Monocyte and Hematopoietic Progenitor Reprogramming as Common Mechanism underlying Chronic Inflammatory and Cardiovascular Diseases

Running title: Reprogramming of monocytes and their progenitors in CID and CVD

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Abstract

A large number of cardiovascular events are not prevented by current therapeutic regimens. In search for additional, innovative strategies, immune cells have been recognized as key players contributing to atherosclerotic plaque progression and destabilization. Particularly the role of innate immune cells is of major interest, following the recent paradigm shift that innate immunity, long considered to be incapable of learning, does exhibit immunological memory mediated via epigenetic reprogramming. Compelling evidence shows that atherosclerotic risk factors promote immune cell migration by pre-activation of circulating innate immune cells. Innate immune cell activation via metabolic and epigenetic reprogramming perpetuates a systemic low grade inflammatory state in cardiovascular disease that is also common in other chronic inflammatory disorders. This opens a new therapeutic area in which metabolic or epigenetic modulation of innate immune cells may result in decreased systemic chronic inflammation, alleviating cardiovascular disease and its co-morbidities.

Introduction

Cardiovascular diseases (CVD) are a major health challenge for modern societies. An estimated number of 17 million people die due to CVD each year, representing ≈ 30% of all deaths worldwide. The high burden of CVD is attributable to the increasing incidence of atherosclerosis, caused amongst others by worldwide adoption of the Western lifestyle[1]. In parallel, the incidence of chronic inflammatory diseases (CID) such as rheumatoid arthritis (RA) is rising. Since CID are accompanied by a 2 to 3-fold higher CVD-risk[2], the increased CID prevalence further contributes to the overall CVD burden. Traditionally, risk factors for atherosclerosis are considered to be dyslipoproteinemia, as well as smoking, hypertension, diabetes and obesity. Subsequently, therapeutic measures have focused on lowering the most atherogenic cholesterol, predominantly low-density-lipoprotein cholesterol (LDL-C), which has successfully lowered CVD-risk by 25 to 35%[3]. This success rate, however, also discloses a large residual risk not adequately addressed by current cholesterol-lowering treatment alone.

Atherosclerosis is a chronic inflammatory disease

Atherosclerosis, formerly considered a lipid storage disease, involves a chronic, low-grade inflammatory response of the arterial wall, initiated by lipid accumulation in the intimal layer[4]. Subsequent activation and recruitment of innate immune cells contributes to plaque progression, and eventually plaque destabilization[5]. In more detail, it has been demonstrated that following accumulation of cholesterol in the arterial wall, the subendothelial lipids are modified leading to the formation of active signaling moieties. Particularly oxidized derivatives trigger a variety of inflammatory pathways, immune cells and mediators[6] that drive atherogenesis and co-morbidities of this disease. As the atherosclerotic lesion advances, the presence of immune cells in the lesion increases proportionally, creating a localized pro-inflammatory milieu within the subendothelial compartment[7]. In addition to excessive amounts of lipids, atherosclerotic lesions thus harbor all classes of immune cells and moreover, serum levels of inflammatory mediators are linked to

coronary heart disease[8]. Recent advances in preclinical research have established a fundamental role for cellular inflammation throughout all stages of this disease from initiation through progression and, ultimately, the thrombotic complications following plaque rupture or erosion[5, 9-11]. A key role for innate immunity is illustrated by studies showing that monocytes and macrophages are abundantly present in atherosclerotic plaques[12], and moreover, inhibiting monocyte-entry into the plaque drastically attenuates atherogenesis[13] as well as CVD-risk[7], whereas immune cell stimulation accelerates atherosclerosis[10].

In parallel, systemic monocyte production and circulating monocyte number increase progressively with advancing disease[14, 15]. These innate immune cells help drive the formation of an extensive microvascular network penetrating the more advanced atherosclerotic lesions. These leaky, immature vessels provide an easy communication and access network for both cellular as well as humoral elements[16]. The extravasated immune cells also have an intricate impact on plaque phenotype by contributing substantially to endothelial dysfunction, oxidative stress and extracellular matrix degradation[7]. From a therapeutic perspective, several randomized clinical trials are currently underway that will determine whether nonspecific anti-inflammatory therapies with for instance interleukin-1-neutralising antibodies or methotrexate can reduce the risk of cardiovascular events in patients with atherosclerosis[17-19].

