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Review

The epigenetic landscape of innate immunity

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Abstract: The inflammatory response is the first line of defense against infectious agents or tissue damage. Innate immune cells are the crucial effectors regulating the different phase of inflammation. Their ability to timely develop an immune response is tightly controlled by the interplay of transcriptional and epigenetic mechanisms. The immunological imprinting elicited by exposure to different concentrations and types of infectious agents determine the functional fate of immune cells, forming the basis of innate immune memory. In this review we highlight the best-characterized examples of gene reprogramming occurring during different phases of inflammation with particular emphasis on the epigenetic marks that determine the specificity of the immune response. We further review the potential of cutting edge experimental techniques that have recently helped to reveal the deep complexity of epigenetic regulation during the inflammatory response.

Keywords: inflammation; histone modification; DNA methylation; non-coding RNAs; epigenetics

1. Introduction

Innate immunity relies on the ability of myeloid cells to efficiently sense and react to various extracellular stimuli in order to protect the organism from pathogenic infections or tissue damage. This prompt response is achieved through the engagement of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) with microbial pathogens. This activates downstream molecular signaling pathways that culminate in the induction of specific gene expression programs (for review

see [1-4]. Once the stimulus is exhausted, the innate immune system restores homeostatic conditions. Strict control of this response is required to avoid either systemic infection (due to an inadequate response) [5,6] or autoimmune disease (arising from chronic inflammation) [7,8]. This process is made possible by the tunable epigenetic control of the differentiation of myeloid cells into immune effectors [9].

The term epigenetics, initially suggested by Waddington in 1942 and derived from the Greek word "*epigenesis*", originally described the influence of genetic processes on development [10]. Nowadays, epigenetics defines the study of the molecular mechanisms promoting altered gene expression patterns, manifesting at phenotypic rather than genotypic level. Recently, significant advances in the development of high-throughput techniques have shed light on the epigenetic landscape of innate immunity. Genome-wide studies profiling transcriptional and epigenetic modifications during inflammation identified differences in non-coding RNAs (ncRNAs), histone modifications and DNA methylation patterns regulating myeloid cell differentiation, inflammation and innate memory (Figure 1) [11-13]. Epigenetic regulation of innate immune memory has been recently uncovered by elegant studies showing that memory characteristics are not confined to adaptive immunity, as was previously thought. Cells of the innate immune system, such as monocytes, macrophages and natural killer (NK) cells, retain a memory of past stimulations and actions, allowing them to enhance the immune response when facing new challenges (for review see [14,15]). So-called "trained immunity" is not antigen-specific, allowing a more robust and heterogeneous response

