

Targeting epigenetic modifiers to reprogramme macrophages in non-resolving inflammation-driven atherosclerosis

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Received 19 April 2021; revised 28 June 2021; editorial decision 13 August 2021; accepted 14 August 2021; online publish-ahead-of-print 17 August 2021

Handling Editor: Daniel FJ Ketelhuth

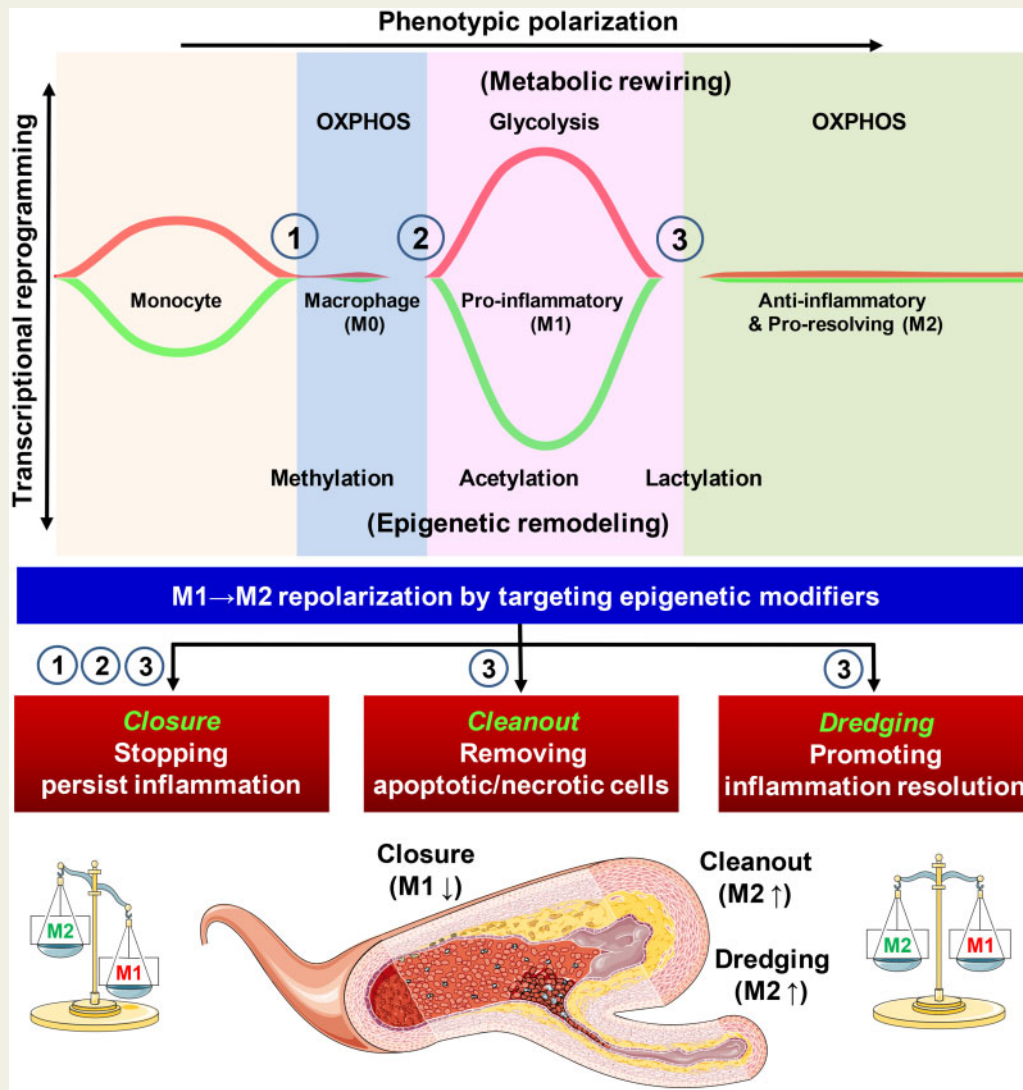
Epigenomic and epigenetic research has been providing several new insights into a variety of diseases caused by non-resolving inflammation, including cardiovascular diseases. Atherosclerosis (AS) has long been recognized as a chronic inflammatory disease of the arterial walls, characterized by local persistent and stepwise accelerating inflammation without resolution, also known as uncontrolled inflammation. The pathogenesis of AS is driven primarily by highly plastic macrophages via their polarization to pro- or anti-inflammatory phenotypes as well as other novel subtypes recently identified by single-cell sequencing. Although emerging evidence has indicated the key role of the epigenetic machinery in the regulation of macrophage plasticity, the investigation of epigenetic alterations and modifiers in AS and related inflammation is still in its infancy. An increasing number of the epigenetic modifiers (e.g. TET2, DNMT3A, HDAC3, HDAC9, JMJD3, KDM4A) have been identified in epigenetic remodelling of macrophages through DNA methylation or histone modifications (e.g. methylation, acetylation, and recently lactylation) in inflammation. These or many unexplored modifiers function to determine or switch the direction of macrophage polarization via transcriptional reprogramming of gene expression and intracellular metabolic rewiring upon microenvironmental cues, thereby representing a promising target for anti-inflammatory therapy in AS. Here, we review up-to-date findings involving the epigenetic regulation of macrophages to shed light on the mechanism of uncontrolled inflammation during AS onset and progression. We also discuss current challenges for developing an effective and safe anti-AS therapy that targets the epigenetic modifiers and propose a potential anti-inflammatory strategy that repolarizes macrophages from pro- to anti-inflammatory phenotypes.

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Graphical Abstract



Targeting epigenetic modifiers to repolarize macrophages in non-resolving inflammation-driven atherosclerosis. Based on an alternative model proposed for macrophage polarization, targeting epigenetic modifiers (e.g. for DNA methylation and histone methylation, acetylation, and lactylation) may repolarize macrophages straight from pro-inflammatory (M1) to anti-inflammatory/pro-resolving (M2) phenotype by transcriptional reprogramming of gene expression via epigenetic remodelling and metabolic rewiring, which thus represents a therapeutic strategy for developing novel anti-inflammatory therapy to treat non-resolving inflammation-driven diseases such as atherosclerosis.

Keywords

Epigenetic modifier • Macrophage • Polarization • Inflammation • Atherosclerosis

Introduction

Atherosclerosis (AS) is the common cause of cardiovascular and cerebrovascular diseases. AS-related acute events, such as heart attack and stroke remain the leading causes of death worldwide.¹ AS has been long recognized as a chronic inflammatory disease of arterial walls stemmed from abnormal lipid metabolism,² traditionally a main risk factor of AS. Recently, non-traditional risk factors, such as air

population, noise, disturbed sleep, age, and age-related clonal haematopoiesis of indeterminate potential (CHIP), have been emerging as novel drivers of AS, which is changing the landscape of AS.¹ Of note, inflammation drives AS by linking both traditional (e.g. lipoproteins, high blood pressure, smoking, obesity, and diabetes) and these non-traditional risk factors to the alterations of virtually all cell types in AS plaques, underlining the role of inflammation in atherogenesis. Unlike infection (e.g. caused by bacteria and viruses) or acute injury where

inflammation is self-limiting in most cases, AS is featured by persistent and stepwise worsening inflammation without resolution, primarily due to lacking the capability to transform inflammation into inflammation resolution, therefore known as non-resolving or uncontrolled inflammation.^{3,4} In this context, it is generally conceded that atherogenesis is intrinsically an inflammatory process driven primarily by the disproportionate polarization of macrophages, the most abundant type of immune cells in AS plaques. As a major type of innate immune cells in AS lesions, the alterations of macrophages result in the imbalance between inflammation and inflammation resolution, which plays an essential role throughout AS onset and progression until plaque rupture.^{5,6} With their high plasticity, macrophages can polarize to pro-inflammatory or anti-inflammatory/pro-resolving phenotype in response to different environmental stimuli.⁷ Emerging evidence supports a notion that, although the mechanism may vary upon diverse environmental cues, the direction of macrophage polarization to a distinctive phenotype is determined at the transcriptional level, a process so-called reprogramming.^{8,9} Moreover, transcriptional reprogramming is primarily governed by the epigenetic machinery via a mechanism named epigenetic remodelling, which involves DNA methylation and histone modifications (e.g. methylation, acetylation, and recently lactylation) as well as their epigenetic modifiers (including writers, erasers, readers, and remodelers), without genetic alterations of the DNA sequence.¹⁰ Despite recent findings on epigenetic alterations associated with the risk of AS, the investigation of functional epigenetics in AS remains relatively sparse.¹¹ Here, we present the current understanding of the epigenetic mechanisms regulating macrophage polarization and functions, as well as discuss the functions of epigenetic modifiers in governing macrophage polarization and the challenges of targeting them as an anti-inflammatory therapy in AS.

An overview of macrophage functions and anti-inflammatory therapies in AS

Phenotypic heterogeneity of macrophages in AS lesions

A traditional concept is that pro-inflammatory macrophages play a pivotal role in orchestrating the process of atherogenesis by initiating and accelerating inflammation response, while anti-inflammatory macrophage prevents disease progression or promotes regression by promoting resolution and tissue repair.⁶ While this concept was originated from an *in vitro* model that macrophages could polarize to either pro- (M1) or anti-inflammatory (M2) phenotype in response to Th1 or Th2 cytokines, this concept is over-simplified to describe the diverse roles of macrophages in atherogenesis because emerging findings indicate multiple functionally-different macrophage phenotypes in AS plaques. For example, various context-specific intermediate phenotypes between M1 and M2 have been observed, including M2 subsets (e.g. M2a, M2b, and M2c), M4, and Mox, among many others.¹²

Recently, the application of single-cell RNA sequencing (scRNAseq) and cytometry by time of flight (CyTOF) has not only confirmed high heterogeneity of macrophages in AS plaques but also

enabled more precise characterization of macrophages and identification of new macrophage subtypes,¹³ collectively called AS-associated macrophages (AAMs), leading to marked advances in the understanding of macrophage phenotypes as well as other immune cell types in AS lesions.^{14,15} In an elegant review article published recently, a meta-analysis of multiple scRNAseq and CyTOF studies involving three mouse models of AS (i.e. *Ldlr*^{-/-} mice, *ApoE*^{-/-} mice, and C57Bl/6 mice with progressing and regressing AS lesions after treatment with PCSK9 adeno-associated virus)^{16–20} has been performed to distinguish diverse immune cell types and subtypes in AS plaques.²¹ While the healthy aorta contains only resident macrophages, the number and diversity of macrophages increase with AS progression. At least five subsets of AAMs have been identified, including three major populations (i.e. resident-like, inflammatory, and TREM2 foamy macrophages) and two relatively small populations (i.e. IFN-inducible and cavity macrophages). Each of these subsets displays a unique transcriptomic profile (although there is some overlap between them), indicating their different functions in atherogenesis. Inflammatory macrophages (~47%; located in the intima and plaques shoulder) highly express the genes encoding chemokines (thus also named chemokine^{hi} macrophages)²⁰ and inflammatory cytokines (e.g. IL-1 β and TNF, two typical M1 cytokines); TREM2 foamy macrophages (~19%; located in the intima and necrotic core) are closely associated with lipid-laden foam cells,¹⁸ while lacking pro-inflammatory activity¹⁶; resident-like macrophages (~35%; located in the adventitia) are associated with aortic tissue-resident macrophages and express the genes functionally involved in endocytosis and proliferation.^{15,21} Unlike resident macrophages that are originated from yolk sac-derived embryonic precursors, both inflammatory (M1-like) and TREM2 foamy macrophages are most likely stemmed from circulating monocytes (e.g. CX3CR1⁺ cells).²⁰ Although the number of resident-like macrophages is increased in AS lesions compared with the healthy aorta, there is however no difference in their population proportion between progressive and regressive plaques,²⁰ arguing against a possibility that they play a primary role in the course of AS. In contrast, both inflammatory and TREM2 foamy macrophages are closely associated with atherogenesis. Moreover, scRNAseq and CyTOF analyses have revealed similar, although not identical, phenotypes of AAMs (e.g. inflammatory and TREM2 foamy macrophages) in human AS plaques as well.^{22,23}

In line with the imbalance between pro-inflammatory (M1-like) and anti-inflammatory (M2-like) macrophages observed in AS lesions, scRNAseq analysis of macrophages derived from CX3CR1⁺ monocyte precursors has also demonstrated heterogeneous activation states during AS progression, including IFN signature^{hi} macrophages with M1 features and Retnla^{hi}Ear2^{hi} macrophages with IL-4 signature (M2 feature).²⁰ Surprisingly, Retnla^{hi}Ear2^{hi} macrophages observed in progressing plaques are absent in regressing plaques. Of note, no anti-inflammatory (M2-like) phenotype has been demonstrated in other scRNAseq studies involving either mouse or human AS plaques,^{16,18,19,21–23} suggesting that this population may be too small to be identified or even lack in AS plaques. This possibility is consistent with the fact that M1 macrophages prevail over M2 in AS progression.²⁴ Together, these scRNAseq studies provide further evidence for phenotypic heterogeneity and functional diversity of AAMs. They also provide novel insights into the roles of macrophages (particularly pro-inflammation and foam cell formation) in atherogenesis,

therefore consolidating the basis for the ‘inflammation theory’ of AS. In sum, [Figure 1](#) outlines the main phenotypes and their functions of macrophages in AS lesions, together with a conceptual model for macrophage polarization and its regulatory epigenetic modifiers possibly involved in atherogenesis. High heterogeneity of plaque macrophages unveiled at single-cell level strongly challenges the dichotomic (M1 vs. M2) classification previously used to describe the phenotypes of AAMs. Functionally, earlier studies have shown that certain classical M2 inducers (e.g. IL-4 and IL-10) might have no anti-AS property but even be pro-atherogenic in some circumstances. For example, systemic deficiency or exogenous administration of IL-4 did not significantly affect AS development.^{25,26} Myeloid deletion of IL-10 receptor 1 (IL-10R1) decreased AS lesion size and severity by reducing intestinal cholesterol absorption, although it results in polarization of macrophages to a pro-inflammatory phenotype *in vitro*.²⁷ Therefore,

it is worth mentioning that M1 and M2 are hereinafter used as general terms for pro- and anti-inflammatory phenotypes of macrophages throughout this article (including figures and legends) only to facilitate reading.