To date, little is known on the mechanism(s) maintaining the chronic inflammatory state in CV patients. Recent data have moved our focus from immune cells within the atherosclerotic plaque towards a systemic pro-inflammatory state, characterized by increased production and mobilization of innate immune cells by hematopoietic organs[10, 20], lipoprotein-driven activation of innate immune cells already within the plasma compartment[21], and rapid, increased influx of immune cells into atherosclerotic plaques in patients[22], collectively driving the inflammatory state in the atherosclerotic plaque.

Whereas the contribution of immune cells to atherogenesis has been well established, it remains elusive why the strong inflammatory response in the arterial wall persists over time. In the adaptive

arm of the immune system, dendritic cells in the plaque promote adaptive immune responses by presenting antigens, leading to enhanced T-cell function both in experimental and clinical atherosclerosis[23]. The interplay between activated T-cells and resident macrophages perpetuates a local inflammatory cascade within the subendothelial compartment[4, 24]. In addition, increasing body of evidence directs towards a role for impaired resolution of inflammation[25-27].

From a therapeutic standpoint, however, interventions targeting the adaptive immune system directly are hampered by a markedly increased incidence of infectious complications[28, 29]. A better understanding of the role of the innate immune system in the chronic inflammatory characteristics of atherosclerosis may overcome these limitations. However, boosting the adaptive immune system using vaccination strategies has therapeutic potential and is currently being studied for atherosclerosis treatment[23, 30].

Multi-level systemic immune cell activation contributes to atherogenesis and cardiovascular risk

The challenges in targeting inflammation in any chronic disease lie in three properties that are critical for evolutionary survival: redundancy, compensation and necessity. Targeting one component may not be sufficient to attenuate a pro-inflammatory reaction. Inflammation is a carefully tuned process that has multiple feedback loops; thus, inhibition of a critical pathway may trigger a compensatory pro-inflammatory response. Finally and most importantly, the inflammatory response is critical for host defense, and even if the previous two challenges have been met, the risk-benefit ratio may still be unacceptable, exposing for example to an elevated risk of infections. Accordingly, broad immune pathway targeting is unlikely to confer therapeutic benefits. Therefore, developing anti-inflammatory therapies involves identifying suitable pathways in mice, pre-clinical testing of whether the pathway can be neutralized without causing collateral damage, translational studies identifying similarly relevant pathways in humans, and, finally, clinical trials. This challenging drug discovery path can be negotiated more efficiently with the help of immune system imaging, especially of immune cells, their subsets and their migration, production and function.

The rate of progression of atherosclerotic lesions is critically dependent on the number and characteristics of the circulating monocyte pool[31]. In view of the short half-life of innate immune cells[11], attention has shifted towards a more prominent role of bone marrow mobilization and activation of immune cell progenitors[10] and local proliferation[11]. Recently, acute events, both myocardial infarction[10] and stroke[32] were found to elicit a spike in neutrophil and monocyte production in experimental models, which subsequently accumulated in the infarcted areas. In apoE$^{-/-}$ mice with advanced atherosclerosis, acute local events were also found to augment inflammation in atherosclerotic plaques at a distance, increasing plaque size and inducing a more vulnerable lesion morphology with higher inflammatory cell content. An increased supply of innate immune cells was identified as the principal driver for this phenomenon[10]. However, the residence time of innate immune cells in ischemic tissue was estimated to be 19 hours, implying the continued need for new immune cells to maintain the high cell number in atherosclerotic lesions[33]. To support this demand, in mice after coronary ligation, increased sympathetic nervous activity released upstream precursors from bone marrow niches[10]. Markedly, both the increased monocyte count and the pro-inflammatory changes in systemic plaques persisted for several months, the mechanism for which remains yet to be established.

