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Epigenetic regulation of innate immune dynamics during inflammation

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Abstract

Innate immune cells play essential roles in modulating both immune defense and inflammation by expressing a diverse array of cytokines and inflammatory mediators, phagocytizing pathogens to promote immune clearance, and assisting with the adaptive immune processes through antigen presentation. Rudimentary innate immune "memory" states such as training, tolerance, and exhaustion develop based on the nature, strength, and duration of immune challenge, thereby enabling dynamic transcriptional reprogramming to alter present and future cell behavior. Underlying transcriptional reprogramming are broad changes to the epigenome, or chromatin alterations above the level of DNA sequence. These changes include direct modification of DNA through cytosine methylation as well as indirect modifications through alterations to histones that comprise the protein core of nucleosomes. In this review, we will discuss recent advances in our understanding of how these epigenetic changes influence the dynamic behavior of the innate immune system during both acute and chronic inflammation, as well as how stable changes to the epigenome result in long-term alterations of innate cell behavior related to pathophysiology.

Summary Sentence:

Review of the epigenetic pathways controlling innate immune memory and their impact on acute and chronic inflammation.

Keywords

Inflammation; innate immune system; epigenetics; innate immune memory; histones; DNA methylation

1 - Introduction

The myeloid compartment is a critical regulator of inflammation during infection and injury.[1,2] As the first line of host defense, myeloid cells play important roles in immune cell recruitment, inflammatory modulation via cytokine release, and pathogen clearance through

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cytotoxic degranulation or phagocytosis. Rapid engagement of these cellular pathways is essential for proper immune clearance and necessitates that innate cells are poised for the activation of key immune genes. Likewise, negative feedback mechanisms must exist to dampen innate immune activity over time, thereby preventing tissue injury that results from prolonged inflammation. Dysregulation of these mechanisms is associated with systemic immune hyperactivation and organ damage, as observed in septic shock, as well as chronic inflammatory disorders such as multiple sclerosis, atherosclerosis, and ulcerative colitis.[3,4]

Much like the adaptive immune system, pathogen exposure can elicit long-term alterations to innate immune behavior through a process known as innate immune memory.[5] In contrast to cellular differentiation, during which irreversible changes to the morphological and behavioral characteristics of a cell are accomplished through lineage committments, innate immune memory enables cells to modify their activation profiles in response to varying danger/damage signals, thereby preserving their fundamental identity while adopting distinct functional states.[6] Dependent upon the nature, strength and duration of danger/damage signals, distinct states of innate immune memory can develop that range from training and tolerance to immune exhaustion (Figure 1). During innate immune training, exposure to select pathogen-associated molecular patterns (PAMPs) results in a cell-intrinsic state of heightened immunity such that future challenges provoke stronger immune responses. This mode of innate immune memory is typified by responses to fungal compound β-glucan or Bacille Calmette-Guérin (BCG) vaccine derivative trehalose 6,6'-dimycolate (TDM), which have been shown to confer broad immune enhancing effects in recipient individuals.[7–11] Tolerance, by contrast, produces the opposite effect; exposure to tolerizing compounds such as endotoxin lipopolysaccharide (LPS) diminishes innate cell responses to future immune challenges, thereby limiting the impact of unrestrained inflammation. On the other hand, prolonged immune stimulation such occurs in sepsis or severe Covid-19 infection results in monocyte exhaustion, a pathogenic form of memory defined by paradoxical pro-inflammatory and immunosuppressive gene signatures coupled with cellular de-differentiation and emergency hematopoiesis.[12–15] Persistent low-grade inflammation, as is observed in subclinical endotoxemia, can also result in the expansion of low-grade inflammatory monocytes that interfere with wound healing and atherosclerotic lesion clearance.[16–18] Such pathogenic memory states can have long-term effects on innate behavior, contributing to chronic inflammatory processes or infection-induced immunoparalysis.[19,20]

Like most forms of transcriptional memory, innate immune memory is regulated by epigenetics, or changes to chromatin above the level of DNA sequence.[5,21–23] Coordinated alterations of epigenetic features such as histone tail modifications, DNA methylation, long non-coding RNA interactions, or nucleosome remodeling help establish open chromatin landscapes that facilitate the binding of regulatory transcription factors or, conversely, generate closed chromatin environments to limit the impact of transcription factors on their target genes. In the innate immune system, epigenetics carries the dual important roles of priming immune genes for rapid activation in response to immune stimuli and fine tuning their expression to alter cellular responses to future immune challenges. Importantly, many of these epigenetic features also demonstrate mitotic heritability, meaning their patterns can

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be stably maintained across cell divisions and contribute to long-term alterations in immune behavior.

In this review we will discuss the contribution of epigenetics to the regulation the myeloid compartment during inflammation. We will focus particularly on chromatin features such as DNA methylation and histones, and will conclude with a discussion of their contribution to long-term innate immune behavior.

2 – Histones

In the eukaryotic genome, histones comprise the core protein octamer of nucleosomes, thereby assisting in the functional compartmentalization of DNA into transcriptionally active (euchromatic) or tightly bound and silent (heterochromatic) regions.[24] Given their close structural association with DNA and availability for covalent modification, histones represent an effective target for epigenetic regulation. This regulation can take one of several forms (Figure 2). In the first, covalent modification of histone tail regions can both alter the local biophysical properties of DNA and serve as a stable platform for the binding of histone "reader" proteins. This mode of epigenetic regulation is typified by histone H3 lysine (H3K) modifications, in which active (e.g. H3K27ac, H3K4me1/3) and repressive (e.g. H3K9me3, H3K27me3) histone marks modulate the transcriptional activity of their affiliate promoter and enhancer regions. In a related fashion, regulated expression of histone reader proteins can be utilized to modulate the impact of histone modifications on their linked genes. Finally, in addition to the canonical H2A/H2B and H3/H4 dimers that comprise the nucleosome octamer, histone variants can be integrated to influence the local chromatin landscape. For example, integration of histone variant macroH2A interferes with NF- κB binding and nucleosome sliding, thereby inhibiting local gene transcription.[25]

Regulation of immune gene expression via histone modification represents the best studied mechanism for epigenetic control of innate immune memory. Early reports demonstrated H3K4me3 remodeling at tolerized genes in LPS-stimulated macrophages and at the promoters of *Tnf*, *Il6*, and *Tlr4* in peripheral blood mononuclear cells (PBMCs) collected from patients four months after BCG vaccination.[26,27] Subsequently, in a landmark study conducted by the BLUEPRINT Consortium, it was demonstrated that active histone marks such as H3K27ac and H3K4me1/3 are broadly repatterned in both tolerized and trained macrophages.[28] Further studies expanded these observations to additional disease contexts, including systemic lupus erythematosus (SLE), endotoxemia, atherosclerosis, and systemic sclerosis, as well as broader immune triggers such as flagellin, oxidized low-density lipoprotein, and monophosphoryl lipid A.[29–35] Critically, it was demonstrated that immune training via histone reprogramming has the capacity to reverse endotoxemia-induced tolerogenesis and age-related immunosenescence, highlighting the therapeutic potential for innate training compounds not only in conferring broad spectrum resistance to infection, but also for the treatment of innate immune dysfunction.[36,37]

Given the existence of several excellent reviews covering the topics of histone modifications in innate immune memory and inflammation,[3,21,38] we will instead focus our discussion on recent evidence for alternative forms of histone regulation influencing myeloid

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behavior during inflammation, including histone variant incorporation, Polycomb-mediated epigenetic priming, and histone modification during neutrophil NETosis.