Current status on the development of anti-inflammatory therapies in AS

The current standard of care for the treatment of cardiovascular AS diseases includes cholesterol-lowering agents (e.g. statins and recently approved PCSK9 inhibitors),²⁸ angiotensin-converting enzyme inhibitors, β -blockers, and aspirin, which are not specifically designed to target macrophages and thus may have limited effects on macrophage polarization. In the past two decades, numerous efforts have been made in developing anti-inflammatory therapy for AS, primarily aiming to prevent or reduce inflammation, including blocking

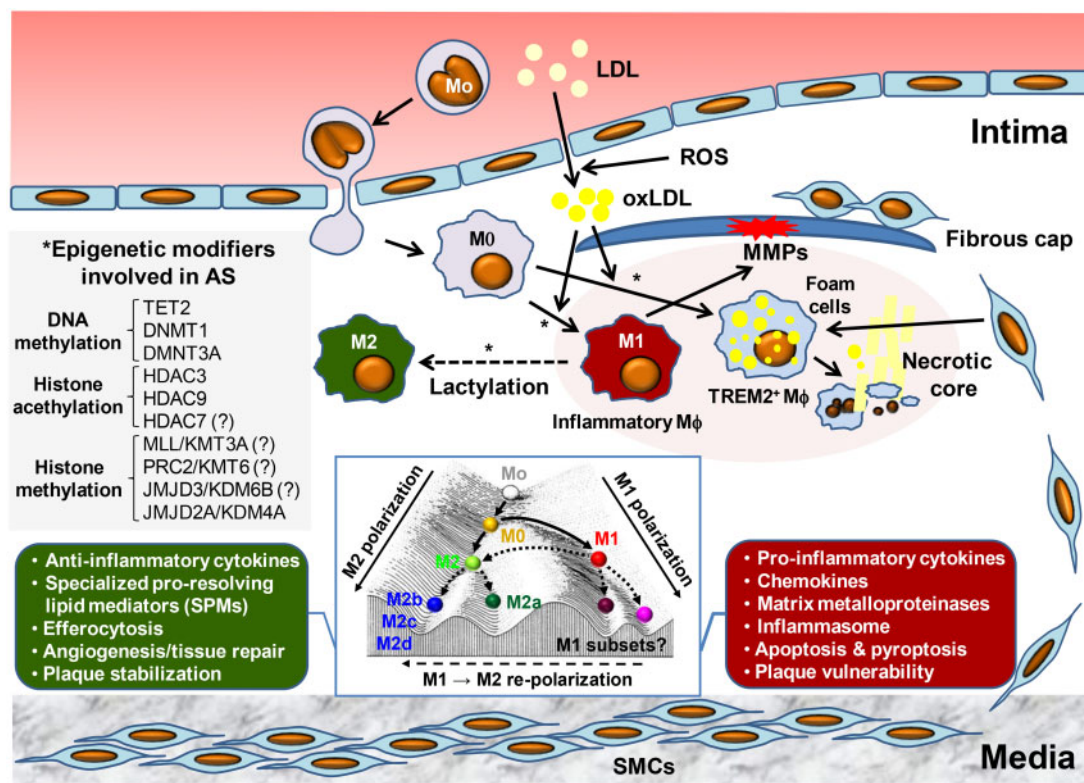


Figure 1 Dysregulated macrophage polarization and its related epigenetic modifiers in non-resolving inflammation of AS. In AS lesions, macrophages display heterogeneous phenotypes due to their high plasticity, which allows them to polarize towards either pro-inflammatory (M1) or anti-inflammatory and pro-resolving (M2) phenotype (the traditional model), or inflammatory (M1-like), TREM2⁺ foamy, and other subsets of macrophages (recently identified by scRNAseq; note: another major subset named resident-like macrophages with uncertain functions in AS are likely located in the adventitia, thus not shown in this graph) in response to different microenvironmental stimuli (e.g. oxLDL, hypoxia, ROS, etc.). In general, predominant polarization of macrophage to M1, together with the impaired capability to repolarize to M2 (homeostasis), leads to an imbalance between these two phenotypes of macrophages with multiple pro- (red box) vs. anti-AS functions (green box), which in turn drives atherogenesis and disease progression until plaque rupture. The direction of macrophage polarization is determined via transcriptional reprogramming of gene expression by the epigenetic machinery, including epigenetic modifications of DNA and histones (e.g. methylation, acetylation, and lactylation) or their regulatory enzymes (named epigenetic modifiers). The epigenetic modifiers identified thus far to be involved in the regulation of macrophage polarization and functions are listed in the grey box, while those with unknown functions in AS are indicated by question marks. Based on the inter-phenotypic transition (or trans-differentiation) described in the updated Waddington's epigenetic landscape, a possibility then arises that targeting these or other epigenetic modifiers (remaining to be explored) may repolarize M1 macrophages straight to M2 phenotype, which provide a strategy for developing anti-inflammatory therapy in AS.

inflammatory cell recruitment (e.g. by antagonists of chemokine receptors or adhesion molecules), stabilizing plaques (e.g. by inhibitors of matrix metalloproteinases), and neutralizing pro-inflammatory factors (e.g. by specific monoclonal antibodies against various cytokines and chemokines).²⁹ In a large randomized study (the CANTOS trial) involving more than 10 000 patients with previous myocardial infarction (MI) and high C-reactive protein levels, administration of the interleukin-1 β (IL-1 β) monoclonal antibody canakinumab reduced C-reactive protein levels and lowered the incidence rate of recurrent cardiovascular events, without affecting the low-density lipoprotein (LDL) cholesterol level; however, no significant difference in all-cause mortality was observed, probably due to increased risk of fatal infection and sepsis with canakinumab.³⁰ Thus, the CANTOS study has approved, for the first time, the 'inflammation hypothesis' of AS in the clinical setting, though IL-1 β might not be an ideal target.³¹ Based on the same consideration, several large clinical studies have recently been conducted to investigate the efficacy and safety of various anti-inflammatory agents, including colchicine (the COLCOT and LoDoCo2 trials),^{32,33} cyclosporine (the CIRCUS trial),³⁴ rosuvastatin (the JUPITER trial),³⁵ and methotrexate (the CIRT trial),³⁶ in patients with AS diseases. The majority of them (e.g. colchicine, cyclosporine, and rosuvastatin) have shown to lower the risk of cardiovascular events, in association with reduced levels of inflammatory factors in the blood, while a few (e.g. methotrexate) fail to do so likely due to their incapability to reduce inflammation.

As abnormal levels of lipoproteins (e.g. LDL, VLDL, and particularly triglyceride-rich lipoproteins) are closely associated with inflammation in AS lesions, the anti-AS activity of cholesterol-lowering agents is derived, at least in part, from their anti-inflammatory property. In addition to statins, many novel agents have been investigated to lower 'bad' cholesterol (e.g. LDC-C) in AS patients with hypercholesterolaemia. For example, ezetimibe (a non-statin drug that reduces intestinal cholesterol absorption via inhibition of the sterol transporter NPC1L1)³⁷ and PCSK9 monoclonal antibodies (e.g. evolocumab and alirocumab,²⁸ which reduce circulating PCSK9 that binds to and degrades the LDL receptors on hepatocytes)³⁸ have been approved to treat AS-related diseases. Other approaches seem also promising, including bempedoic acid (an adenosine triphosphate-citrate lyase inhibitor that reduces cholesterol synthesis),³⁹ pemafibrate (a PPAR α modulator),⁴⁰ evinacumab (a monoclonal antibody against ANGPTL3 that inhibits lipoprotein lipase, an enzyme involved in the hydrolysis of triglyceride-rich lipoproteins),⁴¹ AKCEA-APO(a)-LRx [a hepatocyte-directed antisense oligonucleotide that reduces lipoprotein(a)],⁴² Icosapent ethyl (purified eicosapentaenoic acid ethyl ester that lowers triglyceride levels),^{43,44} and eicosapentaenoic acid (an omega-3 fatty acid).⁴⁵ Alternatively, infusion with reconstituted high-density lipoprotein (HDL) also has an anti-inflammatory effect on AAMs in *ApoE*^{-/-} or *Ldlr*^{-/-} mice fed a Western diet,⁴⁶ suggesting an anti-AS therapeutic benefit. However, several approaches that raise 'good' cholesterol (e.g. HDL-C), including niacin (also known as vitamin B₃), cholesteryl ester transfer protein (CETP) inhibitors,⁴⁷ and HDL mimetics,⁴⁸ have no clear benefit in the reduction of cardiovascular events. One possible explanation is that HDL particles may lose their atheroprotective functions in patients with hypercholesterolaemia, an event named HDL remodelling.⁴⁹

An effective anti-inflammatory therapy for AS may need both to inhibit inflammation (e.g. by reducing M1 macrophages) and to

promote resolution (e.g. by increasing M2 macrophages). This can then break the inflammatory vicious circle underlying non-resolving inflammation in AS lesions.⁵⁰ Meanwhile, M2 macrophages remove apoptotic or necrotic cells via efferocytosis, a step critical for inflammation resolution. In this context, while deficiency of efferocytosis due to high expression of the 'don't eat me' signalling molecule CD47 in advanced plaques is correlated with AS progression, the CD47-blocking antibodies significantly prevent disease progression by restoring the capability of M2 macrophages to remove apoptotic cells via efferocytosis.⁵¹ In fact, the impairment of resolution may be the leading reason for uncontrolled inflammation, which drives AS progression and plaque vulnerability or rupture that triggers acute ischaemic events.²⁻⁴ Therefore, while lesion regression is clinically desirable in AS, this goal may be achieved by redirecting the polarization of macrophages from M1 to M2 phenotype,^{52,53} a process called repolarization hereinafter.

Macrophage repolarization as a potential basis for developing a novel anti-inflammatory therapy in AS

To understand how macrophages are reprogrammed during their differentiation and polarization, we performed a genome-wide survey to compare the gene expression profiling (GEP) of M0 (a resting state), M1, and M2 macrophages, based on transcriptomic data from the macrophage maturation and polarization experiments [GSE5099; Exp Macrophage (Polarization)—Estrada—15—MAS5.0—u133a],⁵⁴ public available on the R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>). The phenomena observed are as follows: (i) the majority of differentially expressed genes (DEGs) are found between M0 and M1 (reflecting M1 polarization), most of which (~84%) are reversed in M2 compared to M1 (presumably reflecting M1→M2 repolarization) (Figure 2A); (ii) among these DEGs, up-regulated genes are the most significantly enriched for proteasome, nuclear factor- κ B (NF- κ B), JAK/STAT, and apoptosis, while down-regulated genes are the most significantly enriched for lysosome, oxidative phosphorylation (OXPHOS), and PPAR (Figure 2B); (iii) interestingly, these pathways are also involved in the differentiation from monocytes to macrophages (Figure 2C); and (iv) of note, the DEGs include various epigenetic modifiers involving histone PTMs (e.g. acetylation and methylation; Figure 2D), among which there are extensive interactions (Figure 2E) that may be required for precise regulation of macrophage polarization. These observations are summarized in a conceptual graph to illustrate the dynamic changes in transcriptional reprogramming for macrophage differentiation, M1 polarization, and potentially repolarization that transforms M1 straight to M2 likely by turning off the M1 programme (Figure 3A). As shown in Figure 3B, an alternative model is proposed (middle panel) by integrating this inter-phenotypic transition (M1→M2 repolarization, an event similar to trans-differentiation described in the updated Waddington's landscape) into the traditional model of macrophage polarization (left panel). Moreover, a comprehensive model (right) can cover the entire process of macrophage evolution, including differentiation/maturation, polarization, and repolarization. This theoretical model raises speculation that the deficiency of repolarization may be a cause for the imbalance between M1 (increased) and M2 macrophages (decreased), a mechanism underlying non-resolving