In humans, several lines of evidence support similar pathways. The number of circulating innate immune cells was shown to be a strong predictor of future CV-risk in patients[34]. Following an acute myocardial infarction, bone marrow activity as well as inflammatory signals in distant atherosclerotic lesions were found to be increased[20], implying a systemic cellular mobilization comparable to experimental data[10]. In advanced atherosclerotic lesions, immune cell accumulation is also markedly increased, implying a role for systemic immune cell activity in maintaining the increased inflammatory state in the arterial wall[22]. Furthermore, not only the monocyte number, but also the monocyte phenotype may be affecting atherogenesis. In vitro, several atherogenic risk factors were shown to induce a persistent activated state in monocytes[35], which can further contribute to arterial wall inflammation.

Trained innate immunity and epigenetic reprogramming: a memory for innate immune cells

In the classical immunological paradigm, activation of the innate immune cells (monocytes and macrophages) provides a rapid first line defense against infectious episodes, followed by a 'memory' response mediated by the adaptive immune system. Recently, Netea *et al*. challenged the classical dichotomy of innate versus adaptive immunity[36-38] by showing that brief exposure to microbial products induces a long-term pro-inflammatory phenotype in monocytes, which was found to be linked to metabolic and epigenetic reprogramming. After vaccination of healthy subjects with BCG, monocytes showed a profound pro-inflammatory phenotype, that persisted even three months after vaccination and could be observed even several months after the initial exposure[39, 40]. The concept that the innate immune system is incapable of mounting adaptive responses[41] has already been contradicted by studies showing that organisms lacking a specific immune system are still capable of responding adaptively to infections[42]. Epigenetic reprogramming, accompanied by markers of histone modifications such as methylation of histone 3 at lysine residue 4 (H3K4) or H3K27 acetylation, has been proposed as the molecular mechanism responsible for long-term memory of innate immunity and this process has been termed 'trained immunity'[37, 40, 43].

Epigenetic control denotes the regulation of gene transcription without altering the nucleotide sequence of the DNA by the modification of the chromatin structure. These modifications result in specific chromosomal regions becoming more or less accessible to transcription factors, leading to prolonged alterations in downstream gene-products. There are many different epigenetic modifications that are tightly regulated by a wide array of specific epigenetic writers and erasers that function in a lineage specific manner[44, 45]. Importantly, these modifications are reversible, making chromatin modifying enzymes a potentially interesting therapeutic target[46]. The drug development area of inhibitors targeting histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) in various disease states is expanding rapidly[47, 48], though not yet in the cardiovascular arena. Interestingly, the epigenetic modulators suberoylanilide hydroxamic acid (SAHA, or Vorinostat) and

valproic acid (HDAC inhibitors) have already been approved by the FDA for cancer treatment and epilepsy, respectively[49, 50]. In the past years, the potential for epigenetic intervention by targeting specific histone methyltransferases has increasingly received attention[51].

In the cardiovascular domain, pro-atherogenic stimuli such as oxLDL and lipoprotein(a) [Lp(a)] have recently been shown to induce a prolonged, 'primed' or 'trained' state of the innate immune cells, already in the plasma compartment. In patients with elevated Lp(a), a major CV risk factor, monocytes showed a primed state by increased pro-inflammatory cytokine production upon ex vivo stimulation. *In vitro* priming of healthy monocytes with Lp(a) showed a training capacity: six days after first exposure to Lp(a), monocytes showed an increased pro-inflammatory phenotype. In vivo, this trained state coincided with activated monocytes which accumulated more rapidly into the arterial wall in vivo[52]. In line, oxLDL also induced trained immunity via epigenetic reprogramming, eliciting an activated monocyte phenotype. Incubation of monocytes with oxLDL was associated with increased H3K4me3[35]. The time course for the induction of these pro-inflammatory changes remains to be established. Because monocytes and macrophages have life spans in the order of hours to days, their upstream progenitors, including bone marrow hematopoietic progenitor cells (HSPCs), should also be investigated. Interestingly, recent findings by van der Valk *et al*. and Nahrendorf *et al.* did reveal a persistent inflammatory state in the arterial wall, as well as increased metabolic activity in the bone marrow more than 3 months after an acute myocardial infarction[53]. These findings, supported by experimental data, imply that epigenetic changes in short-lived monocytes are likely to be maintained by similar changes in the hematopoietic precursor cells in the bone marrow (HSPC)[53]. Detailed in vivo studies on HSPCs are, however, absent to date.

