stimulation of the cells 7 days later with TLR4 or TLR2 agonists. Additionally, IL-18, MMP2, and MMP9 mRNA-expression was significantly higher in the trained monocytes and increased foam cell formation with higher expression of the scavenger receptors CD36 and SR-A and decreased expression of the cholesterol efflux transporters ABCA1 and ABCG1 was found. Importantly, oxLDL-induced trained macrophages showed no significant differences in expression of TLR4, TLR2 and nitric oxide synthase ('M1' markers); arginase1 and CD163 ('M2' markers); and HLA-DR (human leukocyte antigen-DR), DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin), and CD83 expression (DC markers). These data illustrate that the trained innate immune phenotype does not merely represent skewing of the cells into the classical 'M1' but rather represents a unique pro-inflammatory phenotype. Pathway analysis revealed that oxLDL-induced training is dependent on TLR2 and TLR4 activation, and activation of extracellular regulated kinase (ERK) and phosphoinositide 3 kinase (PI3K) [36].

#### 3.2. *in vivo* studies

Corroborating the human *in vitro* data, it has previously been reported that subcutaneous immunization of cholesterol-fed New Zealand White rabbits with a human dose of BCG enhanced peripheral leukocyte activation, aortic monocyte recruitment and atherogenesis [37]. Although this finding fits within the concept that trained innate immunity contributes to atherogenesis, a contributory role for the adaptive immune system cannot be excluded in these experiments. In addition, several papers provide evidence



**Fig. 1.** Cellular stressors can influence the epigenetic landscape of innate immune cells. Depending on the chromatin modification the outcomes lead to different immune responses of the cells towards secondary stimuli, a concept that has been termed 'innate immune memory', with alternative fates being polarization, cell death or increased proliferation.

that glucose can also induce a persistent immunological memory in various cell types that are relevant in the context of atherosclerosis, including EC, vSMC, and monocytes. In a mouse model of diabetes, long-term activation of several key inflammatory genes in isolated vSMC was described, which correlated with increased H3K4 methylation and suppressed H3K9 di- and tri-methylation at the nuclear factor  $\kappa$ B (NF- $\kappa$ B) subunit p65 promoter, which persisted even after *ex vivo* cell culturing [38,39]. Also, brief exposure to high-glucose medium in cultured EC induced a persistent pro-inflammatory phenotype, even after reversal to normoglycemic conditions. This was associated with enrichment of the activating mark H3K4me1 in the NF- $\kappa$ B subunit p65 [40]. These results highlight the surprisingly long-lasting effects that short-term hyperglycemic spikes can execute on vascular cells, suggesting the idea of memory formation as a result of methyl-writing and methyl-erasing histone modifications [41]. In line, the concept of epigenetically mediated metabolic memory was recently also illustrated in patients from the Diabetes Control and Complications Trials (DCCT). Isolated monocytes from patients without diabetic microvascular complications, that had received intensive diabetes regulation in the past, showed overall hyperacetylation of H3K9 compared to patients with microvascular complications who had received less-intensive diabetes regulation in the past [42]. A link to glucose induced alterations in the bone marrow niche has recently been illustrated by the observation that a high-glucose environment induces stable intrinsic pro-inflammatory changes in HSC in mice [43].

Further *in vitro* and *in vivo* studies are required to corroborate the concept of trained innate immunity due to persistent epigenetic changes in response to 'epigenetically toxic' nutrient and/or microbial metabolites such as high fat, high cholesterol or high glucose in the context of metabolic diseases and its complications including atherosclerosis. Nevertheless, one supporting issue in the theory of epigenetic reprogramming and development of a trained memory is the *trans*-generational transmission of epigenetic traits, independently of genetically inherited risk factors [44]. In this regard, several studies in humans and experimental animal models have shown that parental adverse diet feeding (under- or over-nutrition) not only influences the individuals environment,