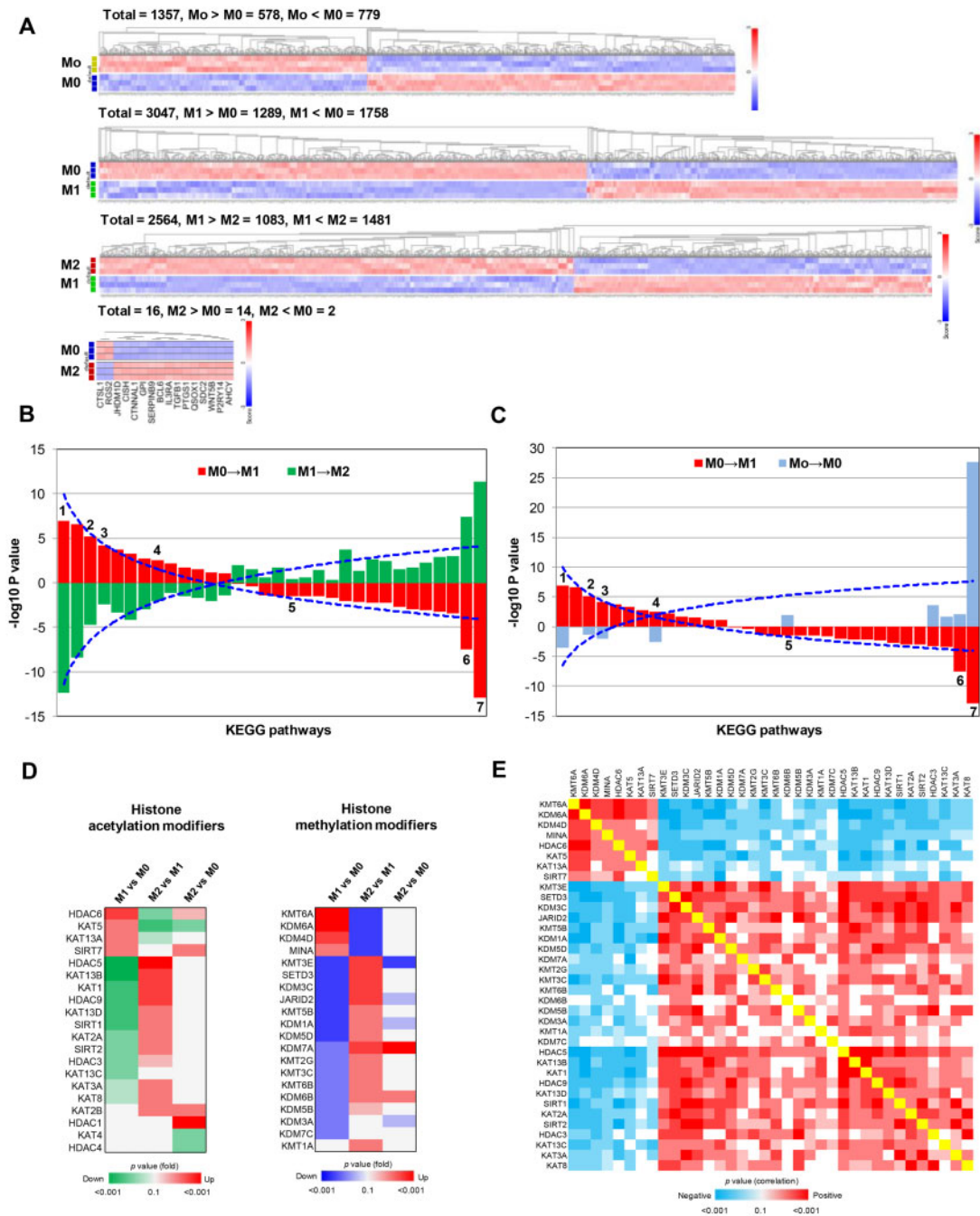


Figure 2 Gene expression profiling for understanding transcriptional reprogramming of macrophage polarization. The analysis is based on a transcriptomic dataset [GSE5099; Exp Macrophage (Polarization)—Estrada—15—MAS5.0—u133a] public available on the R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>). The data were acquired by transcriptional profile analysis using the Human Genome U133 A and B arrays (HG-U133; Affymetrix, containing a total of 39 000 transcripts) from freshly isolated human monocytes (Mo), which were then cultured in the presence of M-CSF (100 ng/mL) for 7 days to differentiate into macrophages (M0), followed by incubation for an additional 18 h with IFN- γ (20 ng/mL) plus LPS (100 ng/mL) for M1 macrophages or with IL-4 (20 ng/mL) for M2 macrophages (see Reference⁵⁵). (A) Mo, M0 (a resting state), M1, and M2 phenotypes display distinct gene expression profiles (GEPs), containing numerous differentially expressed genes (DEGs), with the most DEGs in M1 when compared to either M0 (reflecting M1 polarization) or M2 (presumably reflecting M1 to M2 repolarization) but the least in M2 compared to M0 (reflecting M2 polarization). (B) Functionally, up-regulated DEGs involving M1 polarization are enriched for proteasome (1), NF- κ B (2), JAK/STAT (3), and apoptosis (4), while down-regulated DEGs are enriched for PPAR (5), oxidative phosphorylation (6), and lysosome (7). However, these changes are reversed during M1 to M2 repolarization. (C) These pathways (1–7) are also involved in the differentiation of Mo to M0 macrophages, although to a lesser extent. (D) Among these DEGs, there are many histone epigenetic modifiers, which may represent candidate targets in epigenetic remodelling of macrophage phenotypes. (E) There are also extensive correlations among these epigenetic modifiers, suggest an extensive network across two different types of histone PTMs (e.g. methylation and acetylation) in transcriptional reprogramming of macrophage polarization and repolarization.

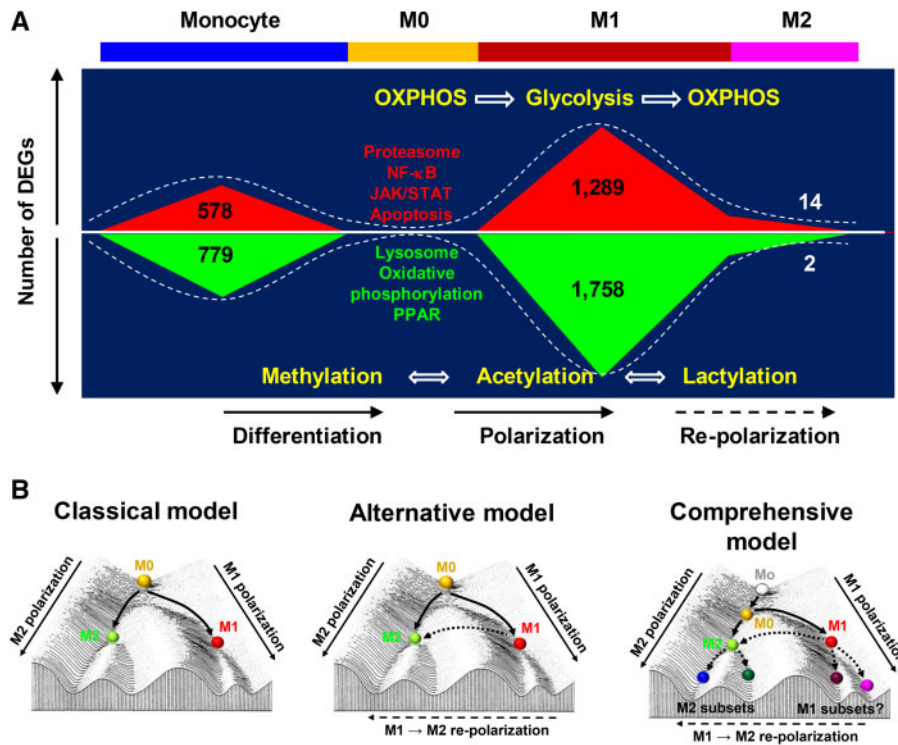


Figure 3 An alternative model for macrophage polarization proposed based on transcriptional reprogramming. (A) Following dynamic changes of the DEGs (mainly involving seven pathways) identified in Figure 2, a continuous process is hypothesized to illustrate the flow of transcriptional reprogramming for macrophages differentiation, polarization, and repolarization, which matches with metabolic rewiring during phenotypic transitions of macrophages from M0 (OXPHOS) to M1 (glycolysis) and M1 to M2 (OXPHOS). Values indicate the number of DEGs. (B) According to these changes of DEGs, an alternative model (middle) is thus proposed by integrating M1→M2 repolarization into the classical model of macrophage polarization (left) based on Waddington's landscape. Furthermore, a comprehensive model (right) is presented to cover the full process of macrophage evolution from maturation through polarization to repolarization.

inflammation in AS lesions. However, this problem may be fixed via restoration of M1→M2 repolarization (e.g. by targeting epigenetic machinery that governs this event).

One of the mechanisms that drive macrophage repolarization seems to be associated with histone lactylation, a novel form of epigenetic modification.⁵⁵ While histone lactylation occurs in the late stage of M1, it is related to the expression of M2 genes, accompanied by the silencing of M1 genes. Notably, histone lactylation in macrophages is mediated primarily by lactate, a 'byproduct' of glycolysis. During M1 polarization, the lactate level in macrophages is increased due to a metabolic paradigm shift from OXPHOS to glycolysis, an event named metabolic rewiring; in turn, increased intracellular level of lactate triggers histone lactylation, promoting M2 gene expression,⁵⁶ an event in agreement with M1→M2 repolarization. Therefore, these findings support a possibility to switch M1 directly to M2 by targeting the key modifiers or modulators that govern epigenetic remodelling or metabolic rewiring during macrophage polarization. In this context, although IL-4 fails to repolarize M1 into M2, likely due to M1-associated inhibition of OXPHOS, therapeutic inhibition of nitric oxide (NO) production can restore mitochondrial function (OXPHOS) to drive metabolic reprogramming and thus

phenotypic repolarization toward M2.⁵⁷ Nevertheless, it is important to understand the mechanisms underlying AAM (re)polarization and thereby identify the potential targets (e.g. epigenetic modifiers) as a molecular 'switch' that controls the reprogramming of repolarization from pro-AS phenotypes, including inflammatory (M1-like) and TREM2 foamy (foam cell-prone) macrophages identified at the single-cell level, to anti-AS phenotype(s).

Epigenetic modifiers in macrophages emerging as anti-inflammatory targets in AS

Both transitions of cell phenotypes and maintenance of cell identity are governed by the epigenetic machinery via reprogramming of gene expression at the transcriptional level.⁵⁸ Epigenetics has been studied in the context of chromatin modifications on either DNA or histones.⁵⁹ They include cytosine methylation of DNA at CpG sites (e.g. 5mC, 5hmC, 5fC, 5caC, 3mC, and 6mA) and histone post-translational modifications (PTMs) primarily at lysine residues (e.g.

methylation, acetylation, phosphorylation, ubiquitination, sumoylation, butyrylation, formylation, propionylation, citrullination, crotonylation, proline isomerization, ADP ribosylation, succinylation, 2-hydroxy isobutylation, and lactylation). Other epigenetic mechanisms also involve non-coding RNA, such as lncRNA, microRNA, and circRNA. DNA methylation is regulated by DNA methyltransferases (e.g. DNMT1, DNMT2, DNMT3A, and DNMT3B) and ten-eleven translocation methylcytosine dioxygenases (e.g. TET1, TET2, and TET3). The most common histone PTMs are methylation and acetylation, both of which are reciprocally regulated by two classes of histone-modifying enzymes, i.e. 'writer' [lysine methyltransferase (KMT) and histone acetyltransferase (HAT)] and 'eraser' [lysine demethylase (KDM) and histone deacetylase (HDAC)].⁶⁰ Histone PTMs result in a 'loose' (open) or 'tight' (close) chromatin configuration, which affects the accessibility of transcriptional factors to the promoter or enhancer regions of target genes, thereby controlling their expression. In addition, the third category of the epigenetic modulators is named 'reader', which recognizes epigenetic codes and recruit transcription-regulatory factors to initiate the transcription of target genes. They consist of two families, i.e. bromodomain and extraterminal protein (BET) and malignant brain tumour domain protein (MBT, including MBT, chromodomain, and Tudor domain, which recognize histone methylation). BET can recognize histone acetylation at the promoter regions of target genes through their distinct bromodomain (BRD) and then recruit positive transcription elongation factor b (P-TEFb, a complex of cyclin-dependent kinase 9/CDK9 and cyclin T), which in turn phosphorylates the C-terminal domain (CTD) of RNA polymerase II to trigger transcription initiation and mRNA elongation.⁶¹ With recent advances in the development of specific BRD4 inhibitors (e.g. JQ1 and I-BET151) in cancer treatment, targeting the BET family members has also attracted a lot of attention in various inflammatory disorders including AS. Another emerging category of epigenetic modulators is called 'remodeler' (also known as nucleosome remodelling factor/NURF), including at least four subfamilies: the switch/sucrose non-fermenting (SWI/SNF), imitation switch (ISWI), inositol requiring 80-like (INO80-like), and chromodomain helicase DNA-binding families.⁶² The remodelers are recruited to their target regions by sequence-specific regulatory proteins (e.g. transcription factors) or non-coding RNAs and then form multiple ATP-dependent chromatin remodelling complexes that coordinately regulate DNA structure precisely and accurately in time and space to facilitate transcription. Although the role of the remodelers in macrophages remains unclear, recent characterization of their structures may, however, facilitate drug development for various diseases including inflammatory diseases, such as AS.^{63,64} Together, all epigenetic molecules can functionally be re-divided into three categories⁵⁹: (i) epigenetic modifiers, which directly control DNA methylation (e.g. DNMTs and TETs), histone PTMs (e.g. KMTs and KDMs for methylation; HATs and HDACs for acetylation), or higher-order chromatin structures; (ii) epigenetic mediators, which are targets of epigenetic modifiers and in turn act to govern cell plasticity and phenotypes via cellular reprogramming; and (iii) epigenetic modulators, which influence the activity or subcellular localization of epigenetic modifiers and link the environment to the epigenome.