How do these pro-atherogenic stimuli modify epigenetic markers? Accumulating evidence points to a pivotal role for rewiring of intracellular metabolism in innate immune cells (Stienstra et al, Cell Metab 2017 in press). Various intermediate metabolites act as important cofactors for epigenetic enzymes, including NAD+, acetylCoA, SAM, FAD, fumarate[54-56]. Trained immunity by BCG or beta-glucan requires a shift from oxidative phosphorylation to aerobic glycolysis[57, 58]. In addition,

accumulation of fumarate due to anaplerotic replenishment of the TCA cycle by glutamine projects onto epigenetic changes by inhibition of the lysine demethylase KDM5[59]. The relevance of these metabolic changes for the development of atherosclerosis is illustrated by the upregulation of glycolytic pathways in monocytes and macrophages isolated from patients with atherosclerosis[60, 61]. For a more detailed description of the role of immunometabolism in the (epigenetic) reprogramming of innate immune cells in the context of atherosclerosis, we refer to recent excellent reviews on this topic[62] (Stienstra et al, Cell Metab in press 2017).

In parallel, in CID, the increased inflammatory responsiveness of monocytes and macrophages due to trained immunity is likely to play a central role[63]. The abundantly present danger associated molecular patterns (DAMPs) in CID and particularly in RA, including biglycan[64], S100 proteins[65], HMBG1[66], citrullinated proteins[67], heat shock proteins[68], and tenascin-C[69] might induce a trained state of innate immune cells, leading to prolonged hyper responsiveness mediated at least partly via histone modifications. Interestingly, synovial fibroblasts from affected joints in RA also showed distinct global and promoter-specific changes in DNA methylation[70], collectively pointing towards a role for epigenetic modulation being involved in the persistent pro-inflammatory state in CID.

From this perspective, the capacity of innate immunity to mount adaptive responses both redefines the function of innate immunity and provides a potential therapeutic target in chronic inflammation, including atherosclerosis and RA. This also echoes the highly feared recurrent CV-events in the first months after an initial CV-event, attributed largely to persistent inflammatory activity[71]. As active participants in arterial wall inflammation, 'trained' innate immune cells may represent promising therapeutic targets[72-74]. Targeting innate immune cells is likely to also offer a wider therapeutic window compared to the adaptive immune system, since patients with innate immune deficiencies are much less prone to infectious complications compared to those with disturbances in the adaptive immune system.

In the Horizon2020 project REPROGRAM[75], we propose that trained immunity is an important pathway promoting an activated state of innate immune cells in the context of atherosclerotic

cardiovascular disease. Hence, modulation of the trained immunity pathway may offer an attractive strategy to effectively attenuate the chronic inflammatory state in atherosclerosis as well as other CID (figure 1).