2a – Histone variant incorporation in innate immunity

While perhaps less well-studied than covalent tail modifications, histone variant incorporation represents an essential feature of epigenetic regulation, with at least 30 identified variants in eukaryotes.[39] These variants have critical roles in DNA repair, mitotic spindle assembly, genome stability, and transcriptional regulation, and have been implicated in the pathogenesis of numerous human diseases, including neurodevelopmental disorders such as alpha thalassemia X-linked intellectual disability syndrome and various cancers.[40,41]

In the innate immune system, several studies have implicated H3 variant H3.3 in the epigenetic regulation of myeloid behavior during infection or immune challenge. In contrast to canonical H3, H3.3 is incorporated into nucleosomes in a replication-independent manner and carries a unique S31 residue.[40] In LPS-stimulated bone marrow-derived macrophages (BMDMs) and dendritic cells (DCs), H3.3S31 is phosphorylated at induced genes to promote gene transcription through enhanced SETD2-mediated H3K36 methylation and eviction of transcriptional corepressor and H3K36me3 reader ZMYND11.[42,43] H3.3 also has potent antiviral activity. In response to innate immune signaling during herpesvirus infection, the HIRA histone chaperone complex silences viral gene expression through H3.3 deposition onto the viral genome in promyelocytic leukemia nuclear bodies.[44–46] Similarly, loss of H3.3 in hematopoietic stem progenitor cells (HSPCs) leads to dysregulated endogenous retroviral expression and aberrant interferon signaling, as well as predisposing granulocyte/macrophage precursor differentiation.[47] Lastly, while not explicitly an H3 transcriptional variant, widespread integration of N-terminal-cleaved H3 (H3 N) in monocytes regulates chromatin accessibility and is lost during macrophage differentiation; this mark is believed to have an anti-inflammatory role, as H3 N is depleted in monocytes of systemic juvenile idiopathic arthritis patients.[48]

In addition to H3.3, several H2A variants play critical roles in innate cell behavior. In monocytes, the histone chaperone NAP1 converts the *Tnf* promoter to transcriptionally permissive state through H2A.Z incorporation in response to LPS stimulation.[49] Consistent with this pro-inflammatory role, H2A.Z and H3.3 were found to be elevated in PBMCs of rheumatoid arthritis patients, with a positive correlation being noted between patient Disease Activity Scores and H2A.Z expression.[50] In addition to its role in macrophage NF-κB signaling, the H2A variant macroH2A has been shown to modulate inflammatory signaling between innate immune cells and in tumor microenvironments. MacroH2A deficient cancer-associated fibroblasts have increased myeloid chemoattractant activity due to hyperinducible expression of inflammatory genes, and macroH2A deficient hepatoblastomas have increased NF-κB signaling in response to TNF-α.[25,51,52] Similarly, the histone variant H2A.J activates pro-inflammatory gene expression in senescent fibroblasts, augmenting the senescence-associated secretory phenotype.[53] By contrast, the contribution of innate cell-intrinsic H2A variant regulation in chronic inflammatory disorders remains largely unexplored.

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2b – Bivalency and epigenetic priming in innate immune behavior

Epigenetic priming describes the process by which gene regulatory sites are maintained in an inactive but poised state, thereby allowing for transcriptional responses to signaling cues while simultaneously protecting against stable heterochromatin formation (Figure 3).[54,55] As the first line of defense against invading pathogens, innate immune cells are particularly reliant on this mode of epigenetic control to facilitate rapid immune engagement independent of previous challenges. At the chromatin level, primed enhancers and promoters are typically distinguished by bivalent signatures of active H3K4me1/3 and repressive H3K27me3 or H4K20me3 histone marks.[56] The major regulator of H3K27me3 deposition, Polycomb repressor complex 2 (PRC2), plays an essential role in this process by maintaining genes in an inactive state.[57] Additionally, the Polycomb repressor complex 1 (PRC1) acts as a reader of H3K27me3 and promotes chromatin compaction and H2AK119 ubiquitination, a mark that enables synergistic PRC2 recruitment through accessory proteins.

Numerous studies have reported a link between PRC2 regulation and inflammatory signaling in innate immune cells. In a recent small molecule inhibitor screen of epigenetic regulators in BMDMs, it was demonstrated that suppression of H3K27 methyltransferase and PRC2 component EZH2 significantly increased the expression of *Tnf* following LPS exposure.[58] Intriguingly, the researchers also demonstrated a global depletion of H3K27me3 in LPS-stimulated BMDMs, suggesting PRC2 inhibition may be a salient feature of tolerogenesis. Consistent with this model, the H3K27me3 demethylase JmjD3 is potently upregulated by NF-κB signaling in macrophages stimulated with LPS or IL-10; in these contexts, it plays a critical role in activating pro-inflammatory gene expression, including *Tnf*.[59–62] JmjD3 is also crucial for M2A macrophage polarization in response to IL-4 signaling or infection by helminth or *N. brasiliensis*, thus demonstrating the broad dependence of innate cells on Jumonji family histone demethylases to overcome Polycomb repression at bivalent regions.[61,63] Conversely, in response to IFN-γ signaling, EZH2 deposits H3K27me3 in the promoters of anti-inflammatory genes to stabilize macrophage activation.[64] In a similar fashion, PRC2 component EZH1 has been shown to promote TLR-mediated cytokine signaling in DCs and macrophages through promoter H3K27me3 deposition and silencing of TLR repressor *Tollip*.[65] Thus, PRC2 functions in both the positive and negative regulation of inflammatory signaling in myeloid cells.

In addition to its role in regulating immune signaling during infection, several studies have implicated PRC2 activity in chronic inflammatory disorders. In the context of atherosclerosis, myeloid-specific deletion of EZH2 in *Ldlr*$^{-/-}$ mice fed a high fat diet exhibit diminished atherosclerotic lesions and reduced macrophage foam cell inflammatory responses.[66] By contrast, transplantation of bone marrow from mice with a myeloid-specific deletion of H3K27 demethylase KDM6b into lethally irradiated *Ldlr*$^{-/-}$ mice accelerates collagen deposition and atherosclerotic lesion necrosis.[67] PRC2 dysregulation has also been demonstrated in several autoimmune disorders. EZH2 depletion reduces macrophage activation and attenuates autoimmune inflammation through the dysregulated expression of immunosuppressive *Socs3*, reducing TLR-mediated NF-κB activation and proinflammatory gene expression.[68] Macrophages deficient for H3K27me3 reader SP140 exhibit a severely attenuated response to LPS, and SP140 SNPs are associated with inflammatory disorders

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such as Crohn's disease and multiple sclerosis.[69–72] In monocytes collected from SLE patients, elevated levels of α-ketoglutarate, an essential cofactor for Jumanji family H3K27 demethylases, results in decreased H3K27me levels at interferon stimulated gene promoters and elevated expression in response to IFNα stimulation; critically, GSK-J4 inhibition of KDM6A/B activity in Balb/c SLE mice reduced autoantibody production, interferon stimulated gene expression, and kidney pathology.[29]

Finally, while less well-studied than PRC2, PRC1 has also been shown to contribute to innate immune regulation during inflammation. LPS-stimulated BMDMs rapidly upregulate the expression of PRC1 complex component and H2A ubiquitin E3 ligase component BMI1; deletion of *Bmi1* increases IL-10 expression in response to LPS stimulation, suggesting the enzyme plays a role in anti-inflammatory gene suppression.[73] Interestingly, PRC1 activity can also promote inflammatory gene expression, as PRC1 component and H3K27me3 reader CBX2 enhances viral-induced type 1 interferon responses in macrophages by recruiting H3K27me3 demethylase Jmjd3 to interferon gene promoters.[74] Conversely, in DCs, PRC1 component PCGF6 recruits the H3K4me demethylase JARID1d to genes involved in DC activation in order to maintain quiescent state; this effect is reversed by LPS stimulation, which causes a rapid downregulation of PCGF6 expression.[75] Thus, as with PRC2, PRC1 can positively or negatively impact inflammatory signaling depending on its interacting accessory components.

2c – Histone modifications in neutrophil NETosis

Neutrophil extracellular traps (NETs) are web-like fibrous structures comprised of DNA, histones, and granule proteins that exert potent antimicrobial and inflammatory activity.[76] The chromatin component of NETs is derived from neutrophil nuclear or mitochondrial DNA extruded through either programmed cell death ("suicidal" NETosis) or vesicular exocytosis ("vital" NETosis).[77–79] As the major structural component of NETs, chromatin plays several key roles in NET-mediated inflammation, including pathogen immobilization, cytotoxicity, and immune signaling.