but can also influence the metabolic phenotype in the offspring thereby raising metabolic and cardiovascular disease susceptibility [45–48]. However, dissecting the effects induced by an unfavorable intra uterine environment the fetus is exposed to during gestation from direct effects on the parental germ line remains a challenging task. To determine whether epigenetic information can be transmitted through the germ line, multiple animal studies have tested the question of whether the offspring responded to changed paternal diet conditions. Indeed, paternal low-protein diet induced an up-regulation of genes involved in lipid and cholesterol biosynthesis in both male and female offspring, which was associated with a changed DNA methylation pattern in enhancer regions of peroxisome proliferator-activated receptors (PPAR)  $\alpha$  [49]. Likewise, chronic HFD feeding of male rats led to altered expression of genes involved in glucose metabolism due to a changed DNA methylation pattern, subsequently impairing glucose tolerance as well as insulin sensitivity in female offspring [50]. A recently published study demonstrates that a high-fat/high-fructose diet, starting at young age, leads to the development of obesity and to the progression of metabolic syndrome, accompanied by altered DNA methylation patterns in male and female Wistar rats [51]. HFD feeding was shown to affect 5-mC levels differently in both genders with a 20% reduction in males and a 15% increase in females. Interestingly, DNA methylation patterns were modified as well in the F1 generation, thus proposing that HFD-induced epigenetic modifications – as mediator of metabolic memory – can be transmitted to the next generation, subsequently increasing the susceptibility for disease development. Three recent studies support the idea of epigenetic inheritance by the paternal germ line in demonstrating that nutritional change can directly remodel the epigenetic signature of spermatozoa. A comprehensive profiling of the sperm epigenome showed that mal-nutrition as well as over-nutrition altered small RNA levels, including tRNA fragments, and DNA methylation patterns, indicating that sperm (as the direct transducer of genetic information) is vulnerable to environmental changes [52–54]. Whether epigenetic changes in the myeloid lineage can be transmitted to offspring remains unanswered yet.

#### 4. The potential mechanisms leading to trained innate immunity

The more prone pro-inflammatory phenotype that characterizes trained innate immune cells is critically dependent on a complex integration of epigenetic reprogramming and a change in the intracellular immuno-metabolism.

##### 4.1. Epigenetic reprogramming

Epigenetic modulation denotes the regulation of gene transcription independent of the DNA sequence. Epigenetic regulation can occur at the level of DNA methylation, histone modifications, or involves RNA-based mechanisms. Whereas DNA methylation is associated with a condensed chromatin structure that prevents binding of transcription factors and gene silencing, histone acetylation is associated with activation of gene transcription. Histone methylation can either activate or repress gene transcription, depending on the exact location of the methylated lysine residue and on the number of bound methyl groups (mono-, di-, or trimethylation). Macrophage differentiation, polarization, and activation can all be influenced by epigenetic reprogramming, which is reviewed in detail elsewhere [55–57].

A distinguishing hallmark of a trained innate immune cell is its ability to exhibit a quantitatively and qualitatively modified – usually more intense – inflammatory response upon re-challenge with danger signals that differ from the priming stimulus. The underlying molecular basis has thus far only partially been resolved and involves changes in chromatin organization (loosening and stringent accessibility to the transcriptional machinery), but might also comprise alterations in DNA methylation and RNA functionality (miRNAs, lncRNAs, tRNAs) caused by a certain primary stimulus [58,59]. Two recent studies support the concept of trained innate immunity in that they evidenced latent or de novo enhancer formation in stimulated macrophages [60,61]. TLR4-induced NF- $\kappa$ B activation in macrophages resulted in binding of the transcription factors (TF) PU.1/C/EBP to an appropriate enhancer element. TF binding was accompanied by recruitment and binding of the NF- $\kappa$ B-p65 subunit and histone acetyltransferases, and subsequent RNA polymerase II binding and elongation. Finally, histone methyltransferases MLL1, MLL2/4 and MLL3 were recruited, being responsible for H3K4 monomethylation and fully active enhancer formation. Notably, de novo formed enhancer elements remained stable (H3K4me1) upon loss of stimulus, and re-stimulation of macrophages resulted in faster and stronger responses.