Common immune responses in atherosclerosis and CID

Many CIDs are associated with altered monocyte phenotype and function, which may alter the potential of these cells to influence atherogenesis[76]. For example, in patients with well-controlled human immunodeficiency virus (HIV), arterial wall inflammation in the aorta is increased, associated with circulating markers of monocyte and macrophage activation[77]. Furthermore, resident macrophages and Toll-like receptor (TLR) signaling play an important role in rheumatoid arthritis[78], atherosclerotic tissue[79] and acute coronary syndromes[80]. MRP8/14, a physiological TLR4 ligand released by activated macrophages, is a prognostic biomarker in both RA[81] and acute coronary syndromes[82]. In mice, experimental osteoarthritis induced increased levels of Ly6C-high, as well as Ly6C-low monocytes, due to increased bone marrow activity[83]. It has been recently proposed that therapeutically targeting interactions between TLRs and foam cell formation may reduce adverse cardiovascular outcomes in individuals with CID[84]. These studies have contributed to uncover novel molecular mechanisms that modulate the inflammatory response in atherosclerotic lesions, and suggest that a parallel exists with chronic inflammatory diseases, such as rheumatoid arthritis (Figure 2). Particularly, a prominent role for endogenous danger signals, comprising both pathogen-associated molecular patterns (PAMPs) as well as DAMPs, in the immunological pathophysiology is emerging rapidly[85, 86]. In RA tissues and synovial fluids, multiple DAMPs are present, most of which have the capacity to act as TLR2 and/or TLR4 agonists.

Several DAMPs have now been shown to contribute to the 'memory' response in monocytes, mediated by distinct histone modifications[87]. The correlation between RA disease activity/duration and the markedly increased inflammatory activity in the arterial wall in RA patients[88] lends further support to a common underlying pathophysiology, eventually translating into a 2 to 3-fold higher CV-

risk in RA patients which is only partly attributable to known CV-risk factors[89]. In support of immune cell hyperactivity as a causal factor of CVD in patients with RA, Bernelot Moens et al recently observed that circulating monocytes in RA patients in remission requiring continued biological therapy are characterized by increased expression of activation/adhesion markers, which coincides with increased arterial wall inflammation in RA patients[88]. A potential contribution of epigenetic modulation to the persistent inflammatory state in RA as well as its cardiovascular co-morbidity has been put forward[90], yet the evidence for this concept remains to be delivered.

Multimodal imaging in atherosclerosis and CID

The optimal method to evaluate the presence or progression of atherosclerosis is to directly visualize the target organ for atherosclerosis: the arterial wall. In line with this, imaging modalities have been used in patient studies to measure the dimension of the arterial wall, including ultrasound[91] and magnetic resonance imaging[92]. With the new insight that inflammation is a key component dictating both the progression of atherosclerotic lesions as well as the vulnerability of advanced plaques, attention has shifted towards novel imaging modalities able to quantify the functional aspects in the arterial wall/atherosclerotic lesions, including the inflammatory activity. In this context, 18-Fluordeoxyglucose positron emission tomography with computed tomography ($_{18}$F-FDG PET/CT) imaging is increasingly applied to serve as a measure of arterial wall inflammation[93]. Although its primary target is to detect overall metabolic activity, $_{18}$F-FDG imaging in patients with atherosclerosis robustly correlates with macrophage density as measured by histology[94] and correlates to plaque macrophage content and distribution[95]. It is increasingly used to monitor inflammation in vascular beds as a function of therapy in patients, particularly in the aorta and carotid arteries[96-99].

Clinical credence for PET-based imaging emerged after several retrospective[100, 101] and small-scale prospective studies showing that PET can identify active culprit lesions[102, 103] and predict the risk of a recurrent event. In addition, the first intervention studies have been performed using changes in $_{18}$F-

FDG uptake PET to monitor therapeutic efficacy of novel anti-atherosclerotic strategies[99, 104]. More recently, several small ^{18}F-FDG PET/computed tomography studies in patients with AMI reported increased PET signals in ischemic myocardial regions in association with higher ^{18}F-FDG uptake in remote non-culprit atherosclerotic lesions, as well as in hematopoietic organs[105, 106], highlighting the systemic inflammation following acute ischemic events, as previously described in pre-clinical models. A retrospective trial in patients with atherosclerosis reported that increased splenic ^{18}F-FDG also predicted higher cardiovascular event rates[20]. These trials exemplify the opportunities generated by whole-body imaging, including the ability to sample more than one organ system. In order to unravel systems-wide immune actions as well as connections between cardiovascular and hematopoietic organs, imaging studies allow integration of data from cardiovascular organs, non-culprit atherosclerotic lesions, spleen, and bone marrow, which can be combined with data derived from blood and bone-marrow analyses. In this scenario, whole-body multimodal imaging can translate preclinical findings, serve as companion imaging in clinical trials, and help guide individualized therapy.