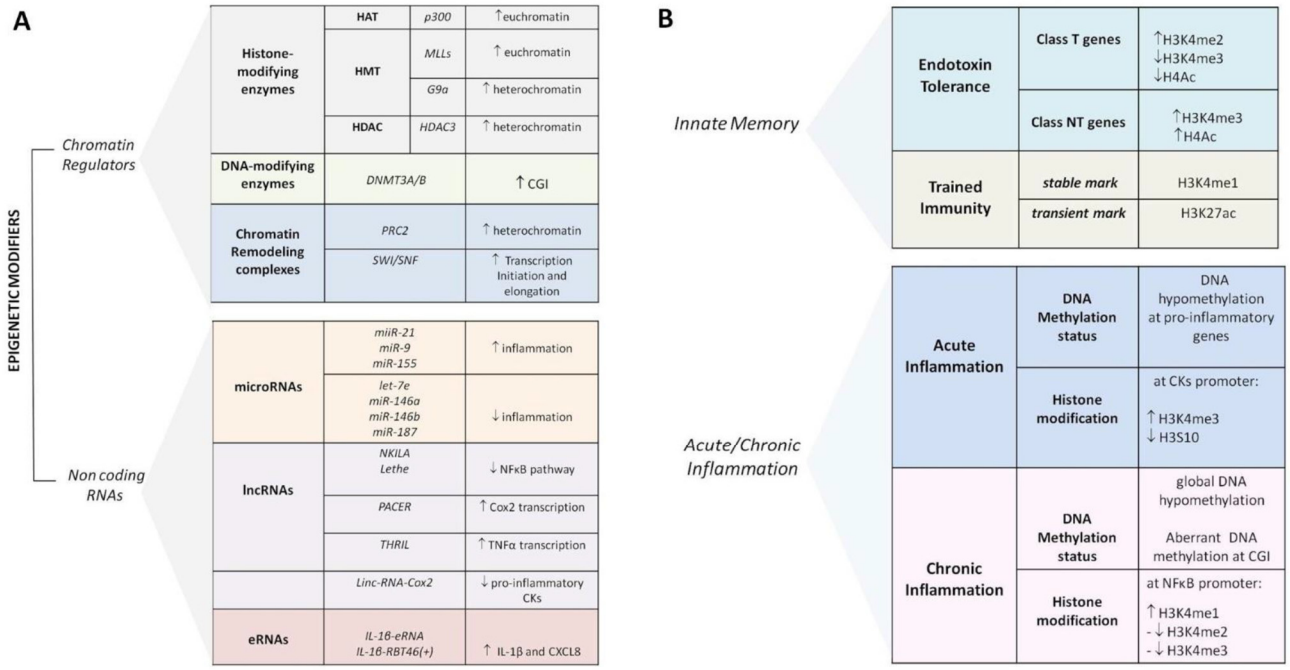

Figure 1. Epigenetic modifications of Innate Immunity. (A) Scheme of the epigenetic modifiers involved in the regulation of innate immune response. Some of the best characterized epigenetic players involved and their relative effect on inflammation are here illustrated. (B) Epigenetic changes (histone modification; DNA methylation) in innate memory and acute vs chronic inflammation. For a more comprehensive description of chronic inflammation see ref (X and Y).

against a larger spectrum of pathogens in the secondary challenge [16-18]. However, the innate immune system is not always primed for an enhanced immunological response. There are times when, in order to maintain a fine-tuned homeostasis, a decreased or attenuated response is instead affected. "Endotoxin tolerance" is the state when innate immune cells display reduced response to lipopolysaccharide (LPS) challenge after previously exposure to the same endotoxin (Table 1).

In this review, we discuss how epigenetics, specifically, chromatin remodeling and ncRNAs, play a central role in the tunable functionality of innate immune response and how recent technological advances have contributed to our understanding of this cellular program.

Table 1. Immunological memory of adaptive vs innate immune system. Summary of the adaptive and innate immune memory main properties.

Features of immune response		
	Adaptive	Innate
Cell type	B and T lymphocytes	Phagocytes (monocytes, DCs, neutrophils), ILCs, epithelial cells
Response time	Slow (1–2 weeks)	Rapid (4 hours to 4 days)
Specificity	Highly specific: Ab production and T-cell clones generation is specific towards a particular Ag: lymphocytes recognize and distinguish between 10^6–10^9 antigens	Semi-specific: activation of innate immune cells through recognition of PAMPS and DAMPs
Ligand quality	Crucial for the activation	Not crucial, ligand concentration is determinant
Receptors	BCRs, Abs, TCRs	TLRs, RLRs, NLRs, DNA sensors
Pathogens protection	Limited spectrum (Ag-specific)	Large spectrum (not Ag-specific)
Protection duration	Lifelong protection	Short and long memory
Functional role	Eradication of the infection and induction of a state of protective immunity against exposure to the same pathogen	Initiation of adaptive immune response through release of inflammatory mediators, Ag capture and presentation to lymphocytes
Outcomes	Immunologic memory and immunologic tolerance	After a first stimulation, cells can enter a stage of long-term immunotolerance (ET) or increase the long-term responsiveness to microbial stimuli (TI) conferring resistance to secondary infections
Development	Reported in vertebrates and invertebrates	Described only in vertebrates

2. Impact of chromatin remodeling on inflammation

2.1. Epigenetic marks

Epigenetic modifications are heritable changes in gene activity and expression occurring without any alteration in DNA sequence composition. They mainly involve covalent modifications of

cytosine residues on DNA and reversible post-translational modification of histones (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, etc.). A further epigenetic mechanism controlling gene expression relies on dynamic nucleosome repositioning along gene regulatory regions, such as promoters and enhancers. The final outcome of such non-genetic alterations is an open or closed conformation of the chromatin structure, leading to induction or repression of specific gene expression modules (Figure 1A).