For oxLDL-induced trained immunity in isolated human monocytes, epigenetic reprogramming at the level of histone methylation is crucial. Brief stimulation of human monocytes with oxLDL leads to the acquirement of a ‘trained innate memory’, which was associated with a pro-atherogenic and pro-inflammatory phenotype, and was characterized by enriched H3K4 trimethylation – a prototypical long-term activating epigenetic mark – on the promoters of pro-inflammatory cytokines and chemokines [36]. Moreover, trained innate immunity was completely prevented by co-incubation with a nonspecific histone methyltransferase inhibitor.

For training with  $\beta$ -glucan, an unbiased genome-wide experimental approach revealed changes in the activating H3K4me3 levels on promoters, H3K4me1 levels on enhancers, and H3K27ac levels on promoters and enhancers. Indeed, most dynamic promoters showed an enrichment of these marks [62]. Moreover, pathway analysis of the promoters that were potentiated by  $\beta$ -glucan identified several innate immune and signaling pathways upregulated in trained cells that are responsible for the induction of trained immunity.

##### 4.2. Immuno-metabolism and epigenetic programming

Epigenetic modifications are indispensable in cell reprogramming, however, it still remains an open question which cellular processes act to initiate or maintain these modifications. Increasing evidence supports a close connection between systemic and cellular metabolic processes and epigenetic reprogramming with a crucial role for cellular metabolites, e.g. S-adenosylmethionine (SAM), flavin adenine dinucleotide (FAD),  $\beta$ -hydroxybutyrate ( $\beta$ -OHB), acetyl-CoA, NAD<sup>+</sup>,  $\alpha$ -ketoglutarate, and adenosine triphosphate (ATP) as co-modifiers for epigenetic ‘writing’ (DNA and histone methyltransferases, lysine acetyltransferases) and ‘erasing’ (DNA demethylases, histone deacetylases, lysine demethylases) enzymes [63,64]. The metabolic state of an immune cell represents a highly dynamic process and changes between homeostasis and immune activation (tolerance versus inflammation), all of which has been documented in different macrophage phenotypes. Generally, resting macrophages and the ‘M2’ phenotype (involved in tissue repair and wound healing) utilize energetically efficient processes including an intact Krebs cycle with ATP generation via oxidative phosphorylation. Upon immune cell activation and a switch towards the ‘M1’ phenotype there is a rapid shift towards aerobic glycolysis, which enables de novo lipogenesis, cholesterol and amino acid synthesis for subsequent fast cell division and growth, and an increased production of inflammatory mediators (eicosanoids, IL-1 $\beta$ , reactive oxygen species) [65,66]. Hence, the cellular transcription machinery and its chromatin associated proteins process cellular metabolic signals to regulate gene expression.

The importance of immuno-metabolism in macrophage polarization and reprogramming suggests that similar mechanisms take place during the long-term acquirement of a trained innate memory phenotype. Indeed, a shift from oxidative phosphorylation towards aerobic glycolysis through the AKT-mTOR-HIF-1 $\alpha$  pathway, accompanied by changes in histone marks such as H3K4me1, H3K4me3 and H3K27ac, has recently been described to be important for training of human monocytes with  $\beta$ -glucan. Pharmacological inhibitors of glycolysis, such as 2-deoxy-D-glucose and inhibitors of the mTOR pathway, such as metformin, prevented  $\beta$ -glucan-induced trained innate immunity [62,67]. LPS-priming induced a shift towards aerobic glycolysis in murine macrophages, which resulted in the accumulation of the Krebs cycle intermediate succinate, which itself induced the expression of the inflammatory cytokine IL-1 $\beta$  through the transcription factor HIF-1 $\alpha$  [68]. These studies emphasize that Krebs cycle metabolites (such as succinate,  $\alpha$ -ketoglutarate or NAD<sup>+</sup>) are important in the induction or inhibition of histone-modifying enzymes, thus inducing long-term changes in the monocyte or macrophage phenotype.