Emerging evidence supports a notion that the epigenetics may bridge the gap between risk factors (either traditional ones, e.g. lipoproteins, blood pressure, smoking, obesity, diabetes, hyperlipidaemia,

metabolic stress, hypoxia, and oxidative stress, or non-traditional ones, e.g. air pollution, noise, disturbed sleep, age, and CHIP)¹ and non-resolving inflammation observed in AS. The following sections summarize the epigenetic modifiers and their functions identified thus far to be associated with the regulation of macrophage polarization, particularly those involved in AS. These and other epigenetic modifiers, many of which remain to be identified, may be considered as candidate targets for the development of anti-inflammatory therapy to treat non-resolving inflammation-driven diseases like AS.

Epigenetic modifiers for DNA methylation

Fast-accumulating evidence indicates a close relationship between epigenetic alterations and the risk of AS. AS lesions display a distinguishable whole-genome landscape of DNA cytosine methylation and a specific methylation profile involving 1895 CpG sites, almost completely different from nearby normal blood vessel tissue.⁶⁵ An EWAS (epigenome-wide association study) analysis has also revealed a different methylation pattern involving 211 CpG sites associated with MI, but not a stroke,⁶⁶ suggesting location- or disease-specific DNA epigenetic alterations in AS. A genome-wide DNA methylation sequencing analysis has revealed a significant correlation between aberrant DNA methylation and the histological grade of AS lesions, indicating a disease progression-specific CpG methylation profile.⁶⁷ Another DNA methylome analysis has demonstrated that widespread demethylation occurs after cerebrovascular events and correlates with the post-cerebrovascular event time, in association with the increased expression of genes involved in anti-inflammation and plaque stabilization, although only a few CpGs display different methylation between carotid plaques from symptomatic and asymptomatic patients.⁶⁸ Together, these findings suggest a positive relationship between global DNA methylation and disease progression, as well as the potential significance of dynamic epigenetic alterations during AS pathogenesis from onset to vascular events and even post-event plaque stability.

In addition to global DNA methylation alterations, an increasing number of studies have also indicated the relationship between gene-specific DNA methylation and AS or its treatment. For example, lower methylation at a CpG site mapped to *TRAF3* (tumour necrosis factor receptor-associated factor 3) gene (cg03548645) is associated with disease recurrence after clopidogrel treatment in AS patients; in contrast, higher methylation of *PPM1A* (protein phosphatase Mg²⁺/Mn²⁺-dependent 1A) gene (cg04985020) correlates with disease recurrence after aspirin treatment in AS patients.⁶⁹ Taking advantage of rapid advances in single-cell sequencing technology, cell-type-specific profiles of either global or target gene-specific DNA methylation at single-cell resolution (e.g. macrophage, endothelial cell/EC, smooth muscle cell/SMC, and lymphocyte) may soon provide even more meaningful insights into the functional and clinical significance of DNA methylation in AS.

Although a paradigm shift from profiling epigenomics to functional epigenetics represents a current trend in many research fields of epigenetics,⁷⁰ the functional roles of the epigenetic modifiers for DNA methylation in macrophages remain largely uncertain in non-resolving inflammation-driven diseases including AS. Macrophage-specific expression of DNMT1 (a DNA

methyltransferase responsible for maintaining *de novo* DNA methylation) aggravates AS progression by promoting the production of M1 cytokines (e.g. TNF- α , IL-1 β , and IL-6) but suppressing the expression of M2 genes (e.g. IL-10) in *ApoE*^{-/-} mice, in association with down-regulation of PPAR γ or KLF4 (Krüppel-like factor 4) via enhancing methylation of their promoters.^{71,72} The expression of DNMT3A (a DNA methyltransferase catalysing *de novo* DNA methylation) is inhibited in M2 macrophage, likely by a lncRNA named DNMT3aos that locates on the antisense strand of DNMT3A.⁷³ Notably, DNMT3A represents one of the most commonly mutated genes in coronary heart disease (CHD) patients carrying CHIP.⁷⁴ Furthermore, transplantation of *Ldlr*^{-/-} mice with bone marrow from DNMT3A^{-/-} mice results in a marked increase in AS lesion size.⁷⁵

TET2 catalyzes DNA demethylation to promote gene expression,⁷⁶ TET2 also modifies histone O-GlcNAcylation,⁷⁷ suggesting its dual roles in the regulation of both DNA methylation and histone PTMs. In macrophages, TET2 specifically represses the expression of IL-6 via recruiting HDAC2, an event required for inflammation resolution.^{78,79} Partial reconstitution of bone marrow clonal haematopoiesis by transplanting *TET2*-mutant cells increases plaque size in *Ldlr*^{-/-} mice, in association with the production of IL-1 β by *TET2*-mutant macrophages via the NLRP3 (NLR containing family pyrin domain containing 3) inflammasome.⁸⁰ In line with this finding, loss-of-function mutations of *TET2*, the second most common mutation in CHD patients carrying CHIP, are associated with increased risk of CHD (e.g. early onset of MI).⁷⁴ Interestingly, the phenotype for *TET2* inactivated mutations resembles that for *DNMT3A* deficiency described above, despite the opposite functions of these two epigenetic modifiers in the regulation of DNA methylation. Nonetheless, these findings may link genetic alterations (e.g. somatic mutations) with abnormal epigenetic regulation in macrophages, in association with non-resolving inflammation. They also provide direct evidence supporting that the epigenetic modifiers (e.g. TET2 and DNMT3A) of DNA methylation in macrophages represent potential targets for anti-inflammatory therapy in AS.

Whereas most epigenetic modifications are reversible, they can also propagate the epigenetic memory of past cellular states and perturbations without changing the DNA sequence, thereby rapidly triggering an enhanced inflammatory response once cells encounter the next pro-inflammatory cue.¹¹ In this context, atherogenic Western diets induce innate immune memory via NLRP3 in monocytes and macrophages, which ultimately promotes inflammation and AS progression.⁸¹ Such epigenetic memory may explain persistent and deteriorating inflammation in AS plaques, presumably due to recurrent pro-inflammatory stimuli (e.g. oxLDL) within the lesional microenvironment. On that note, this phenomenon, named trained immunity, has recently been confirmed in AS,⁸² which may provide a novel insight into uncontrolled inflammation in AS.

Epigenetic modifiers for histone PTMs

Histone PTMs, including at least 15 types of modifications and 130 sites identified so far,⁷⁰ regulate gene expression via chromatin remodelling. Functionally, histone epigenetic codes (mainly methylation and acetylation) can be divided into two categories, i.e. activating

and inhibitory PTMs, which are reciprocally regulated by the writer (KMT and HAT) and eraser (KDM and HDAC) in a PTM type-specific manner. Among numerous histone epigenetic modifiers, only a small part of them have been characterized to regulate macrophage polarization and functions thus far.

Histone acetylation

Histone lysine (K) acetylations generally activate gene expression by loosening the chromatin conformation to increase the accessibility of transcription factors. This type of histone PTMs includes the acetylation of histone H3 at the K27 site and H4 at multiple K residues, which are specifically regulated by HATs and HDACs (Table 1). Thus far, only a few HATs have been reported to be involved in macrophage regulation, including the classical (e.g. EP300/KAT3B and CREBBP/CBP/KAT3A)⁸³ and novel HATs (e.g. MOF),⁸⁴ While it remains unknown whether and which HAT contributes to AS. In contrast, multiple HDACs have been demonstrated in the regulation of macrophage polarization and functions relevant to AS.

HDAC3

HDAC3 catalyzes the deacetylation of H4K9-Ac and H4K14-Ac. Among all HDACs, HDAC3 has attracted the most attention because of its role in the regulation of macrophage polarization and functions, particularly in AS. An integrated genomic analysis has revealed that lipopolysaccharide (LPS) fails to activate the expression of nearly half of the inflammatory genes in *HDAC3*^{-/-} macrophages, suggesting that HDAC3 is required for M1 polarization and pro-inflammatory gene expression.⁸⁵ Loss of HDAC3 results in hyperacetylation of histone H4 in 681 regions in the genome of macrophages. In *HDAC3*^{-/-} macrophages, the number of hyperacetylated regions (2591 regions) is more than tripled after LPS stimulation. Interestingly, a large number of regions in both untreated (1037 regions) and LPS-treated (1094 regions) *HDAC3*^{-/-} macrophages display reduced H4 acetylation, representing an indirect consequence due to *HDAC3* deletion. The most enriched binding sites in these hypoacetylated regions are the recognition motifs for the IRF (interferon regulatory factor) family proteins and STAT1. However, the pro-inflammatory IFN β -STAT1 axis,⁸⁶ but not IRF3 known to directly control transcription of *IFNB1* (interferon beta 1), is impaired in *HDAC3*^{-/-} macrophages exposed to LPS. This event is associated with the up-regulation of *PTGS1* (prostaglandin-endoperoxide synthase 1), a gene encoding cyclooxygenase-1 (COX-1) that is a constitutive isoform of prostaglandin-endoperoxide synthase and generates pro-AS thromboxane A2 (TXA2). Thus, HDAC3 may trigger M1 gene expression via an indirect mechanism involving activation of the pro-inflammatory signalling pathway (e.g. IFN β -STAT1) by activating target genes (e.g. *PTGS1*). This function is independent of HDAC3 enzymatic activity that catalyzes histone deacetylation, which is supposed to repress gene expression. During activation of the NLRP3 inflammasome, HDAC3 translocates to mitochondria, which restricts fatty acid oxidation (FAO) by deacetylating a non-histone protein named mitochondrial trifunctional protein subunit α (HADHA) at K303 and thus reducing its FAO enzyme activity; this promotes IL-1 β production by shaping mitochondrial adaptation rather than affecting the gene expression in the nucleus.⁸⁷ Therefore, HDAC3 plays a crucial role in the activation of the pro-inflammatory

Table 1 Histone acetylation and their epigenetic modifiers

Code	Function	Writer	Eraser
H1K25-Ac	I	Unknown	SIRT1
H2AK5-Ac	DDR	KAT5, HAT1 (KAT1)	Unknown
H3K4-Ac	Unknown	Unknown	HDAC3
H3K9-Ac	A	CBP (KAT3A), p300 (KAT3B), ELP3 (KAT9), PCAF-b (KAT2A)	HDCA3, SIRT1, SIRT6
H3K18-Ac	A	CBP (KAT3A), ELP3 (KAT9), p300 (KAT3B)	SIRT7
H3K14-Ac	A	CBP (KAT3A), p300 (KAT3B), MGEA5, CLOCK (KAT13D), GTF3C4 (KAT12), KAT2A, MYST3 (KAT6A)	HDAC3
H3K23-Ac	A	Unknown	Unknown
H3K27-Ac	A	CBP (KAT3A), p300 (KAT3B)	HDAC1/2
H3K56-Ac	DDR	CEB (KAT3A), p300 (KAT3B)	HDAC1, SIRT2/3/6
H4K5-Ac	A	CBP (KAT3A), p300 (KAT3B), PCAF-b (KAT2A), KAT5, MYST2 (KAT7), HAT1 (KAT1)	Unknown
H4K8-Ac	A	CBP (KAT3A), p300 (KAT3B), MGEA5, KAT2A, KAT5, MYST2 (KAT7)	Unknown
H4K12-Ac	A	CBP (KAT3A), p300 (KAT3B), PCAF-b (KAT2A), KAT5, MYST2 (KAT7), HAT1 (KAT1)	Unknown
H4K16-Ac	A	CBP (KAT3A), p300 (KAT3B), PCAF-b (KAT2A), MYST2 (KAT7)	Unknown

A, activating; Ac, acetylation; DDR, DNA damage response; I, inhibitory.

(M1) gene expression programme in macrophages, likely independently of its deacetylase function as an epigenetic modifier.