Therapeutic implications of epigenetic reprogramming of innate immune cells

CID are the most common diseases of ageing and represent one of our major health threats. These include most forms of CVD, RA, type 2 diabetes and virtually all neurodegenerative diseases. In these disease states, a non-autoimmune primary pathological process determines disease progression; for example, inflammation promotes the formation of oxidized phospholipids that may serve as DAMPs in atherosclerosis[86]. As with autoimmune diseases, inhibition of inflammation could reduce the rate of disease progression to the point of substantial clinical benefit despite not altering the underlying pathogenic process. In contrast, in primary inflammatory or autoimmune diseases there is little evidence as yet for efficacy of this approach in humans.

The increased inflammatory responsiveness of monocytes and macrophages due to trained immunity is likely to play a central role in CIDs. From this perspective, the adaptive capacity of the

innate immune system provides a potential therapeutic target in human diseases. It is thus essential to improve our understanding of the pathophysiology and cellular and molecular mechanisms common to chronic inflammation, starting with atherosclerosis. Moreover, it is essential to understand the cellular and molecular mechanisms that mediate trained immunity, in hopes of harnessing their therapeutic potential. An important finding in that respect is that trained immunity is characterized by a metabolic shift from oxidative phosphorylation to aerobic glycolysis, which closely interacts with the epigenetic reprogramming[57]. This opens doors for new potential therapeutic possibilities. To prevent training, this mechanism can be targeted, for example using inhibitors of glycolysis[107], or inhibitors of micro RNAs that dictate the balance between glycolysis and OXPHOS[108].

New knowledge about inflammatory signaling, particularly in the areas of endogenous homeostatic pathways and inflammation resolution also provides the promise for new therapeutic options that can adequately meet the therapeutic challenges in CID and atherosclerosis. The abundant presence of epigenetic alterations in both CID and atherosclerosis underlines the potential for clinical applications. In line with this, the potential of epigenetic alterations as molecular biomarkers are being explored for CVD risk evaluation, early detection, prognosis stratification, and treatment response prediction. On the other hand, unlike genetic mutations, epigenetic changes, including DNA methylation and histone modifications, are pharmacologically reversible, making them attractive targets in therapeutic strategies.

Novel insights that will develop by a systems biology approach of trained monocytes is highly likely to identify novel therapeutic targets to prevent or treat atherosclerosis[72]. Several preclinical studies have already provided proof-of-concept data that drugs that modulate the activity of epigenetic writers or erasers, such as HDAC inhibitors, can modulate the development of atherosclerosis[109, 110]. Furthermore, BET-inhibitors such as RVX-208, JQ1 and I-Bet that inhibit the interaction of BET proteins with acetylated histone tails, showed a repression of pathways that contribute to cardiovascular disease and inhibition of atherogenesis in mouse models[111-113]. Major

advances have been made in the field of oncology with aberrant DNA methylation profiles and alterations in histone modification being linked to specific cancers and tumor progression, some of which are already used in the clinic[114]. The discrepancy between major advances in the oncology field and scarcity of data in the cardiovascular and rheumatology field illustrates that an organized effort to address the potential of epigenetic modulation in atherosclerosis and CID is long overdue.

In conclusion, innate immune cell activation via epigenetic reprogramming perpetuates a systemic low grade inflammatory state in cardiovascular disease that is also common in other chronic inflammatory disorders. This opens a new therapeutic area in which epigenetic modulation of innate immune cells will result in a decrease of systemic chronic inflammation, alleviating cardiovascular disease and its co-morbidities.

Legend to the Figures

Figure 1. Innate immune cell activation via epigenetic reprogramming as a common pathway perpetuating the upheld inflammatory state in atherosclerosis and other chronic inflammatory disease states.

Figure 2. Different chronic inflammatory disease states and atherosclerotic DAMPs can trigger monocyte phenotype to change into a long-term activated monocyte. In the bone marrow, these PAMPs and DAMPs might even alter the phenotype of the HSCs.