Histone regulation has been demonstrated to be an essential facet of NET assembly and function. In response to inflammatory signals such as LPS or TNF-α, neutrophils engage H3 citrullination by peptidyl arginine deiminase PAD4 prior to chromatin decondensation and extrusion.[80–83] Regulation of histone acetylation has been proposed to facilitate this process: histone H4 acetylation was shown to promote chromatin decondensation and NETosis, while a separate group reported that H3 deacetylation is required for citrullination by PAD4.[84–86] Similarly, neutrophil H3 undergoes N-terminal cleavage in response to various inflammatory stimuli for incorporation into NETs; this is thought to represent an alternative mechanism to mitigate positive charge of histone tail, thereby promoting chromatin decondensation.[87–89] Such histone charge neutralization by tail cleavage or arginine citrullination is also critical for enabling cell-to-cell communication, as interactions between positively charged histone tails and negatively charged cell membranes are profoundly cytotoxic.[89–91] Interestingly, recent evidence suggests that this involvement in cell signaling, rather than driving NET assembly, is the primary role for histone citrullination, as citrullinated histones in NETs promote cytokine induction in monocytes through TLR4 binding and activation.[92]

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Additional forms of histone modification may also factor into immune signaling. Relative to healthy donors, NETs derived from SLE patients have elevated levels of acetylated H2B and H4 as well as H3K27me3, resulting in heightened NET immunostimulatory potential.[93,94] These modified histones are also target epitopes for SLE autoantibodies, further amplifying the NET inflammatory signal.[95,96]

Beyond SLE, dysregulation of NET-associated histone marks contributes to the pathology of numerous inflammatory disorders. In rheumatoid and juvenile idiopathic arthritis, NETs are also a source for carbamylated H3 and H4, which drive rapid monocyte osteoclastogenesis and pathogenic inflammation.[97–99] Consistent with reports of NET-derived histone cytotoxicity, citrullinated histones were shown to disrupt gut epithelial barrier function and integrity in a mouse inflammatory bowel disease model.[100] In the context of infectious disease, inhibition of class I/IIb HDAC activity alleviates NET-mediated inflammatory cytokine signaling during pneumonia and septic shock, and peripheral blood citrullinated H3 levels serve as an early biomarker for septic liver damage.[86,101] During viral infections, the NET signaling apparatus can also be hijacked, as NET histones were recently shown to enhance infectivity of SARS-CoV-2 by promoting host cell attachment and viral entry.[102] Finally, studies have linked NET histones to atherosclerosis progression. Citrullinated histones were shown to promote low-density lipoprotein (LDL) aggregation and foam cell formation, while PAD4 deficiency reduces the atherosclerotic burden in *ApoE*$^{-/-}$ mice.[92,103] These results track with previous reports that citrullinated H3 levels are elevated in heparin-induced thrombocytopenia/thrombosis patients, and that PAD4-deficient mice are protected from IgG thrombosis.[104] In sum, NET histones have potent inflammatory activity in diverse human disorders, and thus represent a compelling target for therapeutic intervention.[78]

3 – DNA Methylation

DNA methylation in the form of 5-methylcytosine (5mC) represents one of the most common forms of epigenetic modification, with approximately 70% of CpG dinucleotides in the human genome existing in a methylated state.[105] As an epigenetic regulator, DNA methylation is typically associated with gene silencing through its inhibition of methyl-sensitive transcription factor binding at promoters and enhancers.[106] However, DNA methylation can exert its regulatory effect through many additional mechanisms, including recruitment of 5mC readers, occlusion of cryptic splice or transcription start sites, and protection of genomic integrity against transposable elements or repetitive heterochromatin translocation.[107–110]

In the innate immune system, DNA methylation has a well-established role in regulating both myeloid cell differentiation and modulating gene expression in response to immune challenges.[111–116] Less well understood is the contribution of DNA methylation to innate immune memory mechanisms and the influence of DNA demethylation intermediates on innate immune activity. In this section, we will discuss recent advances in our understanding of 5mC regulation in innate cells during inflammation, and how DNA methylation repatterning may affect long-term immune behavior.

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3a – Influence of DNA methylation on myeloid behavior during inflammation

DNA demethylation at heterochromatic regions typically represents one of the earliest events during the epigenetic priming of gene promoters and enhancers.[117] For this reason, modulation of genomic DNA methylation levels is an essential component of innate immunity, facilitating the rapid induction of immune gene transcription in response to inflammatory stimuli. This is most easily observed during DC and macrophage differentiation, during which 5mC is broadly reprogrammed at genes critical for the innate immune response.[113,114] Notably, most of these genes do not exhibit commensurate transcriptional upregulation prior to inflammatory stimulation (e.g. LPS exposure), supporting an epigenetic priming role for DNA demethylation at these sites.

Perturbation of this epigenetic priming system during microbial infections can have a dramatically negative impact on host cell responses. For example, during *M. tuberculosis* infection of macrophages or DCs, the host DNA methylome is repatterned at key immune genes to potentiate a transcriptional profile favoring intracellular bacillary survival.[118–120] Given that transcription factor binding and gene upregulation were observed to precedes DNA demethylation, these changes are thought to stabilize long-term transcriptional alteration in infected cells.[121] Similar 5mC dysregulation was also observed in *A. phagocytophilum*-infected neutrophils, wherein transcriptional reprogramming was achieved through genome-wide DNA methylation repatterning to repress antimicrobial activity and promote inflammation.[122] In the context of severe Covid-19 infection, patient PBMCs were shown to exhibit perturbed DNA methylation at inflammatory cytokine genes and disrupted myeloid differentiation signatures consistent with the release of immature monocytes into the periphery.[123,124] While not explicitly viral-mediated, these results highlight the importance of proper DNA methylome establishment during innate immune differentiation, as disturbed 5mC signatures predisposed pro-inflammatory gene expression in patient monocytes.

Beyond epigenetic priming, DNA methylation alterations play an important role in fine-tuning innate immune behavior during severe immune challenges. Monocytes collected from sepsis patients exhibit broad changes in their DNA methylome, with numerous changes observed at inflammatory cytokine genes correlating with increased organ dysfunction.[125,126] Notably, substantial hypermethylation was observed at NF-κB targets, and treatment of cecal ligation puncture (CLP)-induced sepsis in mice with DNA methyltransferase (DNMT) inhibiting drug decitabine improved sepsis survival. Interestingly, in a separate study, supplementation of methionine, the metabolic precursor for methyl donor S-adenosylmethionine (SAM), inhibited the macrophage inflammatory response to LPS stimulation, suggesting a context-dependent role for DNA methylation in modulating innate immune behavior.[127]

Numerous studies have also implicated innate immune 5mC dysregulation in the pathogenesis of chronic inflammatory disorders, with distinct 5mC epigenetic profiles having been demonstrated in myeloid cells from asthma, cystic fibrosis, multiple sclerosis, and antiphospholipid syndrome patients.[128–132] Interestingly, altered DNMT activity is a recurring feature of chronic inflammatory disorders. Whereas *Dnmt1* expression is elevated in PBMCs of SLE patients, DNMT1 suppression was shown to deplete plasmacytoid DCs

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and slows SLE progression in mice.[133,134] In an idiopathic pulmonary fibrosis (IPF) model, DNMT3B was shown to suppress fibrotic macrophage polarization, consistent with reports that both DNMT3A and DNTM3B regulate macrophage polarization and that lung DNA methylation levels are negatively correlated with IPF severity.[135–139] *Dnmt3a* mutations also represent one of the most common drivers of clonal hematopoiesis of indeterminate potential, a contributing pathology to cardiovascular disease; a single-cell RNA sequencing analysis of peripheral blood monocytes from chronic heart failure patients harboring *Dnmt3a* mutations revealed a gene expression profile promoting inflammatory signaling, endothelial adhesion, and T-cell stimulation.[140–142] Consistent with these observations, treatment with folic acid, an upstream metabolite in SAM biosynthesis, restored global DNA methylation levels in fatty acid-exposed monocytes and suppressed atherosclerotic plaque formation in *ApoE*$^{-/-}$ mice.[143] By contrast, in the context of macrophage biology, DNMT suppression has been shown to be atheroprotective. In both *Ldlr*$^{-/-}$ and *ob/ob* mouse models, DNMT inhibition attenuated macrophage inflammation, slowed atherosclerosis development, and improved insulin sensitivity.[144,145] Given the strong link between PBMC DNA methylation and coronary artery disease (CAD) risk, such studies highlight both the potential for therapeutic interventions targeting DNA methylation in CAD patients as well as the necessity for targeted approaches to ensure 5mC modulation is exerted on the appropriate cell type.[146–150]