DNA methylation is the first recognized and most extensively characterized epigenetic modification; it occurs at carbon-5 of the pyrimidine ring of cytosine, primarily in the context of a CpG dinucleotide sequence. Hypermethylation of CpGs at the promoter or proximal to the transcription start site is associated with reduced transcription of the downstream gene; as a result, CpG methylation is associated with transcriptional silencing (for review see [19]). Different from DNA methylations, which are relatively stable and easy to analyze, histone modifications are more dynamic. They can occur in different combinations and at different locations along the genome, and are critical for regulating chromatin structure and function. The two most common histone modifications are acetylation and methylation of lysine residues at the N-termini of histone tails. Acetylation of lysine relaxes the compact chromatin structure of nucleosomes in chromatin and activates transcription, whereas histone deacetylation represses transcription. Another epigenetic mark associated with transcriptional activation is phosphorylation of histone 3 (H3) on serine residues, namely Ser^{10}. Methylation of H3 on Lys^{9} facilitates DNA methylation, heterochromatin formation and gene silencing [20,21]. Other key histone modifications will be discussed with their epigenetic consequences in the inflammatory context later in this review. A third key mechanism of transcriptional regulation is the dynamic remodeling of nucleosome positions within discrete regulatory regions, especially promoters and enhancers. As the fundamental units of chromatin, each nucleosome is composed of an octamer of the core histone proteins H2A, H2B, H3 and H4, around which 147 base pairs of the DNA wraps 1.65 times. Nucleosome organization dictates the local transcriptional potential of chromatin, allowing or preventing access to regulatory factors and cofactors. This process can result from direct competition between specific transcription factors and nucleosomes for the binding of regulatory regions on DNA, and may involve the participation of chromatin remodeling complexes and histone chaperones.

2.2. Epigenetic regulation of macrophage differentiation

Monocytes and macrophages are responsible for cytokine production, phagocytosis and consequent clearance of the pathogen during inflammation. They exhibit a remarkable ability to sense and adapt to changes in their microenvironment. Simultaneously, they fulfill highly specialized functions, maintaining their differentiated state through homeostatic mechanisms that guarantee robustness in maintenance of cell identity. Linking these two characteristics is the integration of microenvironment- and stimulus-specific signals, which drive tissue- and signal-associated transcription factors to dynamically shape the relative epigenomic landscape. This results in the acquisition of new functional properties in response to different stimuli, and manifests as a spectrum of different functional states, oversimplified by the canonical dual distinction between classically- (M1) and alternatively-activated macrophages (M2) [22]. M1 macrophages are associated with pro-inflammatory responses, as they develop in response to PRR activation and secrete pro-inflammatory mediators. By contrast, M2 macrophages are associated with response to parasite

infection, tissue remodeling, angiogenesis and tumor progression [22]. They develop in response to anti-inflammatory stimuli such as interleukins, especially IL-4 and IL-13 [23,24].

Genome wide studies profiling transcriptional epigenetic modifications occurring in differentiated macrophages reveal profound dynamic changes in histone modifications and DNA methylation patterns associated with macrophage polarization. These modifications are the product of two classes of enzymes: histone deacetylases (HDACs) and DNA methyltransferases. Overexpression of DNA methyltransferase 3B (DNMT3B) or loss of HDAC3 renders macrophages hyper-responsive to IL-4, skewing differentiation towards the M2 phenotype [25,26]. HDAC3 is also required for inflammatory M1 activation of certain LPS-responsive genes, such as *il6* and *tnfα*. An important characteristic of M1 polarization is IFNγ priming of macrophage-mediated expression of pro-inflammatory cytokines, such as IL-6 and IL-12b. This priming induces increased H3K4me3 and increased phosphorylation of H3S10. Moreover, chromatin remodeling complexes, such as the ATP-dependent complex BAF (also termed SWI/SNF) are also required to recruit NFκB and release paused RNA pol II to promote transcription elongation [27,28]. Polarization of M2 macrophages is instead mediated by IL-4 in a STAT6-dependent manner. The histone demethylase JMJD3 is required for M2 gene expression in M-CSF cultured bone marrow-derived macrophages. Its function is to facilitate expression of the key M2 transcription factor IRF4, by removing transcriptionally repressive H3K27me3 histone marks at the *irf4* locus [29].

Altogether, this evidence demonstrates the importance of histone modifications and chromatin remodeling in directing macrophage polarization.

2.3. Remodeling of the macrophage enhancer landscape by inflammation

Stimulation-induced plasticity of myeloid cells is dependent on partial reprogramming of certain cell-specific enhancers, which are largely responsible for cell-type-specific gene expression. Enhancers are regulatory regions of the genome that are distal to the protein-coding gene they regulate. Although their exact mechanism is not fully understood, they are thought to bind transcription factors via formation of a loop structure within the chromatin, bringing the enhancer sequence into close proximity with the promoters of specific genes (for review see [30,31]). Enhancers can generally be classified as inactive, primed or poised. Inactive enhancers are located in heterochromatin regions, devoid of transcription factor binding and histone modifications. Primed enhancers are located in nucleosome-free regions of open chromatin in close proximity of sequences occupied by transcription factors. They become active in a signal-dependent manner, after the recruitment of specific transcription factors or chromatin remodeling complexes. Poised enhancers can be defined as primed enhancers that also contain repressive epigenetic chromatin marks [30]. Moreover, the acetylation state of specific histone tails discriminates between primed and poised enhancers, with H3K27ac marking primed enhancers.