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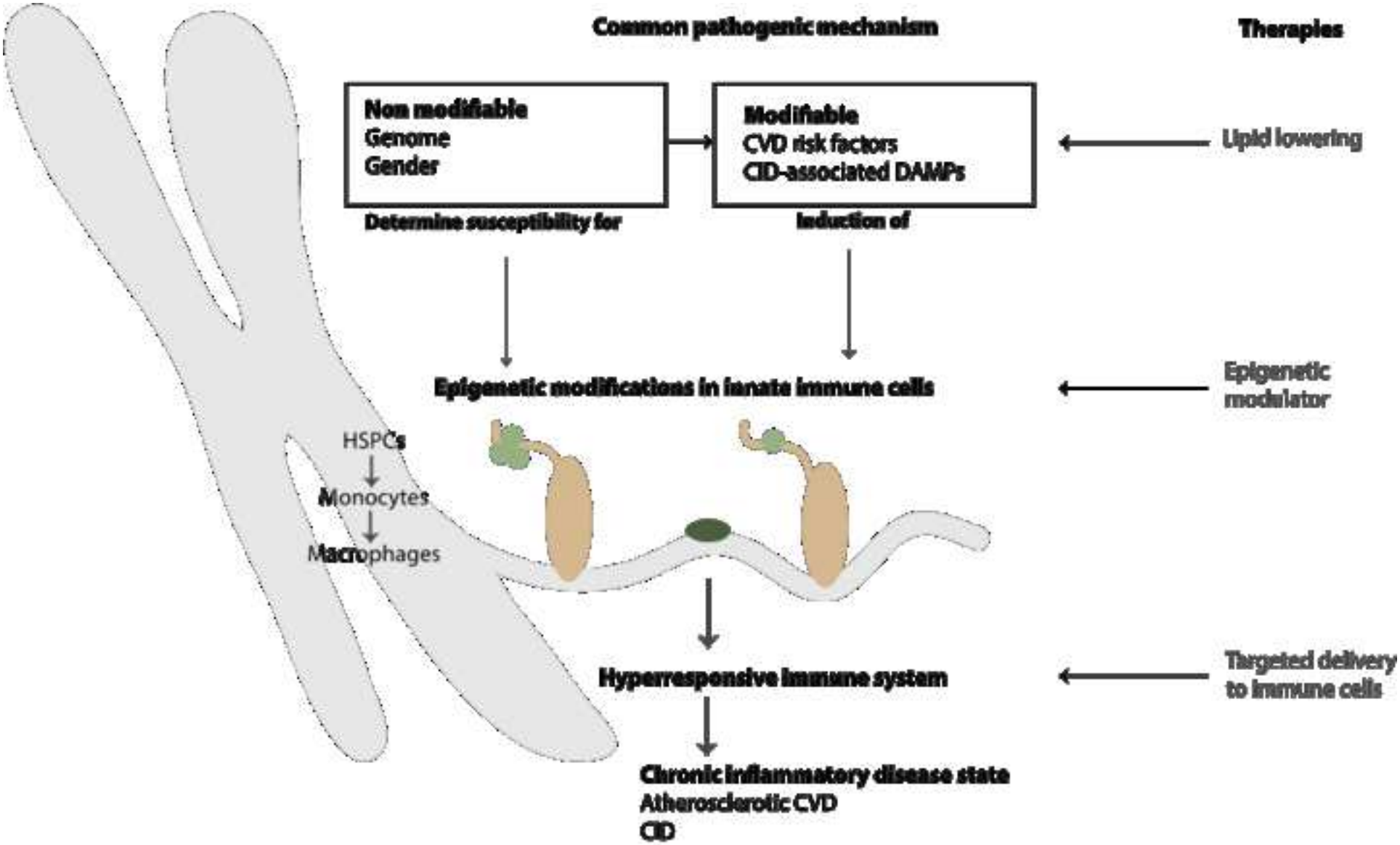

Figure 2

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