3b – TET enzymes and their oxidized derivatives in innate immunity

DNA methylation is catalyzed by DNMTs via the direct addition of a C-5 methyl group to cytosine.[106] This process may occur *de novo* through DNMT3L-mediated recruitment of DNMT3A or DNMT3B to unmethylated regions or through DNMT1 maintenance methylation at hemimethylated sites following DNA replication. By contrast, no direct means of DNA demethylation exists in vertebrates. Instead, DNA demethylation is accomplished through one of several different pathways (Figure 4). Genome-wide DNA demethylation, such as is observed during early embryonic or germline epigenetic reprogramming, occurs through the general suppression of DNMT activity, allowing for the passive dilution of 5mC over the course of several rounds of DNA replication and cellular division.[151] Alternatively, site-specific DNA demethylation is initiated through the iterative oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), or 5-carboxycytosine (5caC) by TET family enzymes. Oxidized cytosine derivatives are exempt from DNMT1 maintenance activity, allowing for their passive dilution over several rounds of cellular division. Additionally, 5fC and 5caC are recognized by thymine DNA glycosylase (TDG), which cleaves these residues to generate an abasic site and thereby start the process of base excision repair to restore the unmodified cytosine.

TET enzymes (particularly the TET2 isoform) have a well-established role in the development and function of the myeloid compartment. *Tet2* deletion in mice enhances the self-renewal capacity of bone marrow HSPCs and permits the premature differentiation of HSPCs toward the myeloid lineage, particularly monocytes and macrophages, via cell-intrinsic mechanisms.[152,153] As a result, compromised TET2 activity is associated with the development of severe myeloid malignancies in both mouse models and human patients.[154,155] This effect is believed to be mediated through TET2's oxidative

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activity, as transient 5hmC enrichment is observed at myeloid lineage enhancers during differentiation and direct inhibition of TET2 catalysis impairs functional myeloid differentiation.[112,114,115,156–158]

In mature myeloid cells, TET enzymes are important regulators of immune gene expression during inflammation. Following LPS exposure, both TET1 and TET2 act as negative transcriptional regulators of *Il1b* and several additional pro-inflammatory genes in DCs and THP-1 cells.[159] Consistent with this anti-inflammatory role, TET2 and 5hmC are generally depleted in PBMCs from multiple sclerosis patients.[160] By contrast, the pro-inflammatory recruitment of TET1 to the *Tnf* promoter in response to LPS stimulation drives local DNA demethylation and transcriptional upregulation in macrophages.[161,162] Several studies have also demonstrated a link between TET activity and NF-κB signaling during inflammation. Whereas TET2 depletion has little effect on the early LPS response in macrophages, LPS exposure induces *Tet2* upregulation for 24 hours via NF-κB signaling; knockout of TET2 results in the constitutive expression of LPS-induced genes in unstimulated peritoneal-derived (but not bone marrow) macrophages and a failure to resolve inflammation after LPS stimulation in BMDMs[163] In DCs, treatment with vitamin C, a cofactor for TET enzymatic activity, promotes TET-mediated demethylation at NF-κB/p65 binding sites and boosts the cells' immunostimulatory potential.[164] On the other hand, evidence also suggests that MAFB and glucocorticoid-mediated recruitment of TET2 to tolerance genes promotes DNA demethylation and DC tolerance acquisition, consistent with the role of NF-κB signaling in tolerogenesis.[165,166] Thus, TET enzymes are crucial not only for immune priming, but also for compensatory tolerizing activity following immune stimulation.

Interestingly, TET enzymes are also able to influence innate immune gene regulation through mechanisms outside of their DNA demethylation activity (Figure 5). First, TET enzymes exhibit non-catalytic activity in their ability to recruit transcription factors and chromatin modifying enzymes to DNA through protein-protein interactions. For example, following IL-6 upregulation in LPS-stimulated DCs and macrophages, TET2 mediates HDAC1/2 recruitment to the *Il6* promoter to drive histone deacetylation and IL6 repression; a similar effect is observed at the *Il1b* promoter, with the result that *Tet2* deletion significantly upregulates *Il1b* expression in response to various inflammatory stimuli.[167,168] TET's non-catalytic activity was also shown to be a critical regulator of mast cell proliferation, as the hyperproliferative phenotype of *Tet2*-null mast cells could be rescued with catalytically dead TET2.[169] Second, TET enzymes have been demonstrated to catalyze the oxidation of 5mC not only in the context of DNA, but also in mRNA.[170,171] As a notable example, TET2-mediated demethylation of the *Socs3* mRNA 3'UTR inhibits its translation, thereby enabling infection-induced emergency myelopoiesis.[172] Finally, there is some evidence to suggest that oxidized 5mC intermediates exhibit their own signaling potential in the genome. Various regulatory mechanisms have been ascribed to these oxidized intermediates, including roles in transcription factor recruitment, transcription fidelity, DNA damage response, nucleosome stabilization, and heterochromatin suppression.[173–184] Whether TET-oxidized intermediates may play similar roles in innate immune cells remains an open area of investigation.

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3c – Contribution of DNA methylation to innate immune memory

In contrast to histone modifications, the contribution of DNA methylation to innate immune memory is less well understood. This may be due in part to the limited evidence for DNA methylation's involvement in innate immune training. In response to BCG vaccination, differential methylation was observed in PBMCs from responders versus non-responders in immune pathway genes that were stable for up to 8 months.[185] Similarly, differences in promoter methylation were shown to underlie adaptive natural killer cell subsets in response to CMV infection.[186] However, in contrast to the broad H3K4me and H3K27ac reprogramming observed in BMDMs trained with β-glucan, altered DNA methylation was limited to only a few hundred genomic loci in trained cells.[28,36] Thus, DNA methylation is thought to be a minor contributor to the epigenetic memory of trained cells.

Instead, DNA methylation seems to play a greater role in innate immune tolerogenesis. Within 24 hours of LPS exposure, BMDMs exhibit stable changes in DNA methylation at gene enhancers that become more extensive as the cells differentiate.[36] Consistent with this observation, treatment with DNMT inhibitors had a negligible impact on TNF-α expression in β-glucan-trained BMDMs, but instead prevented TNF-α suppression in LPS tolerized cells.[58] This process is dependent in part on TET2-mediated DNA demethylation downstream of JAK2/STAT signaling, as JAK2 inhibition interfered with tolerance gene upregulation in LPS-stimulated human monocytes.[187] A similar dependence was observed in DCs tolerized through vitamin D exposure, with TET2-mediated demethylation of tolerance genes being orchestrated through direct interactions with STAT3 and the vitamin D receptor.[188] Finally, while there is a lack of *in vivo* studies to corroborate the longitudinal role of DNA methylation in tolerance memory, there exists correlative evidence to support DNA methylation's influence on tolerance behavior. Cultured blood monocytes collected from sepsis patients exhibit a unique DNA methylome that correlates with a tolerance signature of decreased TNF-α, IL-1β, and IL-6 secretion and increased IL-10.[125] Furthermore, while relatively few changes in DNA methylation were observed in circulating monocytes between acute pneumonia patients and controls, multiomics integration identified a link between DNA methylation and endotoxin tolerance via transcriptional signatures attuned to cholesterol biosynthesis.[189] Thus, altered DNA methylation seems to be a conserved feature of innate immune tolerance.