HDAC3 also serves as an epigenetic brake of M2 polarization. A GEP analysis has revealed that untreated *HDAC3*^{-/-} macrophages exhibit a phenotype similar to IL-4-induced alternative activation in wild-type macrophages.⁸⁸ Among the genes up-regulated by HDAC3 deficiency, most of them are not stimulus-specific as they can be induced by both IL-4 and LPS in wild-type macrophages, while only a few are specifically induced by LPS but not IL-4. However, the majority of the down-regulated genes in *HDAC3*^{-/-} macrophages are the genes up-regulated by LPS in wild-type macrophages. These findings argue that *HDAC3* deletion leads to the skewing of macrophages toward M2, supporting the notion that HDAC3 serves as a suppressor of M2 polarization even in the absence of the stimulus. *HDAC3* deletion in macrophages results in a marked increase in H3K9-Ac in the regions where HDAC3 binds, an event further enhanced by IL-4, indicating that HDAC3 directly represses M2 gene expression via a process dependent on its deacetylase activity. A similar effect on H3K27-Ac also occurs, suggesting that the function of HDAC3 seems not lysine residue-specific, and instead, may influence global histone acetylation in the genome, particularly in HDAC3-enriched regions near the genes activated by IL-4. Both H3K9-Ac and H3K27-Ac are increased in advanced AS lesions, while only H3K9-Ac in macrophages and SMCs is associated with plaque severity.⁸⁹ Taken together, HDAC3 plays dual roles in the regulation of macrophage polarization. On the one hand, HDAC3 acts as a mediator of M1 polarization via activation of certain signalling pathways (e.g. IFN β -STAT1) after being stimulated with pro-inflammatory stimuli (e.g. LPS), which is independent of its deacetylase function. On the other hand, HDAC3 functions as a brake for M2 polarization via global histone deacetylation in specific regions enriched for M2 genes under the basal condition or in response to Th2 cytokines (e.g. IL-4 and IL-13), an event that relies on its deacetylase activity. Therefore, as

HDAC3 deficiency or inactivation may simultaneously turn on M2 genes and shut down M1 genes even in the presence of pro-inflammatory factors or environmental cues, it theoretically represents an ideal target for repolarizing macrophages from M1 to M2 in non-resolving inflammation-driven diseases like AS.

Indeed, a genetic approach targeting macrophage-specific HDAC3 stabilizes AS plaques, suggesting that HDAC3 in macrophages is associated with plaque vulnerability.⁹⁰ In *Ldlr*^{-/-} mice fed a high cholesterol diet, transplantation of bone marrow cells with myeloid *HDAC3* deletion results in a favourable plaque phenotype, featured by the deposition of collagen (particularly red collagen, the most mature and stable among all collagen types) in the fibrous cap, which stabilizes the plaques by thickening the fibrous cap. After exposure to oxLDL, *HDAC3*^{-/-} macrophages secrete more TGF- β , an anti-inflammatory M2 cytokine, than their wild-type counterparts, which in turn promotes the production of collagen by vascular SMCs (VSMCs), increasing plaque stability. This event is associated with increased H3K9-Ac and H3K14-Ac at the *TGF β* locus, suggesting that HDAC3 may directly bind to the regions near the *TGF β* promoter and thus inhibit its expression. Such an adverse property further underscores HDAC3 as a potential anti-AS target. Therefore, HDAC3-selective inhibitors represent a promising approach to treat non-resolving inflammation-driven diseases, particularly AS, as HDAC3 inhibition may reduce plaque vulnerability.

HDAC9

A GWAS (genome-wide association study) analysis has identified a genetic variant in the locus corresponding to *HDAC9*, which correlates with large vessel ischaemic stroke.⁹¹ In a discovery set consisting of 3548 ischaemic stroke cases and 5972 controls, a single-nucleotide polymorphism (SNP) on chromosome 7p21.1 (rs11984041), located within the final intron of the *HDAC9* gene, is significantly associated with large vessel stroke, but not other subtypes of ischaemic stroke.

Both rs11984041 and another variant (rs2107595, which is in linkage disequilibrium with rs11984041) are associated with carotid artery plaque and intima-media thickness.⁹² Interestingly, rs2107595 specifically correlates with the mRNA level of HDAC9, but not its neighbouring genes, such as *TWIST1* (Twist family BHLH transcription factor 1) and *FERD3L* (Fer3 like BHLH transcription factor). However, this genetic variant in peripheral blood mononuclear cells of homozygous and heterozygous carriers of the risk allele has no significant correlation with the specific morphological characteristics of plaques (including asymptomatic, symptomatic, and with fatal events), including calcification, collagen, atheroma size, intraplaque haemorrhage, macrophage, SMC, and vessel density.⁹³ Interestingly, the mRNA level of HDAC9 decreases in unstable plaques, in association with *TWIST1* expression.⁹⁴ These findings suggest a close relationship between *HDAC9* genetic alterations and AS susceptibility, accelerated disease progression, and increased plaque vulnerability. However, the mechanism by which the *HDAC9* genetic variants facilitate AS and reduce plaque stability remains unknown.

HDAC9 is highly expressed in differentiated macrophages. During differentiation of monocyte to macrophage, two major isoforms of HDAC9 derived from alternative splicing are expressed,⁹⁵ including the one containing an HDAC domain and its truncated form lacking this domain. The latter is known as HDAC-related protein (HDRP) or MEF2-interacting transcription repressor protein (MITR). HDRP does not have deacetylase activity due to lack of the HDAC domain, which instead functions to recruit other HDACs (e.g. HDAC1 or HDAC3). In differentiated macrophages, HDAC9 expression can be further induced by oxLDL, acetylated LDL, and toll-like receptor (TLR) signals (e.g. triggered by lymphotoxin alpha/LTA, LPS, and flagellin). In basal condition without stimuli, *HDAC9* deficiency in macrophages promotes M2 polarization (e.g. expression of Arg1 and IL-10) while inhibits the expression of M1 genes (e.g. IL-1 β and MCP-1), likely via up-regulating PPAR- γ through chromatin remodelling in association with increased acetylation of H3K9 but not H3K18 at the promoters of the PPAR- γ gene. *HDAC9* deficiency also increases cholesterol efflux from macrophages, an event negatively correlating with AS progression, by promoting expression of ABCA1 (ATP binding cassette subfamily A member 1) and ABCG1 (ATP binding cassette subfamily G member 1) due to increased H3K9-Ac, rather than H3K18-Ac, at the promoters of these target genes. In *Ldlr*^{-/-} mice fed an atherogenic diet, either systemic or macrophage-specific deletion of *HDAC9* reduces the plasma levels of triglyceride and very-low-density lipoprotein (VLDL), in association with AS lesion reduction and plaque stabilization.⁹² *HDAC9* deficiency also reduces the size and number of AS lesions in *ApoE*^{-/-} mice fed a normal chow diet without increasing plasma cholesterol level, accompanied by reduced acellular cores but increased macrophage proportion. Genome-wide analysis has revealed that HDAC9 is associated with the expression of *MMP12* located in the regions clustered with inflammatory genes in macrophages but not SMCs within AS plaques. Both genes are associated with the expression of M4 markers (e.g. MMP7 and S100A8). However, MMP12, but not HDAC9, positively correlates with the expression of M2 markers in AS plaques.⁹⁶ Owing to its multiple pro-AS roles (e.g. promoting M1 but inhibiting M2), HDAC9 may be considered another potential target in AS.

In addition, the high level of HDAC9 in macrophages is maintained by DNMT3A in innate immunity,⁹⁷ suggesting a cross-talk between

DNA methylation and histone acetylation in the regulation of macrophages. While DNMT3A is also highly expressed in macrophages compared to other types of immune cells (e.g. lymphocytes, natural killer/NK cells, and dendritic cells/DCs), its deficiency selectively impairs the expression of type I interferons (e.g. IFN- γ) induced by pro-inflammatory stimuli (e.g. LPS). This is likely due to inhibition of TBK1 (TANK binding kinase 1)-mediated IRF3 phosphorylation (activation). DNMT3A maintains the level of HDAC9 in macrophages by regulating H3K27me3 at the distal promoter region of the *HDAC9* gene. In turn, HDAC9 binds to TBK1 through its deacetylase domain to deacetylate TBK1 at K241, an event promoting its kinase activity. Activation of the TBK1-IRF3 signalling pathway by HDAC9 results in the production of IFN- γ . However, it remains uncertain whether this mechanism, which involves multiple epigenetic and signalling molecules (e.g. DNMT3A, HDAC9, TBK1, IRF3, and IFN- γ) is applicable in the regulation of macrophage polarization and functions in AS. Presumably, this positive regulation of HDAC9 by DNMT3A may become a challenge for developing an anti-AS therapy by targeting either of these two epigenetic modifiers in macrophages, because HDAC9 and DNMT3A exhibit pro- and anti-AS properties, respectively.

HDAC7

Structurally, HDAC7 is comparable to other HDACs of the same class (class IIa), such as HDAC4, HDAC5, and HDAC9. However, HDAC7 displays minimal intrinsic deacetylase activity and requires the binding of another HDAC (e.g. HDAC3) to suppress gene expression. During cellular reprogramming for transdifferentiation from pre-B cells into macrophages, HDAC7 is selectively down-regulated, while the levels of all other HDACs of the class IIa (including HDAC9) remain unchanged.⁹⁸ Similarly, C/EBP β (CCAAT enhancer-binding protein beta)-mediated conversion of primary B cell precursors to macrophages is also accompanied by HDAC7 down-regulation.⁹⁹ Restoration of HDAC7 expression impairs the expression of numerous genes, including chemokines, cytokines (e.g. IL-18 and IL-15), TLRs, and other genes involving phagocytosis and the TNF pathway. In pre-B cells, HDAC7 binds to and recruits the transcription factor MEF2C (myocyte enhancer factor 2C) to the promoters of its target genes, an event that requires the deacetylase activity. Thus, HDAC7 serves as a brake for transdifferentiation from pre-B cells into macrophages, which can be released via down-regulation of HDAC7 during this phenotypic transition. These observations also suggest an inhibitory role of HDAC7 in the regulation of the genes essential for macrophage functions.⁹⁸

In macrophages, HDAC7 promotes TLR4-induced expression of inflammatory genes.¹⁰⁰ Among all HDACs (HDAC1–11), HDAC7 is the only one member that is up-regulated in murine thioglycolate-elicited peritoneal macrophages with a pro-inflammatory property. Although HDAC7 overexpression does not affect the expression of the classical M1 marker iNOS in macrophages exposed to LPS, it instead up-regulates a subset of TLR4-inducible pro-inflammatory (M1) genes and promotes the production of the corresponding cytokines, such as IL-6, TNF- α , and IL-12p40. This event can be blocked by a relatively selective inhibitor of the class IIa HDACs. Interestingly, HDAC7 synergizes with HIF-1 α (hypoxia-inducing factor-1 α) to promote M1 polarization induced by LPS, similar to its cooperation with HIF-1 α in response to hypoxia.¹⁰¹ In macrophages, HDAC7 and

other class IIa HDACs also link TLR-induced aerobic glycolysis with inflammatory responses.¹⁰² Myeloid-specific HDAC7 expression increases LPS-induced glycolysis (a metabolic landmark of M1 macrophages) and inflammatory response via forming an HDAC7-PKM2 (pyruvate kinase M2) complex and resulting PKM2 deacetylation at K433. These findings raise the possibility that HDAC7 may be involved in immunometabolism, a term to describe the changes in intracellular metabolic pathways that can alter macrophage functions.¹⁰³ In this context, emerging evidence supports the role of immunometabolism in AS.¹⁰⁴ Together, while HDAC7 needs to be silenced during macrophage differentiation, it appears to display a pro-inflammatory function in macrophages, e.g. in TLR-mediated inflammatory response. However, whether HDAC7 contributes to non-resolving inflammation-driven diseases (e.g. AS) remains to be investigated.

HDAC inhibition as anti-inflammatory therapy—pros and cons

Owe to the roles of HDACs in macrophage differentiation, polarization, and metabolism,¹⁰⁵ small molecule HDAC inhibitors have attracted considerable interest in the treatment of infectious and inflammatory diseases, including AS.¹⁰⁶ However, the evidence for the effect of HDAC inhibitors on either inflammation or AS remains controversial. In a survey involving 11 949 patients with ischaemic events, exposure to the HDAC inhibitor sodium valproate used as an anti-epileptic drug is associated with a decrease in the risk of stroke recurrence after a previous stroke or transient ischaemic attack.¹⁰⁷ This finding is consistent with the results of a preclinical study, in which the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, also known as vorinostat, approved for the treatment of cutaneous T-cell lymphoma) attenuates AS progression in hypercholesterolaemic *ApoE*^{-/-} mice, in association with reduced immune cell infiltration and inflammation.¹⁰⁸ However, another pan-HDAC inhibitor named trichostatin A (TSA) has been reported to exacerbate AS induced by an atherogenic diet in *Ldlr*^{-/-} mice, without altering the plasma lipid profile.¹⁰⁹ It has also been reported that HDAC inhibition by TSA in macrophages has either pro- or anti-inflammatory effects *in vitro*.¹¹⁰ On the one hand, TSA markedly impairs the LPS- and IFN γ + LPS-induced expression of most M1 markers (e.g. iNOS) and cytokine genes in macrophages; in contrast, the HAT inhibitor epigallocatechin-3-gallate (EGCG) has only a mild effect. On the other hand, TSA also inhibits the expression of the M2 marker (e.g. Arg1) induced by IL-4. Administration of TSA inhibits HDAC activity (reflected by increased histone H4 acetylation) in most cell types (e.g. foam cells, ECs, and SMCs) of AS lesions, accompanied by a marked increase in macrophage infiltration and AS lesion size. In addition, TSA promotes the expression of CD36, a scavenger receptor of oxLDL, on macrophages via increasing H4 acetylation at its promoters, which in turn promotes oxLDL uptake by macrophages and thus facilitates the formation of foam cells. In macrophages exposed to oxLDL, TSA also increases the expression of other scavenger receptors (e.g. scavenger receptor-A/SRA), ATP-binding cassette transporters (e.g. ABCA1 and ABCG1) responsible for cholesterol efflux, pro-inflammatory TNF- α , and VCAM-1 (vascular cell adhesion molecule 1), while inhibits the expression of other pro-inflammatory factors, such as IL-6, IL-1 β , MCP-1, and E-selectin.¹¹⁰ Together, whereas pan-HDAC inhibition seems to preferentially inhibit the pro-inflammatory property of macrophages, it may however take a

risk to enhance the pro-AS property of macrophages (e.g. foam cell formation by increasing oxLDL uptake). Therefore, it is necessary to first understand the distinctive role of individual HDAC in the regulation of macrophage polarization and functions, and then specifically target those HDAC(s) with a potent pro-inflammatory property but little or no pro-AS activity (e.g. foam cell formation) to effectively execute the anti-inflammatory activity while avoiding unfavourable effects derived from pan-HDAC inhibition.