How immuno-metabolism is altered under conditions of hyperlipidemia and which metabolites potentially contribute to the establishment of a trained myeloid (precursor) cell phenotype in atherosclerosis is still ill-defined. Transcription factors such as sterol regulatory element binding protein (SREBP; regulation of cholesterol biosynthesis and uptake), liver X receptor (LXR; regulation of cholesterol elimination and inflammation) and PPARs (regulation of fatty acid oxidation/energy homeostasis, lipid metabolism and inflammation) strictly control the balance between systemic and cellular lipid homeostasis by regulating genes involved in lipid uptake, storage, efflux, de novo lipid biosynthesis and catabolism, as well as inflammatory processes. Excessive lipid or cholesterol uptake by immune cells has been shown to favor inflammatory processes, which accounts for the exacerbated outcome of diseases associated with chronic metabolic inflammation (obesity, atherosclerosis). For example, overload of modified lipoproteins (oxLDL) in liver Kupffer cells induces a continuous state of intracellular stress and promotes a switch towards

the pro-inflammatory 'M1' phenotype [69]. Hyperlipidemia also modulates the immune system by enhancing bone marrow and extramedullary hematopoiesis through epigenetic reprogramming of genes crucially involved in monocyte proliferation and differentiation [29]. Somewhat unexpectedly, Spann and colleagues have recently shown that peritoneal macrophages isolated from Western type diet fed *Ldlr*<sup>-/-</sup> mice exhibited an overall reduction in pro-inflammatory gene expression compared with macrophages from chow fed mice, which was related to the accumulation of desmosterol, an intermediate metabolite in the cholesterol biosynthesis pathway and activator of LXRs [70]. Desmosterol accumulation in peritoneal foam cell macrophages might be explained by impaired functionality of desmosterol reductase, the metabolic enzyme for desmosterol degradation. Together, these data clearly illustrate that firstly a tight interplay between the different lipid sensing nuclear receptors is important to balance intracellular lipid and cholesterol homeostasis and to control immune signaling pathways, such as NF- $\kappa$ B, or Nlrp3. Secondly, the specific environment crucially contributes to metabolic processes, which critically influences inflammatory responses in different myeloid subsets.

## 5. Nutritional control of immunity

Diet and its components can activate cells of the innate immune system, thus profoundly influencing immune responses, and potentially inducing long-term epigenetic modifications and a 'trained innate memory', especially within the gut resident myeloid subsets. In line, the gut microbiota plays an important role in the development and maintenance of the immune system. The microbiota affects immune cell metabolism and function either through metabolites derived from its enzymatic machinery, such as short chain fatty acids (SCFA), bile acid, or trimethylamine (TMA), or through particular microbial molecules, such as innate immune receptor activating ligands [71–73]. Maternal and early-life dietary habits already play an important role in the development of the immune system, and the prevention of immunological disorders later in life [74]. The composition (quantity and quality) of free fatty acids and lipids (and their oxidative metabolites) in the diet is crucial in maintaining immune cell homeostasis and influences both innate and adaptive immune cell responses. Changes in dietary habits such as increased intake of energy-dense processed food enriched in sugars, fat, phosphatidylcholine (PC) and L-carnitine instead of nutrient-rich foods, induce a shift from commensal to more pathogenic bacterial strains within the gut. These changes in enteric microbiome composition correlate with an increased incidence of immunological disorders, such as asthma, cancer, allergies, autoimmune and cardiovascular diseases [75,76]. In contrast, diets enriched in complex carbohydrates and high fibers, such as found in vegetables, fruits and fish, promote gut homeostasis. These diets can beneficially influence amongst others the production of antimicrobial peptides and mucus, maintenance of epithelial integrity and tissue repair, production of anti-inflammatory cytokines and maintenance of immune tolerance. Mechanistically, the microbial enzyme machinery can process these fiber-rich food into SCFA, including *n*-butyrate, propionate and acetate, and can influence the production of other end products including idole-3-aldehyde, niacin and omega-3 fatty acids. Besides their function as energy sources, these metabolites control gut homeostasis by signaling through different receptors, e.g. the Aryl hydrocarbon receptor (AhR) and G-protein coupled receptors (GPCR), expressed on innate lymphoid cells, innate immune cells and Tregs. Food-induced epigenetic reprogramming in innate immune cells has been supported by a recent study, in which the SCFA *n*-butyrate led to the down-regulation of LPS-induced pro-inflammatory mediators, including nitric oxide, IL-6, and IL-12 [77]. These effects were independent

of TLR-signaling or activation of GPCRs, but were due to inhibition of histone deacetylases by *n*-butyrate. Overall, this rendered lamina propria macrophages hypo-responsive and maintained a tolerogenic environment.