Beyond tolerance and training, there is emerging evidence to support the involvement of DNA methylation in establishing pathogenic innate immune memory states. In a recent study from our lab, we demonstrated that thousands of sites across the genome experience differential methylation in exhausted BMDMs under prolonged LPS stimulation.[190] These changes are conserved across monocyte subtypes, suggesting a common suite of differentially methylated regions that correlate with altered gene expression of exhaustion regulators such as *Plac8*, *Socs3*, *Runx3*, and *Klf4*. Notably, therapeutic intervention in the form of DNMT inhibitors or immune training compounds such as TDM or mycolic acid rescues the exhaustion phenotype at the level of both gene expression and DNA methylation.[190,191] Thus, despite the limited evidence for DNA methylation's involvement in innate immune training, immune training compounds have the potential to counteract

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pathogenic DNA methylation patterns in a similar fashion to β-glucan's effect on disrupted H3K27ac in tolerized cells.[36]

4 – How long is "long-term" innate immune memory?

Epigenetics plays an essential role in shaping cellular behavior, limiting the expression of genes to those required for cell type-specific function and facilitating the rapid activation of genes in response to external stimuli. As the central component of transcriptional memory, epigenetics resides at the interface between genes and environment, integrating information from external stressors and fine-tuning gene expression to maximize organismal fitness. Unlike in the adaptive immune system where long-term memory is mediated through V(D)J recombination specific to a given antigen, innate immune cells instead rely on non-specific signaling queues to either strengthen (train) or diminish (tolerize) their response to future immune challenges. These functionally opposed mechanisms are crucial for immune homeostasis, priming the immune system against future reinfection while limiting the negative impact of sustained inflammation. Whereas these mechanisms are expected to have a tremendous impact on the health of an individual, how long epigenetic memory can shape innate immune behavior remains one of the major outstanding questions in the field of innate immunity.

Perhaps one of the best studied phenomena relating to long-term innate immune memory is innate training linked to BCG vaccination. One month after BCG vaccination, altered H3K27ac patterns are observable in patient monocytes that correlate with trained cytokine induction and reduced yellow fever viremia.[192] At three months post-BCG vaccination, increased H3K4me3 is still visible at gene promoters for pro-inflammatory *Tnf* and *Il6* in circulating monocytes of human patients, as well as genome-wide differential open chromatin environments correlating with transcriptional reprogramming in these cells.[27,193] Interestingly, despite no obvious link between altered DNA methylation and innate immune training in β-glucan-stimulated BMDMs, BCG-linked changes in DNA methylation at interferon-responsive genes were present in circulating monocytes 14 months after vaccination in infants.[194] This is consistent with the high stability of DNA methylation in the genome; for example, longitudinal studies of DNA methylation in peripheral blood leukocytes of inflammatory bowel disease patients found evidence for differential methylation stable for periods as long as 9 years.[195]

Such long-term memory raises questions as to the mechanism by which these epigenetic changes are propagated in innate immune cells. Most of the above studies focused on monocytes, which have subtype-specific lifespans ranging from 24 hours (classical) to one week (non-classical).[196–198] Furthermore, a recent study of H3K4me and H3K27ac propagation in immortalized BMDMs trained with β-glucan found that these marks are progressively lost over the course of 14 rounds of cellular division, calling into question the cell-intrinsic capacity of innate immune cells to retain epigenetic memory.[199] Thus, long-term innate immune memory is expected to derive from sources extrinsic to mature immune cells.

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While the adaptive immune system contributes in part to long-term innate immune memory, multiple studies have also implicated central innate memory of bone marrow HSPCs as a reservoir for epigenetic changes in innate immune cells.[200,201] In mice, β-glucan treatment promotes IL-1β signaling in the bone marrow to drive the expansion of transcriptionally reprogrammed HSPCs conferring lineage trained immunity, thereby reducing the impact of LPS-induced systemic inflammation as late as four weeks after β-glucan administration.[202] This effect is likely mediated through epigenetic changes in stem and progenitor cells; differential open chromatin and H3K4me and H3K27ac peaks observed in circulating monocytes and BMDMs from BCG-vaccinated individuals correlate with patterns observed in hematopoietic stem cells (HSCs), suggesting epigenetic memory is transmissible through myeloid differentiation for at least 3 months.[193,203] In a separate mouse study, single-cell ATAC-seq revealed chromatin remodeling in HSPCs two weeks after the cessation of ligature-induced periodontitis; in this context, however, immune training led to the maladaptive expansion of hyper-inflammatory myeloid cells, exacerbating collagen antibody-induced arthritis development in bone marrow recipient mice.[204] These results underscore the potential hazards of innate immune training, which can result in long-term central memory increasing susceptibility to inflammatory conditions such as atherosclerosis, SLE, and systemic sclerosis.[205,206] Furthermore, HSC-derived epigenetic memory is not limited to immune training, as *Mycobacterium* infection was shown to induce transcriptional alteration of HSCs to suppress myelopoiesis and innate training, leading to bone marrow exhaustion at least one year post-infection.[207] This effect mirrors results from our own lab that demonstrated persistent DNA methylation changes associated with innate immune exhaustion in bone marrow monocytes even after sepsis resolution.[190] Similarly, studies in mice revealed that disrupted H3K4me3 deposition at promoters of *Il1b*, *Il12*, and *Il23* in bone marrow HSPCs 4 weeks after sepsis recovery correlated with impaired wound healing of macrophages in response to tissue injury.[208] Thus, epigenetic changes comprising central innate immune memory represent the likely origin of long-term peripheral innate memory.

Beyond the immune system, research has also demonstrated that epithelial and stromal cells are critical reservoirs for inflammatory memory.[209,210] In response to inflammatory damage, skin epithelial and hair follicle stem cells develop persistent chromatin accessibility to drive heightened inflammasome signaling and wound repair upon reinjury.[211,212] Similar results were demonstrated for pancreatic epithelial cells, which undergo stable epigenetic reprogramming following injury to attenuate future inflammatory responses and limit tissue damage.[213] Such inflammatory memory can broadly impact innate host defenses through alterations in cytokine signaling, immune activation, and infiltration.[214] For example, both lung and synovial fibroblasts exhibit memory in that inflammatory cytokine exposure primes increased IL-6 secretion upon restimulation.[215,216] The resulting web of intercellular interaction becomes quite complex, with crosstalk between immune and non-immune cells each exhibiting their own cell-autonomous forms of inflammatory memory, therefore necessitating care extrapolating results from a single cell type to the organismal level.

In addition to these mechanisms for long-term epigenetic memory, there is increasing evidence to support the involvement of post-transcriptional regulation in the modulation of short-term innate behavior. This mode of regulation encompasses a broad range of

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mechanisms, including alternative splicing and polyadenylation, altered RNA stability, RNA editing, translational re-initiation, and interactions with RNA binding proteins and microRNAs.[217,218] Given that epigenetic control requires significant upstream regulation prior to transcriptional initiation or suppression, the major advantage post-transcriptional control is the speed with which cells are able to alter protein translation to match the immune demands of the cell. Markedly, under LPS stimulatory conditions, altered cell behavior is often linked with broad transcriptional silencing, increasing the cell's reliance on a stable transcript pool.[26] It is worth noting, however, that this transcriptional silencing is frequently linked to a genome-wide expansion of repressive chromatin features, including increased DNA methylation and a depletion of active histone marks such as H3K27ac and H3K4me3.[28,31,58,190] Thus, epigenetic regulation may function in the short-term as a regulatory switch to prioritize mature transcripts over nascent transcript synthesis, although one cannot discount inhibition of immune signaling pathways as a major driver for this transcriptional suppression.[219]

Perhaps more controversially, emerging studies have reported evidence of intergenerational transmission of trained immunity. Two reports noted a link between maternal BCG vaccination status and cytokine production in neonatal samples; however, whether this effect is mediated through germline transmission or maternal environment is unclear.[220,221] Bona fide intergenerational inheritance of trained immunity was first reported for the F1 offspring of male mice infected with *C. albicans*; these mice demonstrate increased inflammatory cytokine secretion and survival during endotoxin challenge, potentially through the persistence DNA methylation patterns inherited from the paternal germline.[222] However, a separate mouse study failed to recapitulate intergenerational immune resistance arising from paternal BCG vaccination, β-glucan exposure, or *C. albicans* infection.[223] Thus, the contribution of parental immunity to the innate immune status of offspring remains unclear.