In addition to histones, many non-histone proteins also serve substrates for the deacetylation by HDACs¹¹¹; even more downstream targets are subjected to be transcriptionally regulated via histone deacetylation mediated by HDACs. Owe to this broad diversity of the substrates or downstream targets, the effects of HDAC inhibition (especially by pan-HDAC inhibitors) on macrophages could vary in a context-specific manner. Its outcomes may depend upon which class of HDACs or individual HDAC is affected more specifically as well as which substrates or downstream targets are influenced more dominantly. For example, MS-275 (also known as entinostat), a selective inhibitor of class I HDACs (particularly HDAC1 and HDAC3), increases the basal level of the M2 marker Arg1, an event analogous to the phenotype of *HDAC3* deletion, while has no significant effect on IL-4-induced Arg1 expression.⁸⁸ Similarly, HDAC3-specific inhibition increases the anti-inflammatory activity of macrophages.¹¹² However, the pan-HDAC inhibitor TSA down-regulates both basal and IL-4-induced expression of Arg1 via an HDAC3-independent process.⁸⁸ Despite this controversy, most HDAC inhibitors seem to display the anti-inflammatory activity by suppressing the expression and production of pro-inflammatory cytokines (e.g. TNF- α , IL-1 β , IL-6, and IFN γ) in macrophages.¹¹³ However, their anti-inflammatory efficacy as a single agent is often limited.

Broad-spectrum inhibition of histone deacetylation with lack of specificity has been considered as a major cause of the side effects of pan-HDAC inhibitors.¹¹⁴ Inhibition of non-histone protein deacetylation by HDAC inhibitors may represent another reason for unwanted 'off-target' effects or adverse outcomes. In this context, several non-histone proteins have been identified as the substrates for deacetylation mediated by HDACs, including many transcription factors (e.g. RelA, p53, and STAT1). Among them, RelA (p65) is the most abundant form of NF- κ B, a well-established signalling pathway essential for the activation of inflammatory response in M1 macrophages, inhibition of which promotes M2 polarization.¹¹⁵ HDAC3 deacetylates RelA at multiple lysine residues (e.g. K310 and K221), a mechanism for silencing the NF- κ B signal after activation by the classical NF- κ B activators (e.g. TNF- α).¹¹⁶ Deacetylated RelA preferentially binds to *de novo* synthesized I κ B α , a direct downstream target of NF- κ B, and thus promotes nuclear export of the RelA/I κ B α complex, which turns off the NF- κ B signal and allowing cells to respond to the next stimulus. This negative feedback loop may explain why acute inflammation is often self-limited. Previously, our group has reported that HDAC inhibitors (especially pan-HDAC inhibitors) induce NF- κ B activation, in associated with differentiation of monocytes (e.g. U937 cells, a human monocytic line) into CD11b⁺ macrophages (a model used for *in vitro* studies of macrophages) via expression of the endogenous CDK inhibitor p21^{cip1/waf1}.¹¹⁷ This event is abolished in cells expressing an S32A/S34A double mutant form of I κ B α (known as 'super repressor'). Unlike the NF- κ B signal triggered by TNF- α , the activation of NF- κ B induced by HDAC

inhibitors is a relatively persistent process due to the blockade of RelA deacetylation, an event that prevents nuclear export of RelA by *de novo* synthesized I κ B α .¹¹⁸ Moreover, we have also observed that exposure to HDAC inhibitors can also trigger NF- κ B signal, via I κ B kinase 2 (IKK2)-dependent S536 phosphorylation of RelA, even in the absence of extracellular stimuli, such as TNF- α .¹¹⁹ RelA phosphorylation facilitates its translocation into the nucleus where it is further acetylated by HATs (e.g. EP300 and CBP) followed by deacetylation by nuclear HDACs (e.g. HDAC3), while the latter can be blocked by HDAC inhibitors. Thus, these findings raise the possibility that the administration of HDAC inhibitors may activate NF- κ B in macrophages, probably promoting or prolonging inflammation. In this context, HDAC inhibitors, especially those targeting the class I HDACs (HDAC1-3), induce IKK-dependent expression and production of pro-inflammatory CXCL8 (C-X-C motif chemokine ligand 8, also known as IL-8) in macrophages, in association with NF- κ B activation.^{120,121} NF- κ B activation in macrophages also promotes oxLDL uptake and foam cell formation via up-regulation of CD36.¹⁰⁹ Moreover, the NF- κ B signal is required for TNF- α -induced CD47 expression, which impairs the capability of M2 macrophages to remove apoptotic cells via efferocytosis, resulting in the accumulation of apoptotic cells in the necrotic core, a feature of rupture-prone plaques.⁵¹ In contrast, macrophage-specific IKK2 deletion increases AS lesion size and leads to more advanced lesions in *Ldlr*^{-/-} mice, in association with reduced production of IL-10, TNF- α , and IL-6 induced by LPS in a time-dependent manner, raising a possibility that NF- κ B promotes AS by influencing the pro- and anti-inflammatory balance.¹²² Even more complicatedly, IL-10 can inhibit NF- κ B activation by preventing ROS-dependent I κ B α degradation,¹²³ suggesting a negative feedback loop between NF- κ B activation and IL-10 production in the regulation of macrophages. Thus, the net outcome of NF- κ B activation in macrophages may depend on its downstream targets. Another example is the transcription factor STAT6, which can be acetylated at K383 by HATs (e.g. CBP), thereby promoting macrophage activation but suppressing M2 polarization.¹²⁴ Presumably, inhibition of STAT6 deacetylation by HDAC inhibitors could impair the M2 polarization of macrophages. Therefore, these 'off-target' effects of HDAC inhibitors on the deacetylation of non-histone proteins may explain, at least in part, their limited efficacy against inflammation, even though histone deacetylation is sufficiently inhibited by them in macrophages.¹¹⁴

Although the observations described above need to be further verified in the setting of non-resolving inflammation, HDAC inhibition may indeed be a double-edged sword for treating inflammatory diseases like AS. On the one hand, inhibition of HDACs (HDAC3, HDAC9, or HDAC7) suppresses inflammation by epigenetically reprogramming macrophage polarization. On the other hand, the 'off-target' effect of HDAC inhibition may facilitate inflammation via activation of pro-inflammatory signalling pathways (e.g. NF- κ B) due to inhibition of non-histone protein (e.g. RelA) deacetylation. A caution should be taken particularly when using HDAC inhibitors as anti-AS agents as HDAC inhibitors (e.g. TSA) may also promote AS progression and increase the risk of plaque rupture by increasing oxLDL uptake and foam cell formation or impairing efferocytosis (e.g. due to CD47 expression).¹⁰⁹ Therefore, an effective and safe epigenetic therapy for AS may need to target a specific HDAC in a specific cell type or even a specific phenotype identified in AS lesions

(e.g. inflammatory and TREM2 foamy macrophages). To this end, many nanomedicine-based strategies have been investigated to specifically target macrophages or their phenotypes (e.g. M1 or M2) for anti-AS therapies.¹²⁵ For example, a type of hyaluronan nanoparticles selectively targeted plaque-associated pro-inflammatory (M1) macrophage phenotype, likely via hyaluronan-immune cell interactions during inflammation, to improve plaque stability in *ApoE*^{-/-} mice.¹²⁶ Moreover, a conjugate of atorvastatin with hyaluronan markedly enhanced anti-inflammatory effects of atorvastatin in *ApoE*^{-/-} mice, due to selective binding of hyaluronan to CD44, an adhesion molecule for macrophage recruitment and retention in AS lesions.¹²⁷ On the other hand, an M2 macrophage-specific strategy has also been reported to prevent AS by using single-walled carbon nanotubes loaded with a chemical inhibitor of the anti-phagocytic CD47-signal regulatory protein alpha/SIRP α signalling axis, which can specifically be taken by lesional macrophages and thus promote inflammation resolution via efferocytosis.¹²⁸ Furthermore, the scRNAseq analysis has revealed that this potential nanotherapy acts like a 'Trojan horse' to suppress the expression of inflammatory genes. This pilot study provides a promising direction for the development of novel and effective therapies that target macrophage-mediated resolution to treat non-resolving inflammation-driven diseases, such as AS in a high cell type- or even phenotype-specific manner.¹²⁹

Histone methylation

Histone lysine methylation can be either activating or inhibitory in the regulation of target gene transcription, depending on which lysine residue is methylated with how many methyl groups.⁸³ Both activating and inhibitory histone methylations are reciprocally regulated by KMTs (writer) and KDMs (eraser), respectively (Table 2).

KMTs

H3K4me3 represents one of the activating histone PTMs, which is regulated by mixed-lineage leukaemia (MLL, also named KMT2A) and KDM5B, respectively. Immunohistochemical analysis of human carotid AS plaques has revealed that H3K4 methylation is constitutively increased in macrophages during disease progression from early to advanced stages, in association with AS severity.⁸⁹ MLL is up-regulated in M1 macrophages, accompanied by enhanced H3K4 methyltransferase activity and increased H3K4me3, while its enzymatic activity is reduced in M2 macrophages, indicating that MLL-mediated H3K4me3 is primarily involved in M1 polarization.¹³⁰ Increased H3K4me3, in turn, leads to the activation of pro-inflammatory genes, such as CXCL10 and IFN- γ via epigenetic remodelling of their promoter sites, an event blocked by the smallmolecule inhibitors targeting the MLL-Menin interaction. Similarly, KMT inhibition by 5'-methylthioadenosine, a broad-spectrum KMT inhibitor, prevents LPS-induced expression of M1 markers and pro-inflammatory genes as well as reduces LPS + IFN- γ -induced production of TNF- α and IL-6, except IL-1 β , which is instead increased.¹¹⁰

Increasing evidence supports the role of H3K4me3 in macrophages in non-resolving inflammation-driven diseases like AS. For example, priming of monocytes with oxLDL, but not native LDL, up-regulates the mRNA levels of various pro-inflammatory genes (e.g. TNF- α , IL-6, MCP-1, and IL-18), scavenger receptors (e.g. CD36 and

Table 2 Histone methylation and their epigenetic modifiers

Code	Function	Writer	Eraser
H1K25me1	I	EZH2 (KMT6A, PRC2), EHMT1/2 (KMT1C/1D)	KDM4D
H3K4me1	A	SETD7 (KMT7)	KDM1A (LSD1)/1B (LSD2)
H3K4me2	A	NSD3	KDM5A/5B/5D, KDM1A
H3K4me3	A	MLL1 (KMT2A), MLL2 (KMT2B), MLL3 (KMT2C), MLL4 (KMT2D), PRDM9 (KMT8B), SETD1A (KMT2F)/1B (KMT2G), SMYD3 (KMT3E)	PHF8 (KDM7B), KDM2A/2B, KDM5A-5D, NO66 (JMJD9)
H3K9me1	I	EHMT2 (KMT1D)	KDM3A/3B, KDM1A
H3K9me2	I	EHMT1/2 (KMT1C/1D), PRDM2 (KMT8)	PHF8 (KDM7B), KDM3A/3B, KDM4B/4C/4D, JHDM1D (KDM7A or KIAA1718), KDM1A/1B, KDM7C (PHF2)
H3K9me3	I	SETDB1 (KMT1E)/B2 (KMT1F), SUV39H1/2 (KMT1A/ 1B), MINA	KDM4A-4D, JMJD2A (KDM4A)
H3K27me1	A	EZH1/2 (KMT6B/6A), EHMT1/2 (KMT1C/KMT1D)	Unknown
H3K27me2	I	EZH1/2 (KMT6B/KMT6A), NSD3	PHF8 (KDM7B), KDM6B (JMJD3), JHDM1D (KDM7A)
H3K27me3	I	EZH2 (KMT6A), NSD3	KDM6A/6B (JMJD3), UTX (KDM6A), UTY (KDM6C), JARID2
H3K36me1	A	ASH1L (KMT2H)	KDM2A/2B
H3K36me2	(-) H3K27me	SETMAR, NSD1 (KMT3B), SMYD2 (KMT3C), ASH1L (KMT2H)	KDM2A/2B, KDM4A/4B/4C, JMJD5 (KDM8)
H3K36me3	(-) H3K27me	SETD2 (KMT3A), NSD2 (WHSC1)	KDM4A/4B/4C, NO66 (JMJD9)
H3K79me2	I	DOT1L (KMT4)	Unknown
H3K79me3	Unknown	DOT1L (KMT4)	Unknown
H4K20me1	(+) H3K9me	SETD8 (KMT5A), NSD2 (WHSC1)	PHF8 (KDM7B)
H4K20me3	I	SUV420H1/2 (KMT5B/5C), NSD2 (WHSC1)	Unknown

(-), inhibition; (+), activation; A, activating; I, inhibitory; me, methylation.