Western type diets are enriched in poly-unsaturated fatty acids, associated with dysbiosis, a state of low-grade chronic inflammation in the intestines through activation of NF- $\kappa$ B and the Nlrp3 inflammasome, and increased intestinal permeability resulting in endotoxemia [78]. Indeed, studies in humans and animal models have shown that an accumulation of lipids and cholesterol, and an impaired lipid clearance in myeloid cells are associated with worse septic shock outcome due to increased TLR2 and 4 activation [79]. A systemic-wide metabolomics approach has recently shown that metabolites (choline, TMA, betaine) derived from the dietary phospholipid PC or L-carnitine represent risk factors for cardiovascular diseases. Indeed, increased systemic levels of these metabolites correlate with more aggravated CVD phenotype [80,81]. Consistent with these observations, *ApoE*<sup>-/-</sup> mice fed a diet enriched in PC or L-carnitine, exhibited worsened atherosclerotic disease outcome [80,81]. PCs and L-carnitine, enriched in red meat, eggs, milk and certain fish, are processed by the gut microbial enzymatic machinery to choline and TMA, which is further metabolized to TMA *n*-oxide (TMAO) by the hepatic flavin mono-oxygenase (FMO). TMAO has been shown to increase the expression of the scavenger receptors CD36 and SR-A1 on macrophages, subsequently promoting foam cell formation. Additionally, it adversely affects reverse cholesterol transport; both processes that promote atherosclerosis.

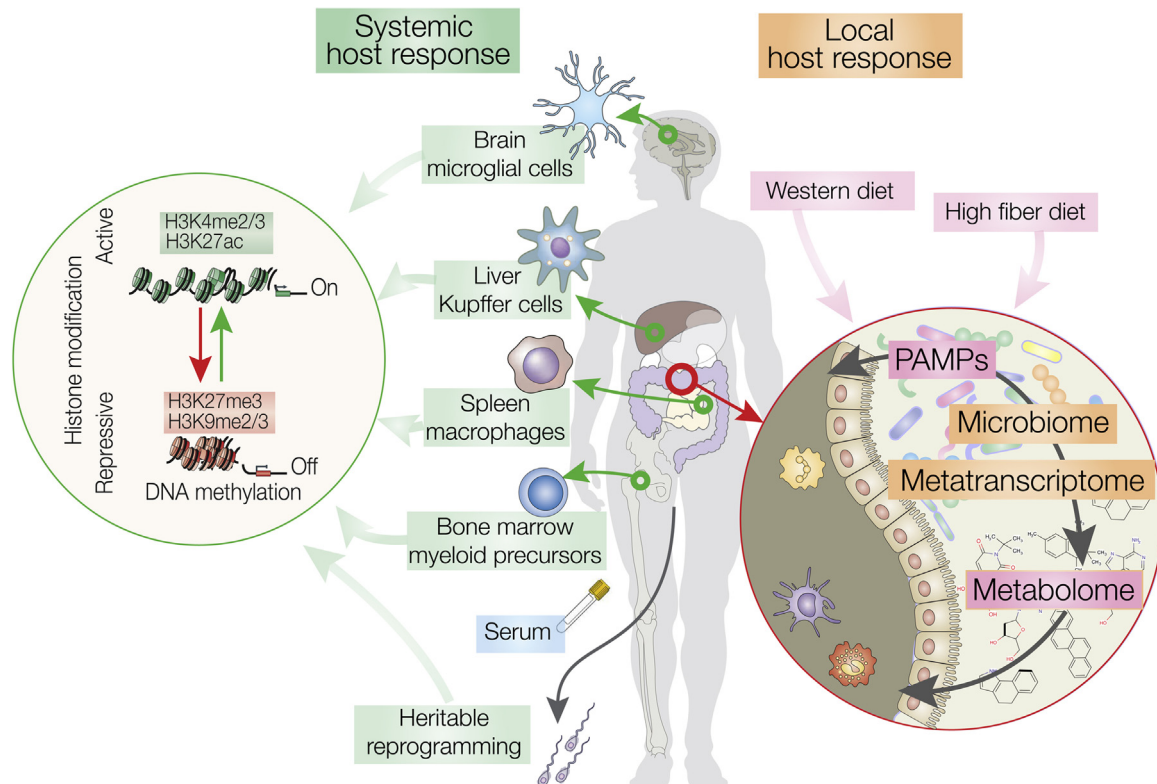
The above-summarized data illustrate the complex network between food composition, the microbiota and host's metabolism linked to immune homeostasis. Consequently, a balanced diet, rich in fibers, keeps a balanced microbiota and an intact intestinal barrier function, which is a requirement for a regulated metabolism and immune homeostasis (Fig. 2). It still remains to be resolved how systemic to cellular immune-metabolic crosstalk, impacted by the gut microbial composition, is working, and how this can lead to a long-term epigenetic reprogramming.