5 – Conclusion and Future Directions

Epigenetic alterations of histones and DNA methylation are major regulators of innate immune behavior during inflammation. These changes can take one of several forms, including epigenetic priming to facilitate rapid transcriptional activation in response to external cues, direct signaling through oxidized 5mC intermediates or NET-associated histones, and long-term alterations to the epigenome to potentiate innate immune memory. Dysregulation of these epigenetic mechanisms can be profoundly deleterious for the organismal health and survival, both during acute infection and chronic inflammatory disorders. For this reason, epigenetic pathways represent valuable targets for therapeutic intervention, with several promising trials underway to test the effect of innate immune training compounds or small molecule epigenetic inhibitors in disease treatment and prevention.[224–227]

Despite major advances in the field of innate immune memory over the past decade, several important avenues of research warrant further consideration. First, given that most of the work during this period has focused on the role of histone modifications in regulating innate memory, alternative forms of epigenetics (e.g. DNA methylation, chromatin remodeling,

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non-coding RNAs) remain critically underexplored. To that end, recent advances in joint (e.g. Chromium Single-Cell Multiome ATAC+Gene Expression) and single-cell (e.g. Joint-snhmC-seq for the simultaneous profiling of 5mC and 5hmC at single-cell level) sequencing techniques offer researchers unprecedented insight into the epigenome, improving our understanding of the influence of epigenetics on gene expression and the hierarchical interplay of distinct forms of epigenetic modification within an individual cell.[228] These techniques also sidestep major technical pitfalls from bulk sequencing approaches, as for example the misattribution of PBMC DNA methylation changes to epigenetic memory rather than shifting cell compositions.[229] Second, whereas the contribution epigenetics to innate immune behavior is now well understood, considerable work remains to determine the underlying molecular mechanisms by which these epigenetic alterations are established and propagated. This is of particular importance given that innate immune memory exhibits distinct phases of epigenetic reprogramming, complicating our identification of molecular targets for therapeutic interventions.[36,199] Equally critical is improving our understanding of the underlying source of long-term innate immune memory, especially in reference to central innate immune memory and the cell non-autonomous influences of adaptive and epithelial or stromal inflammatory memory. Finally, we advocate for an expansion of our molecular toolkit beyond BCG and fungal derivatives. Different immune training compounds exhibit distinct modes of pattern recognition and cell signaling activation upstream of epigenetic changes, some of which may be better suited for a given clinical application.[34,191] Distinct forms of epigenetic memory are also achievable through variation in the strength and duration of training molecule delivery.[12,17] By refining our understanding of innate memory modulators and their downstream phenotypic impact, we can better tailor molecular intervention to specific clinical contexts for both the prevention of inflammatory disease and restoration of chronic immune disfunction.

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Abbreviations

5caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
BCG	Bacille Calmette-Guérin
BMDM	Bone marrow-derived macrophages
CAD	Coronary artery disease
CLP	Cecal ligation puncture

DC	Dendritic cell
DNMT	DNA methyltransferase
H3 N	N-terminal-cleaved H3
H3K	Histone H3 lysine
HSPC	Hematopoietic stem progenitor cells
IPF	Idiopathic pulmonary fibrosis
LPS	Lipopolysaccharide
NET	Neutrophil extracellular trap
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PRC	Polycomb repressor complex
SAM	S-adenosylmethionine
TDG	Thymine DNA glycosylase
TDM	Trehalose 6,6'-dimycolate

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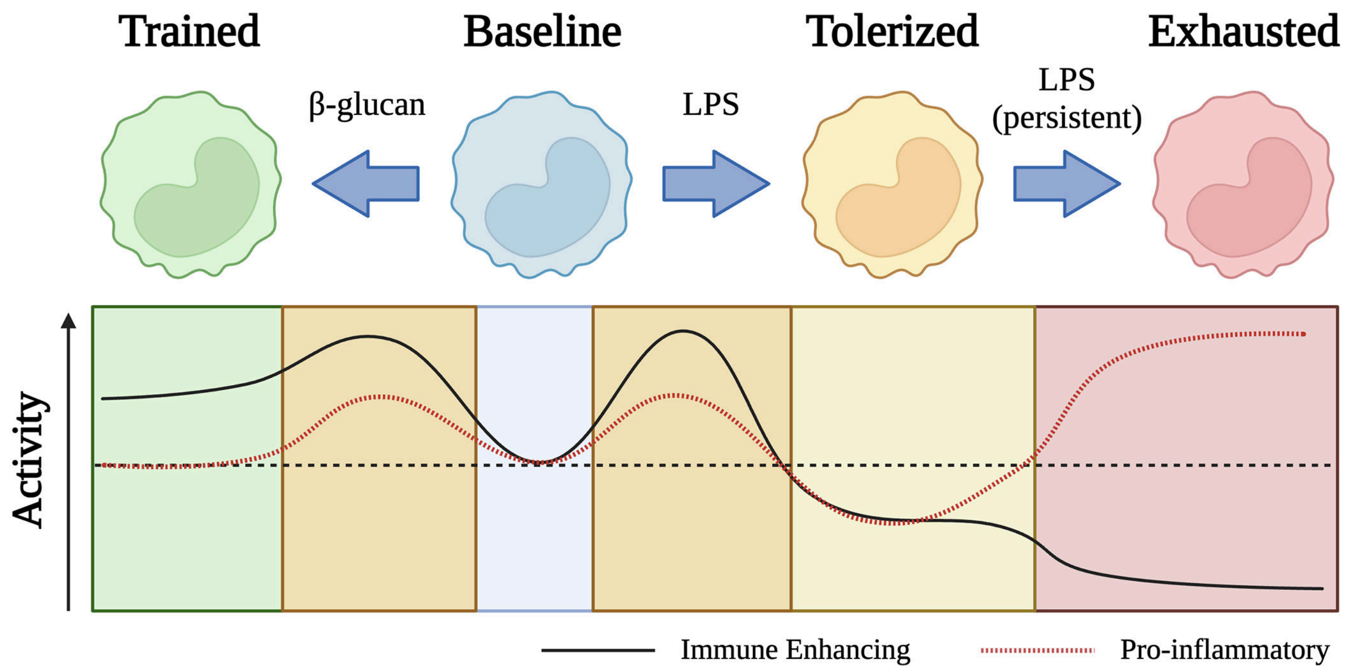

Figure 1 –. Modes of innate immune memory.

Innate immune cell exposure to pathogen-associated molecular patterns alters the cellular response to future immune challenges. Immune training (e.g. β-glucan exposure) exerts an immune enhancing effect that increases the strength of future immune responses. Tolerization (e.g. LPS exposure) produces the opposite effect, diminishing the immune response to future challenges. Prolonged exposure to immune pathogens results in innate immune exhaustion, during which cells exhibit immunosuppressive behavior and normal cellular functions are severely compromised. In contrast to the acute inflammation observed during initial immune stimulation, exhausted cells pro-inflammatory behavior is often chronic and debilitating.