SRA), and MMPs (e.g. MMP2 and MMP9) by increasing H3K4me3 on their promoters via activation of the TLR pathway.¹³¹ In addition to resulting in persistent production of pro-inflammatory cytokines, H3K4me3 also promotes the formation of foam cells in macrophages exposed to oxLDL via up-regulation of scavenger receptors (e.g. CD36 and SRA) but down-regulation of ATP-binding cassette transporters (e.g. ABCA1 and ABCG1), both events accelerating AS progression.¹³² In this context, inhibition of H3K4me3 by pan-KMT inhibitors almost completely abolishes the ability of oxLDL to prime monocytes (e.g. by suppressing pro-inflammatory gene expression) and to augment foam cell formation (e.g. by reducing oxLDL uptake).¹¹⁰ Therefore, although it remains to be defined which specific KMT(s) accounts for H3K4me3 in macrophages exposed to oxLDL, MLL that catalyzes H3K4me3 may represent a potential target for anti-inflammatory therapy in AS, due to its dual properties involving pro-inflammation and pro-AS (e.g. promoting foam cell formation).

In contrast, H3K9me3 and H3K27me3 are two typical inhibitory epigenetic codes. While H3K9 and H3K27 methylations are undetectable in healthy carotid arteries, they are markedly increased in macrophages and lymphocytes in human carotid AS plaques.⁸⁹ Interestingly, these inhibitory codes are significantly reduced in inflammatory cells within advanced plaques compared to early-stage lesions, although their levels are still higher than those for healthy

arteries. Similarly, the global levels of H3K27me3 are also reduced in advanced peri-renal aortic AS plaques.¹³³ However, these alterations do not correlate with expression of the KMTs either for H3K27me3, including PRC2 [polycomb repressive complexes 2, also known as EZH2 (enhancer of zeste 2 polycomb repressive complex 2) or KMT6A, which is known to negatively regulate M1 polarization induced by the genes like *TRPA1* (transient receptor potential cation channel subfamily A member 1) in AS plaques]¹³⁴ and G9a (KMT1C), or for another inhibitory code H3K9me3, including SETDB1 (SET domain bifurcated histone lysine methyltransferase 1; KMT1E), and SUV39H1/2 (suppressor of variegation 3–9 homolog 1; KMT1A/B). Whereas it remains uncertain whether KMTs (writer) for these inhibitory histone methylations are involved in AS, their KDMs (eraser) may instead take this responsibility.

JMJD3

The JMJD (Jumonji C-terminal domain-containing enzyme) family represents a major group of KDMs that remove the methylation codes from the lysine residues of histones. Among them, JMJD3 (also known as KDM6B, which specifically catalyzes the demethylation of H3K27me3)¹³⁵ is the first KDM identified to be involved in the regulation of macrophage activation and functions.¹³⁶ JMJD3 is expressed in macrophages in response to inflammatory stimuli.^{130,136} A ChIP-

seq (chromatin immunoprecipitation sequencing) analysis has revealed that JMJD3 is often recruited to transcription start sites (TSSs) containing high levels of H3K4me3 and RNA polymerase II. In numerous LPS-inducible genes, more than 70% are the downstream targets of JMJD3, suggesting its crucial role in M1 polarization. However, most of these M1 genes are not affected by JMJD3 deficiency, which is uncoupled with increased levels of H3K27me3.¹³⁶ This observation suggests that JMJD3 itself is unable to direct macrophage polarization toward M1, but instead functions to fine-tune the transcriptional programme for pro-inflammatory gene expression in response to LPS. Notably, this function of JMJD3 is independent of its H3K27me3 demethylase activity. In cooperation with KIAA1718 (KDM7A, an H3K9me2/H3K27me2/H4K20me1-specific demethylase) and other proteins involving transcription elongation, JMJD3 demethylates H3K27me3 at the promoters of target genes during differentiation of monocytes into macrophages induced by TPA (12-O-tetradecanoylphorbol 13-acetate, also known as phorbol 12-myristate 13-acetate/PMA), while down-regulation of either JMJD3 or KIAA1718 attenuates the mRNA elongation of these genes.¹³⁷ Therefore, JMJD3 may require collaboration with other epigenetic modifiers (e.g. KIAA1718) and proteins to fully function as an H3K27me3 demethylase in the regulation of macrophage differentiation as well as M1 polarization.

Pro-inflammatory serum amyloid A (SAA), the plasma levels of which are related to the pathogenesis of chronic inflammatory diseases like AS, induces the expression of JMJD3 in macrophages, accompanied by decreased H3K27me3.¹³⁸ SAA-induced expression of pro-inflammatory genes is significantly impaired by *JMJD3* knockdown or inactive mutation, in association with restoration of the H3K27me3 level. Together, these findings suggest that the enzymatic activity of JMJD3 for demethylating H3K27me3 is required for inflammation induced by SAA (chronic) but not LPS (acute). A possibility then arises that JMJD3 regulates M1 polarization or inflammation likely via different mechanisms in a context-specific manner. In addition, JMJD3 deficiency also prevents SAA from promoting oxLDL-induced foam cell formation in macrophages.

Considering the role of the inhibitory H3K27me3 and its epigenetic modifiers (e.g. JMJD3) in various chronic inflammatory diseases,¹³⁹ a selective inhibitor (GSK-J1) of H3K27-specific demethylases [including JMJD3 and UTX (ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome) of the KDM6 subfamily, both demethylating H3K27me3]¹⁴⁰ has been developed.¹⁴¹ GSK-J1 specifically binds to the catalytic pocket of human JMJD3 and thus inhibits its demethylase activity (reflected by increased H3K27me3). Unlike genetic deletion of *JMJD3*, inhibition of its enzymatic activity by GSK-J1 leads to a marked reduction in pro-inflammatory gene expression in human primary macrophages exposed to LPS. This observation supports the role of JMJD3 as an H3K27me3 demethylase in enhancing the pro-inflammatory property of M1 macrophages. It also suggests that the histone demethylase activity of KDMs like JMJD3 is targetable in an epigenetic code-specific manner, which may pave an avenue for the development of highly specific inhibitors targeting histone epigenetic codes (e.g. H3K27me3) as an anti-inflammatory therapy to treat non-resolving inflammation-driven diseases, such as AS.

JMJD3 may also play a functional role in the M2 polarization of macrophages. IL-4 induces JMJD3 expression, resulting in decreased

inhibitory H3K27me2/3 but increased activating H3K4me3 at the promoters of M2 genes.¹⁴² Removal of H3K27me3 by JMJD3 leads to transcriptional activation of M2-specific genes. Unlike in wild-type mice, helminth infection or chitin fails to induce M2 polarization in *JMJD3*^{-/-} mice, in which JMJD3 seems dispensable for M1 polarization.¹⁴³ Similarly, JMJD3 is involved in M2 polarization induced by M-CSF, rather than M1 polarization induced by GM-CSF. Although *JMJD3* deficiency results in an increase in global H3K27me3 at the promoters and in their downstream regions of many target genes, only a small part of genes are however specifically affected by *JMJD3* deletion. For the latter, most loci are instead regulated by UTX in the absence and presence of JMJD3. Among these genes, IRF4 has been identified as a JMJD3-specific target gene that is responsible for M2 polarization.¹⁴³ IRF4 expression in macrophages relies on the demethylase activity of JMJD3. *IRF4* knockout phenocopies *JMJD3* deletion (e.g. preventing chitin-induced M2 polarization), while IRF4 expression can rescue the impairment of M2 polarization in *JMJD3*^{-/-} macrophages. Interestingly, GM-CSF can induce IRF4 expression by enhancing JMJD3 demethylase activity in macrophages, resulting in the production of the pro-inflammatory CCL17 (C-C motif chemokine ligand 17) in a murine model of arthritis, an event also blocked by GSK-J1.¹⁴⁴ Together, these findings suggest that JMJD3 and its downstream targets, such as IRF4 may play a functional role in either M1 or M2 polarization in a context-dependent manner. They also support the notion that JMJD3 itself seems unlikely to determine the direction of macrophage polarization, while cooperation with other factor(s) may be required for it to make such a decision.

Many transcription factors are involved in the regulation of JMJD3 expression. For example, LPS induces JMJD3 expression through TLR4-dependent NF- κ B activation in macrophages, which requires MyD88 (myeloid differentiation primary response 88), an adaptor protein for TLR signalling.¹³⁸ This event can be blocked by the I κ B α super repressor via NF- κ B inhibition. A similar phenomenon has also been observed in ECs exposed to LPS.¹⁴⁵ In microglia, LPS-induced JMJD3 expression is associated with the activation of STATs.¹⁴⁶ Although knockdown of individual STAT has only a mild effect, double knockdown of both STAT1 and STAT3 markedly inhibits the expression of JMJD3 as well as pro-inflammatory genes. Again, this effect of JMJD3 on pro-inflammatory gene expression is independent of its H3K27me3 demethylase activity. Another transcription factor involved in LPS-induced JMJD3 expression is HIF-1 α , which is induced by LPS at the transcriptional level and promotes the expression of many HIF-dependent genes, including *JMJD3*, in macrophages.^{147,148} On the other hand, IL-4 triggers JMJD3 expression via the activation of STAT6.¹⁴² *STAT6* knockout specifically inhibits JMJD3 expression induced by IL-4, but not LPS, suggesting an exclusive role of STAT6-mediated JMJD3 expression in M2 polarization. Indeed, *STAT6* deficiency promotes IL-4-induced M2 gene expression, in association with reduced H3K27me3 demethylation, while does not affect LPS-induced M1 gene expression. Similarly, STAT6-dependent JMJD3 expression also mediates SOD1 (Cu, Zn-superoxide dismutase)-induced M2 polarization of macrophages in pulmonary fibrosis model.¹⁴⁹ These findings suggest that JMJD3 serves a downstream target of STAT6 specifically in M2 polarization. Interestingly, adiponectin (a factor produced from adipose tissues) can switch the direction of macrophage polarization from M1 to M2, an event associated with both JMJD3 and IRF4 up-regulation.¹⁵⁰ This phenomenon

suggests that although the JMJD3-IRF4 axis primarily contributes to M1 polarization as described above, it may also be involved in M2 polarization. However, the upstream signals (e.g. transcription factors) that trigger JMJD3 expression seem selective for M1 vs. M2 polarization of macrophages.¹⁵¹ For example, NF- κ B and STAT1/3 appear to preferentially up-regulate JMJD3 during M1 polarization, while STAT6 is specifically responsible for JMJD3 expression during M2 polarization. Nevertheless, lack of selectivity for JMJD3 as well as its downstream targets (e.g. IRF4) in the regulation of M1 and M2 polarization would make it challenging to target JMJD3 in non-resolving inflammation-driven diseases, such as AS. Indeed, in *Ldlr*^{-/-} mice fed a high-fat diet, transplantation with bone marrow cells from myeloid JMJD3-deficient mice increases the amount of collagen and necrosis in AS plaques, with no effect on lesional macrophage number and lesion size, suggesting that JMJD3 deficiency in macrophages may be associated with AS progression.¹⁵² Thus, a takeaway is that whereas the roles of JMJD3 in the regulation of macrophages have been well-documented, caution needs to be taken to develop an anti-inflammatory therapy that targets JMJD3 in AS.

KDM4A

KDM4A [also known as JMJD2A or JHDM3A (JmjC domain-containing histone demethylase 3A)] represents another member of the JMJD family, which catalyzes H3K9 and H3K36 demethylation.^{153,154} Interestingly, KDM4A demethylates H3K9me3 and H3K36me2 more efficiently than H3K9me2 and H3K36me3, respectively. Moreover, KDM4A also demethylates H1.4K26me3.¹⁵⁵ Although KDM4A has been widely investigated in cancer,¹⁵⁶ its role in macrophages or inflammation remains almost unknown. Our group has identified KDM4A as a novel epigenetic modifier that governs the direction of macrophage polarization in response to oxLDL.¹⁵⁷ While oxLDL triggers KDM4A expression in macrophages, knockdown of KDM4A impairs oxLDL-induced M1 polarization (e.g. reduced expression of iNOS and production of IL-1 β , TNF- α , and MCP-1) and instead promotes M2 polarization (e.g. increased expression of Arg1 and production of IL-10 and VEGF) without removal of oxLDL. These observations raise a possibility that targeting KDM4A may be able to switch the direction of macrophage polarization from M1 to M2 or even repolarize M1 macrophages to M2 phenotype.