## 6. Therapeutic interventions in atherosclerosis and potential clinical relevance

### 6.1. Targeting LDL/cholesterol levels and inflammation in atherosclerosis

So far, statins are still used as the main drug treatment in CVDs to lower plasma cholesterol levels, thus decreasing disease burden. However, despite the LDL lowering effect up to >50%, a significant residual burden of CVDs remains. Alternatively, ApoA-I containing high density lipoprotein (HDL) is being developed as a therapeutic agent to remove excess cholesterol from peripheral tissue, and to dampen inflammation [82–85]. Studies in different animal models as well as in humans have shown the beneficial effect of HDL administration in reducing atherosclerotic plaque size [86–88]. Notwithstanding, infused HDL in two randomized controlled clinical trials in human have not reached the expected beneficial effects on atherosclerosis [89,90].

In the last years scientists have searched for more intelligent ways of drug delivery, but also for novel therapeutic targets to dampen atherosclerotic lesion progression. The use of nanoparticles seems to be an attractive therapeutic tool to deliver anti-inflammatory drugs to specific cell types with the aim to impair immune actions in certain cell subsets e.g. restricting myeloid cell differentiation and migration of pro-inflammatory Ly6C<sup>high</sup> monocytes to the plaque, dampening plaque macrophage immune signaling and increasing macrophage efflux. Some recent preclinical studies have successfully been proposed [91,92].



**Fig. 2.** Schematic representation of the hypothesis that diet can have long lasting effect on the systemic host response via epigenetic reprogramming by microbiome-derived PAMPs and metabolites.

## 6.2. Epigenetic remodeling as a potential therapeutic approach for CVD

The recent insights into possible mechanistic links between epigenetic cellular reprogramming and pathophysiology of atherosclerosis could lead to novel strategies to lower CVD risk [93,94]. Moreover, epigenetic modifications (DNA methylation pattern, modified histone marks) might evolve into useful predictors for disease stage [95] and have recently been linked to atherosclerosis in humans [30,96]. Wierda and colleagues evaluated global H3K27me3 levels, as well as levels of histone-modifying enzymes (EZH2, BMI1 and JMJD3) in vessels representing different stages of atherosclerotic plaque development. Overall, they notified a global decrease in H3K27me3 in the media of more advanced atherosclerotic plaques, which might reflect the dynamic pattern of vSMC differentiation and proliferation associated with atherosclerotic disease [30].

Co-modifying metabolites as well as ‘writing’ and ‘erasing’ enzymes are amenable to pharmacological modulations, and could potentially turn into novel therapeutic targets. A few nonselective HDAC inhibitors are already used in regular patient care in cancer treatment [97]. In addition, nonselective HDAC inhibitors have shown beneficial effects in animal models for arthritis, septic shock and inflammatory bowel disease [98]. Preclinical studies in mice support the idea that ‘epigenetic drugs’ may also be a potential tool in the treatment of atherosclerosis [99]. Administration of the nonselective HDAC inhibitor trichostatin A (a lysine deacetylase inhibitor) into *Ldlr*<sup>-/-</sup> mice induced a more exacerbated neointima formation by increasing CD36 mRNA, protein and surface expression levels thus increasing macrophage foam cell formation.