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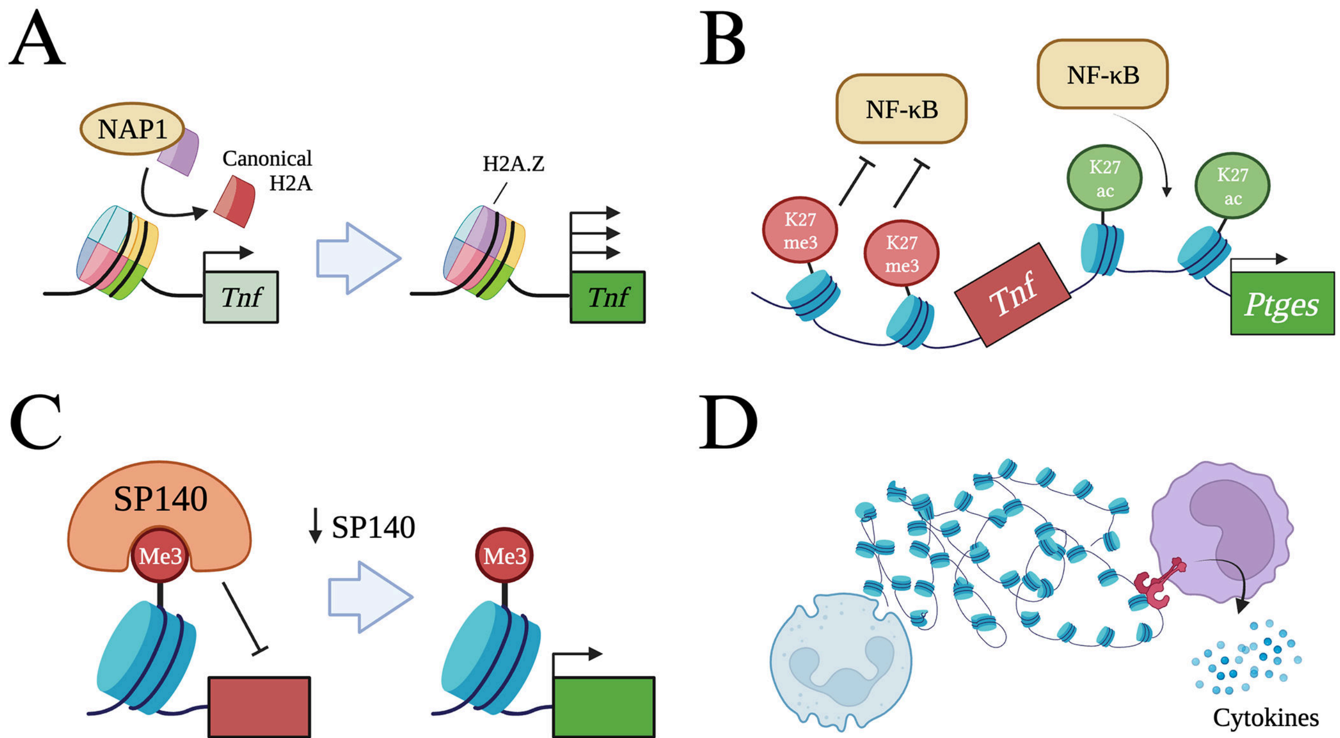

Figure 2 –. Major forms of histone regulation of innate immune behavior.

A) Incorporation of histone variants into nucleosomes alters the local chromatin environment to increase or decrease gene expression. In the provided example, NAP1-mediated integration of variant H2A.Z into the *Tnf* promoter region results in an open chromatin environment conducive to increased *Tnf* expression. **B)** Covalent modification of histone tails regulates gene expression by influencing chromatin compaction and transcription factor recruitment through histone reader proteins. These covalent modifications can be transcriptionally repressive (e.g. H3K27me3) or activating (e.g. H3K27ac). In tolerized cells, H3K27me3 incorporation into the *Tnf* promoter prevents NF-κB binding and gene expression, while retention of H3K27ac in the non-tolerizable *Ptges* promoter permits sustained NF-κB binding and transcriptional activation. **C)** Altered expression of histone reader proteins influences signal integration of covalently modified histones. For example, diminished expression of H3K27me3 reader protein SP140 permits the expression of H3K27me3-marked genes normally repressed through SP140 binding. **D)** Histones incorporated into extruded genomic DNA during neutrophil NETosis signal to nearby immune cells in order to propagate inflammation.

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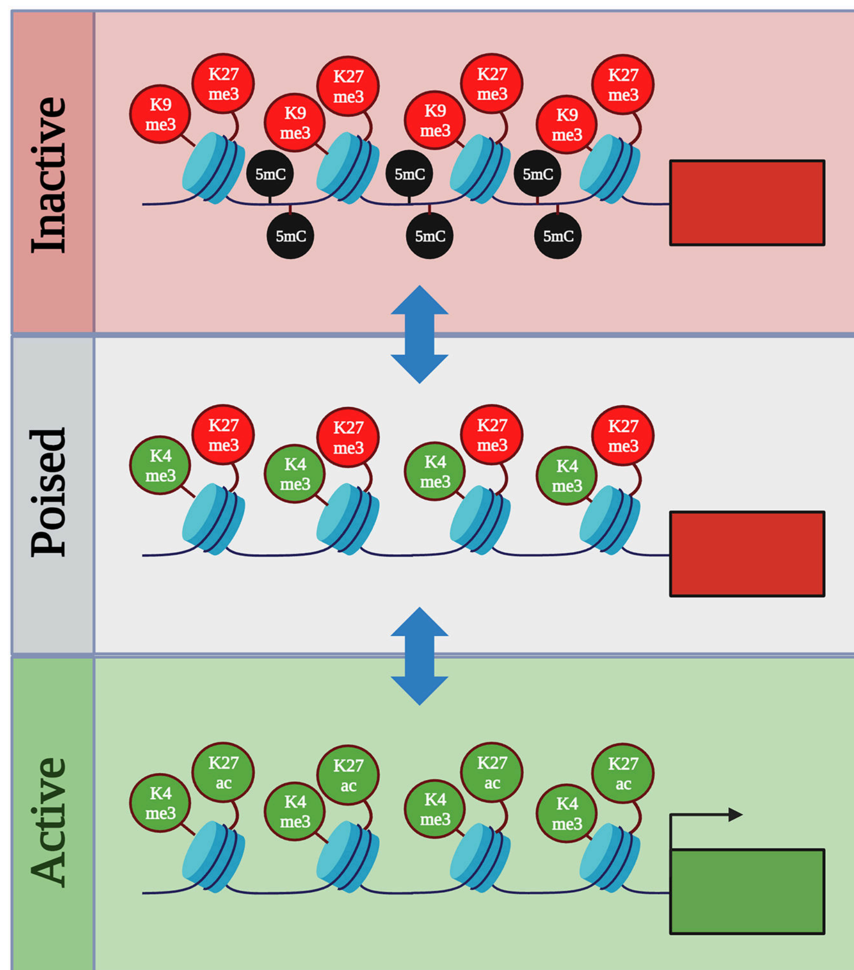

Figure 3 –. Epigenetic priming.

Gene regulatory features are maintained in distinct epigenetic states based on the transcriptional demands of their affiliate genes. Inactive or quiescent genes are often marked with various heterochromatin features, including repressive histone modifications (e.g. H3K9me3 and H3K27me3) and DNA methylation. During epigenetic priming, removal of DNA methylation and H3K9me3 paired with the deposition of activating histone marks H3K4me1 (enhancers) or H3K4me3 (promoters) results in a bivalent epigenetic profile to suppress gene expression while remaining poised for rapid transcriptional activation in response to further signaling cues. Finally, replacement of H3K27me3 with activating H3K27ac creates an open chromatin environment permissive to transcription factor binding and gene expression.