Most members of the JMJD family are hypoxia-inducible. However, unlike other members (e.g. JMJD3/KDM6B, KDM2B, KDM3A, KDM4B/C, and KDM5B) that are induced at the transcriptional level via HIF-1 α activation by hypoxia,¹⁵⁸ the protein level of KDM4A is up-regulated, without changing its mRNA level, in response to hypoxia.^{159,160} Exposure to hypoxia stabilizes KDM4A protein by preventing its ubiquitination and degradation via the SCF-containing ubiquitin ligase complex.^{161,162} As mentioned above, JMJD3 is induced at the transcriptional level via the activation of certain transcription factors including NF- κ B and HIF. In this context, we have observed that oxLDL-induced KDM4A expression is also accompanied by the activation of the HIF pathway likely due to NF- κ B-dependent expression of ARNT (aryl hydrocarbon receptor nuclear translocator, which encodes HIF-1 β , a regulatory subunit required for the active HIF complex).¹⁶³ However, neither NF- κ B inhibition nor ARNT knockdown prevents the increase in KDM4A protein level in macrophages exposed to oxLDL, suggesting that oxLDL

up-regulates KDM4A via a process independent of NF- κ B and HIF activation.¹⁵⁷ A possibility then arises that like hypoxia, oxLDL may also up-regulate KDM4A by stabilizing its protein at the post-translational level. Thus, KDM4A acts to mediate M1 polarization (e.g. induced by oxLDL) via a separate mechanism, likely uncoupled from the activation of known pro-inflammatory signalling pathways, such as NF- κ B and HIF. Therefore, KDM4A may represent a candidate target for developing an effective and probably safe anti-inflammatory therapy that aims to repolarize macrophages straight from M1 to M2 even in the presence of the environmental cues (e.g. oxLDL, hypoxia, and probably pro-inflammatory cytokines as well) in non-resolving inflammation-driven diseases, particularly AS.

Taken together, accumulating evidence supports the functional roles of epigenetic modifiers (particularly histone epigenetic erasers like HDACs for acetylation and KDMs for methylation) in transcriptional reprogramming, metabolic rewiring, and phenotypic polarization of macrophages via epigenetic remodelling (Figure 4). Thus, they may represent promising targets for the development of anti-inflammatory therapy to treat non-resolving inflammation-driven diseases, such as AS.

Conclusions and future directions

In the aetiology of AS, a paradigm shift from a 'lipid', 'metabolic', and 'inflammatory' disorder to an 'epigenetic' disease has recently been emerging, while an increasing number of epigenomic and epigenetic alterations associated with the risk of AS have been unveiled.¹⁶⁴ This conceptual change has evoked great interest in the understanding of the epigenetic basis for chronic non-resolving inflammation and its related diseases from the mechanistic and therapeutic points of view. Although many epigenetic profiling studies have been carried out to identify the abnormalities of genome-wide DNA methylation and histone PTMs (particularly methylation and acetylation) in AS, the investigation of functional epigenetics in this or other non-resolving inflammation-driven diseases remains however in its infancy, especially in contrast to its rapid advance in the field of cancer. As the most abundant immune cell types in AS lesions, AAMs display high heterogeneity (including traditionally M1- vs. M2-like phenotypes and their related subtypes, as well as newly-characterized phenotypes, such as resident-like, inflammatory, and TREM2 foamy macrophages) due to their high plasticity, which is primarily controlled by the epigenetic machinery via transcriptional reprogramming of gene expression, a process named epigenetic remodelling. It has now been widely accepted that owe to their functional diversity and flexibility, AAMs play multifacial roles in non-resolving inflammation and other AS-related events (e.g. foam cell formation), which provides a rationale for developing the anti-inflammatory therapy by targeting the epigenetic modifiers that govern the phenotypic transition of macrophages, especially repolarization from pro-inflammatory (M1-like or inflammatory macrophages) to anti-inflammatory and pro-resolving (M2-like) phenotype as proposed in the alternative model for macrophage polarization. To this end, several macrophage-specific targeted approaches to polarize macrophages toward M2 have recently been investigated in AS or relevant inflammatory diseases, including profferocytic nanoparticles,¹²⁸ injectable silk/sitagliptin gel scaffolds,¹⁶⁵ small molecule compounds,¹⁶⁶ macrophage-engineering vesicles,¹⁶⁷

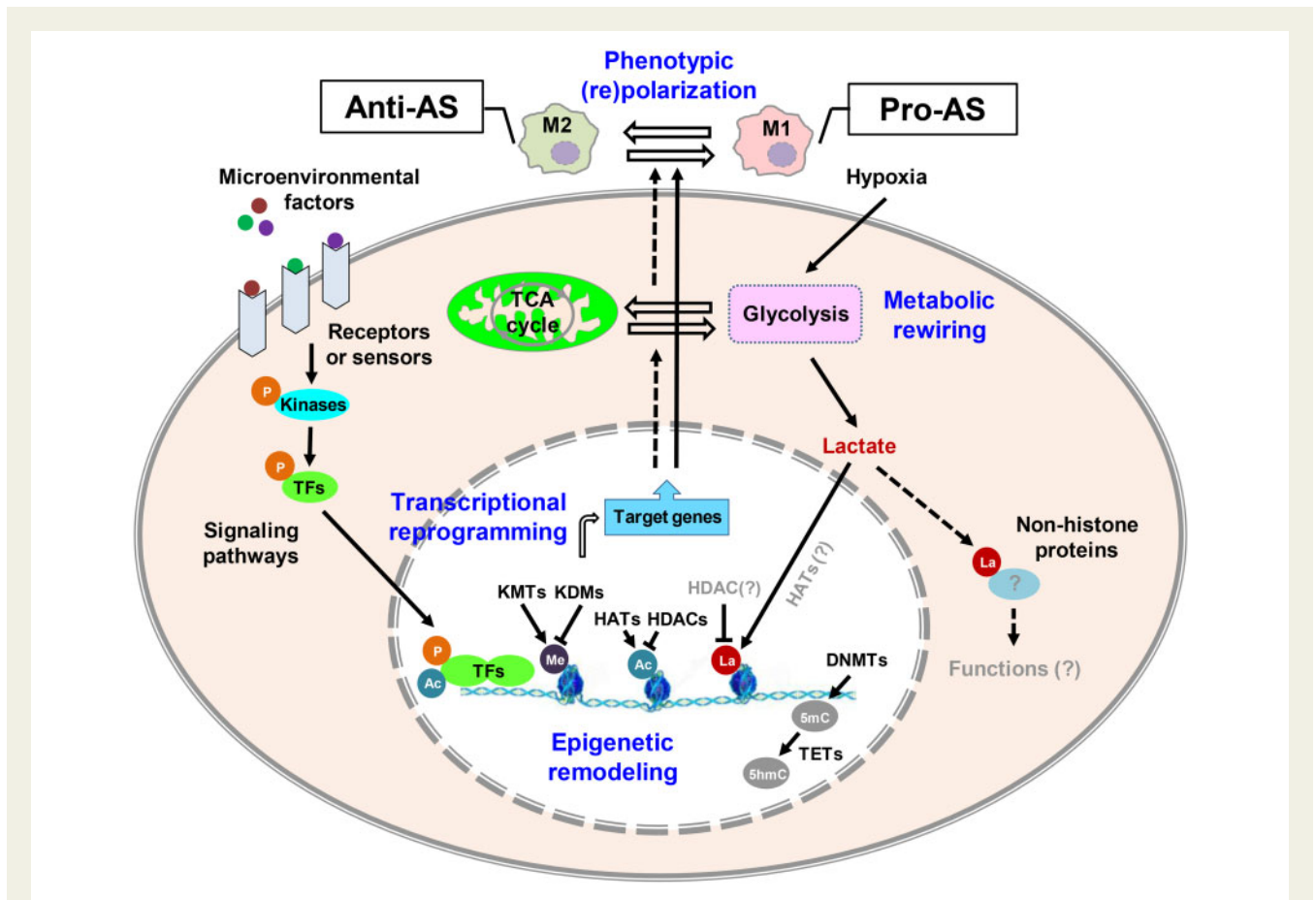


Figure 4 Current understanding of the molecular mechanisms underlying macrophage (re)polarization in AS. To date, many mechanisms have been described to address how macrophage polarization in response to various AS risk factors (including traditional and novel ones) and micro-environmental cues (e.g. oxLDL, hypoxia, and other pro-inflammatory factors) is regulated in inflammation and particularly non-resolving inflammation-driven diseases like AS. Briefly, macrophages are regulated via at least four mechanisms, including epigenetic remodelling, transcriptional reprogramming, metabolic rewiring, and phenotypic polarization. Among them, epigenetic remodelling links environmental stimuli or intracellular alterations (e.g. metabolic changes) to transcriptional reprogramming (either expression or silencing of specific genes), which in turn determine the direction of phenotypic (re)polarization directly or indirectly via metabolic rewiring. Owing to the central role of epigenetic remodelling in orchestrating these mechanisms, epigenetic modifiers that govern epigenetic remodelling by regulating DNA methylation and histone PTMs (e.g. methylation, acetylation, and lactylation) may therefore represent promising targets for the development of novel anti-inflammatory therapies against non-resolving inflammation-driven diseases, especially AS. However, extensive crosstalks among these mechanisms and potential ‘off-target’ effects (e.g. due to affecting PTMs of non-histone proteins, including acetylation and probably lactylation as well) may make this task difficult and complicated, especially in regards to the issue of effectiveness and safety. Thus, further understanding of these mechanisms and the precise roles of the epigenetic modifiers in AS is necessary to address such a challenge in this field.

SAA-containing HDL,¹⁶⁸ and CD137 agonists.¹⁶⁹ Of note, M1 may be less phenotypically plastic than M2, probably due to its greater epigenetic drift (e.g. much more extensive DNA methylation).¹⁷⁰ However, certain approaches (e.g. restoration of mitochondrial function by inhibiting NO production) can bypass or break this barrier, therefore leading to M1→M2 repolarization for therapeutic control of inflammatory diseases.⁵⁷ Alternatively, since polarization and maintenance of the M1 phenotype are primarily mediated by epigenetic remodelling, it is also possible to achieve M1→M2 repolarization by targeting the epigenetic modifiers governing this mechanism. In this case, our transcriptomic analysis reveals that several epigenetic modifiers involving histone acetylation and methylation are either up- or

down-regulated during M1 polarization and then almost completely reversed when repolarizing from M1 to M2, suggesting their potential roles in epigenetic remodelling of macrophages. Although evidence for dysregulated expression of these or other epigenetic modifiers in AAMs is still lacking, the application of scRNAseq can unveil macrophage- or its phenotype-specific expression of relevant epigenetic modifiers in human AS lesions, which allow validating the findings from the *in vitro* and *in vivo* animal studies involving AS. However, only a few epigenetic modifiers (e.g. TET2, DNMT3A, HDAC3, HDAC9, HDAC7, JMJD3, and KDM4A) in macrophages have functionally been characterized thus far to be involved in inflammation, of which those associated with AS are even fewer. Although they can

theoretically be considered the therapeutic targets in AS, caution should be taken in the development of anti-inflammatory or anti-AS agents that target these or other epigenetic modifiers due to their diverse functions in macrophages, which might lead to potential 'off-target' effects (e.g. by affecting the PTMs of non-histone proteins). It is also noteworthy that the epigenetic machinery most likely does not function on its own, while there are considerable crosstalks at the levels of cellular signalling transduction, epigenetic rewiring, transcriptional reprogramming, metabolic rewiring, and phenotypic polarization in macrophages. These include (i) the interactions between epigenetic regulation (e.g. epigenetic modifications and their modifiers) and transcription factors (e.g. NF- κ B, HIFs, IRFs, STATs, PU.1, PPAR- γ , etc.)¹⁷¹; and (ii) the interactions between epigenetic remodelling (e.g. DNA methylation and histone PTMs, such as methylation, acetylation, and lactylation) and metabolic rewiring (e.g. a paradigm shift from OXPHOS to aerobic glycolysis during M1 polarization, and then back to OXPHOS in M2 macrophages,^{172,173} an event consistent with the process of M1 polarization and then repolarization to M2 as described in the alternative model). In the latter case, targeting mitochondrial metabolism in macrophages represents another promising direction to develop anti-inflammatory therapy for treating AS.^{174–176} Therefore, it is necessary to deepen our understanding of the epigenetic mechanisms underlying macrophage-driven non-resolving inflammation in AS and to precisely define the functional roles of the epigenetic modifiers in transcriptional reprogramming of macrophage polarization and repolarization, particularly in the presence of either traditional or novel risk factors relevant to AS. With the application of novel analytic techniques (e.g. scRNAseq, CyTOF, and emerging spatial single-cell transcriptomics) and the advances in functional epigenetics, it can be anticipated that high cell type (e.g. macrophage)- or even phenotype-selective therapeutic targets and their corresponding agents, especially those (e.g. TET2 or DNMT inhibitors for DNA methylation, HDAC or KDM inhibitors for histone PTMs, and BRD inhibitors for epigenetic readers) approved or currently undergoing pre-clinical and clinical investigation for other diseases (e.g. cancer), would soon be added to the armamentarium for developing effective and safe anti-inflammatory therapy to tackle non-resolving inflammation-driven diseases, including AS and other chronic inflammatory diseases, from a distinct angle.

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Acknowledgements

The R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nli>) was utilized for analyses of gene expression profiling in macrophages under specified conditions.

Funding

This work was supported by the National Natural Science Foundation of China (81471165, 81670190, 81971108, 81670189, and 81870160), the Natural Science Foundation of the Jilin Province (20190201042JC and 20190201163JC), Program of the Education Department of Jilin Province (JJKH20190045KJ), Program of Finance Department of Jilin Province (2019SRCJ016), and Program of Jilin Provincial Science & Technology Department (20200201415).

Conflict of interest: none declared.

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