Histone demethylases can also induce a pro-inflammatory macrophage phenotype. For example, Jmjd3, UTX and Uty (which are H3K27 demethylases) are induced upon TLR engagement and are linked to the expression of inflammatory genes [100]. Target-

ing of Jmjd3 and Utx H3K27 demethylases with small molecule inhibitors impaired inflammatory responses in human primary macrophages, which is of high pharmacological interest [101]. However, Jmjd3 also plays an important role in the ‘M2’ polarization in the context of helminth infection, illustrating its dual role dependent on stimulus and environmental context [102]. Selective HDAC inhibitors may be used to dampen inflammatory responses within plaque macrophages. Three recent studies illuminated a functional role for certain HDACs in the context of atherosclerosis. Myeloid deletion of HDAC3, a histone deacetylase which promotes ‘M1’ polarization [103], was shown to augment lesion size, but lesions showed a more stable phenotype characterized by a collagen-rich fibrous cap, reduced lipid content and reduced numbers of plaque macrophages. The more stable plaque phenotype was associated with increased collagen formation by vSMC due to increased TGF- $\beta$  release by HDAC3 deleted myeloid cells [104]. In addition, a genome-wide association study illustrated the association between HDAC9 modifications and atherosclerosis development. In fact, certain HDAC9 transcripts are more related to coronary artery disease susceptibility [105]. Consistent with these data, Cao and colleagues revealed a correlation between HDAC9 deletion in bone marrow cells and limited atherosclerosis development in the *Ldlr*<sup>-/-</sup> mouse atherosclerosis model. HDAC9 deletion in macrophages was linked to an increased expression of the cholesterol efflux transporters ABCA1 and ABCG1, reduced inflammation, and a switch towards the ant-inflammatory ‘M2’ phenotype [106].

The above-mentioned studies support the impact of epigenetic remodeling in the pathophysiology of atherosclerosis, thus encouraging the development of epigenetic tools for beneficial lesion remodeling. Nevertheless, it has to be noted that different isoforms of histone modifying enzymes might have opposing effects within the same cells, but also in different cell types. Additionally, enzymes might function differently in healthy versus acute versus chronic

disease stages of atherosclerosis. A tremendous challenge within the next years will be to better understand epigenetics in the pathophysiology of CVDs and to design cell-specific epigenetic drugs that target cell subsets which only detrimentally contribute to disease progression. Another important challenge will be the correct timing of therapeutic intervention.

In addition to specific epigenetic inhibitors, further elucidation of the immune-metabolic basis of trained innate immunity, such as the metabolic shift that occurs, might provide alternative novel drug targets. Drugs that interfere with glycolysis (such as metformin, direct mTOR inhibitors, glycolysis inhibitors) prevent the occurrence of trained immunity in *in vitro* settings [67]. In animal models of neovascularization it has been revealed that glycolysis inhibition limits neovessel formation [107]. A number of small studies in patients with atherosclerosis have evidenced the systemic anti-inflammatory effect of metformin [108]. In addition, metformin inhibited monocyte to macrophage differentiation, and reduced pro-inflammatory cytokine production [109]. However, a real anti-atherosclerotic effect by metformin administration has not been proven yet. Although metformin treatment reduces cardiovascular morbidity and mortality in patients with diabetes compared to alternative glucose-lowering agents with similar glycemic control [110], metformin could not limit the intima media thickening in a recent trial in patients without diabetes [111].

## 7. Conclusion

There is accumulating evidence that many cardiovascular risk factors, both traditional and non-traditional (e.g. nutrients, microbiota), induce a long-term (epigenetic) reprogramming of cells of the innate immune system, which in turn may provoke a condition of continuous innate immune cell activation. It still remains an open question, which cellular processes act to initiate or maintain these alterations. An increasing number of studies support the idea of a close connection between systemic and cellular metabolic processes and epigenetic reprogramming in the induction of trained innate immunity. These findings pave the road for the identification of novel drug targets. Further mechanistic insights on the induction of trained innate immunity are necessary to make beneficial use in future clinical therapeutic applications. Especially, unraveling the mechanisms in the various stages of atherosclerosis will be a challenging task within the next years. Nevertheless, epigenetic remodeling and the use of 'epigenetic drugs' are a promising tool for future treatment of atherosclerosis.

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