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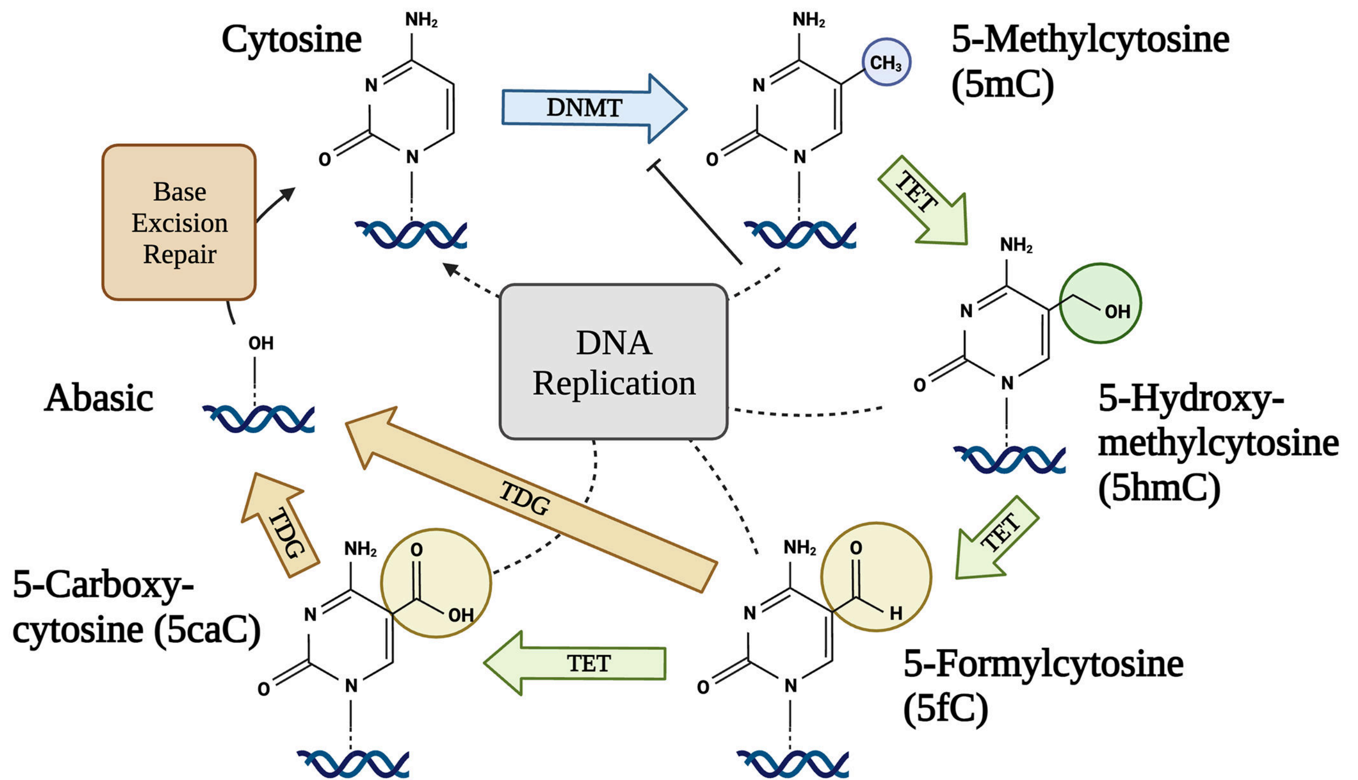

Figure 4 –. Pathways for DNA demethylation.

Following DNA methylation through the enzymatic activity of DNMTs, several pathways exist to restore cytosine to its unmodified state. Suppression of DNMT activity permits the global loss of 5mC in the unmethylated daughter strand following DNA replication. Alternatively, in a site-specific manner, TET enzymes promote the iterative oxidation of specific 5mC residues to 5hmC, 5fC, or 5caC. These oxidized residues are not recognized by maintenance methyltransferase DNMT1, allowing for their passive loss through DNA replication. Alternatively, 5fC and 5caC are recognized by the enzyme TDG, which targets the residue for degylcosylation to generate an abasic site. Base excision repair then restores the unmodified cytosine in a process independent of DNA replication.

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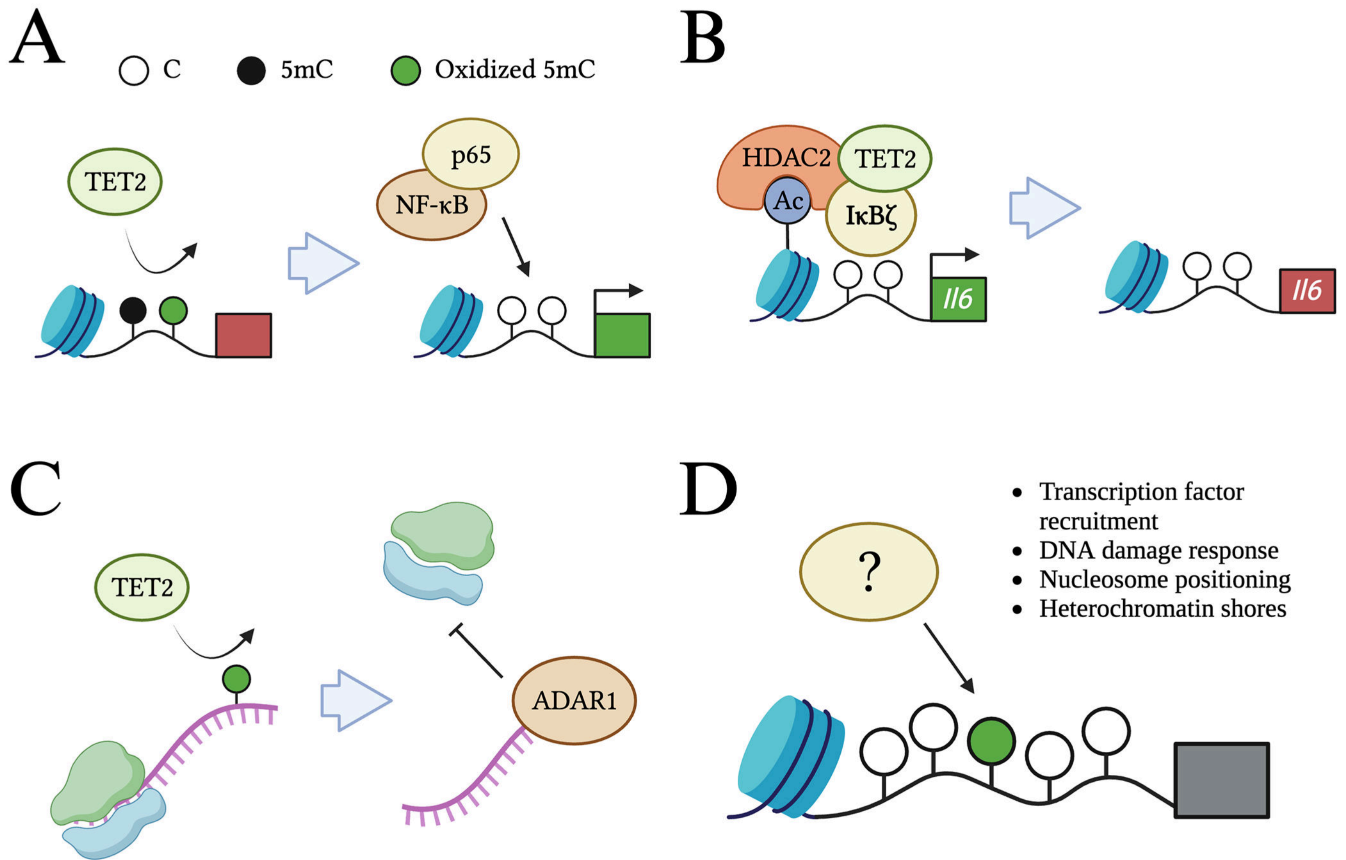

Figure 5 –. Mechanisms of TET-mediated regulation of innate inflammatory processes.
A) TET-mediated DNA demethylation promotes open chromatin formation to permit the binding of methyl-sensitive transcription factors. In the provided example, TET2-mediated demethylation of NF-κB/p65 binding sites promotes transcription and boosts the innate cell's immunostimulatory potential. **B)** Non-catalytic TET activity influences gene expression through the recruitment of transcription regulators via protein-protein interactions. For example, in tolerizing DCs and macrophages, TET2-mediated recruitment of HDAC2 promotes histone deacetylation at the *Il6* promoter to suppress its expression. **C)** TET enzymes oxidize methylated cytosines on RNA molecules to alter their stability and translation. In this example, TET2 oxidation of the 3' UTR of *Socs3* leads to ADAR1 binding and suppressed translation through the enzymes base editing activity. **D)** Various signaling pathways and gene regulatory activities have been proposed to be mediated through readers of oxidized cytosine residues.