According to their response to distinct stimuli, primed macrophage enhancers can be further classified into two main categories: constitutively activated or constitutively repressed. Constitutively active enhancers are primarily associated with the presence of H3K4me1 and H3K27ac histone marks in unstimulated cells. Upon LPS challenge, constitutively activated enhancers show increased acetylation whereas constitutively repressed enhancers display reduction or loss of H3K27ac. Conversely, poised enhancers show basal H3K4me1 without H3K27ac [32]. Most poised enhancers are unaffected by LPS stimulation, and those that are likely represent a small

subset of poised-activated enhancers that acquire H3K27ac in addition to constitutively-repressed elements [32]. TLR4 signaling primarily regulates macrophage gene expression through modulating a pre-existing enhancer landscape. In addition to using pre-primed regulatory elements, inflammatory stimuli alter macrophage gene expression through the selection and activation of about 3000 new enhancers and the inactivation of about 1000 constitutively repressed enhancers. These stimulus-dependent activated enhancers gain H3K4me2, which is correlated, along with H3K27ac, with expression of adjacent genes [32]. The corresponding genomic regions marked by H3K4me2 exhibit significant enrichment for motifs recognized by NFκB, AP1, C/EBP, IRFs/STATs and PU.1 [32]. Thus, the activation of *de novo* enhancers proceeds in a cell specific manner, with lineage-specific transcription factors, such as PU.1 and C/EBPα, operating in a collaborative manner with broadly expressed signal-dependent factors. This is exemplified by the stimulus-induced cooperation between PU.1 and NFκB, both of which are required to activate specific *de novo* enhancers [32]. In this context, it has been shown that H3K4 methylation is dependent on the activity of MLL1, MLL2/4 and MLL3 histone methyltransferases (HMTs), the functions of which appear to be redundant in macrophages. Members of the MLL family of HMTs associate with the C-terminal domain (CTD) of RNA pol II. A proposed mechanism of action suggests MLL recruitment at enhancers in response to CTD phosphorylation by the CDK9 component TEFb [32]. Interestingly, inflammatory signals prompt a dramatic and rapid reorganization of the epigenomic landscape through a global redistribution of chromatin activators to *de novo* clustered enhancers, with the consequent formation of so called “super enhancers” (SEs). These are regions where enhancers are clustered in close proximity to key regulatory genes, and are distinct from typical enhancers in their ability to activate cell type specific and tissue specific genes (for review see [33]). It has been suggested that mammalian genomes evolved SEs to confer higher transcriptional activity and sensitivity to perturbations. In a paper published by Plutzky and colleagues [34], the authors demonstrate cooperativity between NFκB and BRD4 in regulating the formation of *de novo* SEs during pro-inflammatory activation. Therefore, *de novo* SEs may represent a mechanism by which inflammation-related transcriptional regulators coordinate dynamic changes in cell state.

2.4. The “epigenetic dualism” between host and pathogen in controlling the immune response

In addition to myeloid cells, another arm of the innate immune response is represented by innate lymphoid cells (ILCs) and intestinal epithelial cells (IECs). Altogether these different cell populations collaborate with each other and with the adaptive immune system to promote the immune response, inflammation and tissue repair [35-39]. The ILC family includes not just NK cells, but also other newly characterized populations of ILCs classified as ILC1, ILC2 and ILC3. ILCs are enriched at barrier surfaces, common sites of colonization or invasion by pathogens, and together with epithelial cells, they provide multiple antimicrobial effectors against bacteria, viruses, helminths and protozoa. By producing pro-inflammatory cytokines, such as IFNγ, TNFα and type-2 cytokines (e.g. IL-4, IL-5, IL-13), ILCs promote alternative activation of macrophages.

IECs are specialized to function as a dynamic barrier to the environment, which protects the host from infection and continuous exposure to potentially harmful stimuli. Fundamental to maintaining this barrier and immunoregulatory functions is their ability to integrate commensal bacteria-derived signals into anti-microbial and immunoregulatory responses [40]. A bi-directional cross-talk between IECs and ILCs is important for maintaining homeostasis. Cytokines produced by

IECs upon microbiotic stimulation, as well as compounds from diet, support and/or modulate the generation of ILCs and their acquisition of effector functions. Conversely, ILC subsets, when activated preserve epithelial cell homeostasis by promoting tissue repair in response to injury or participating in the induction and activation of the immune response against pathogens. This active cross-talk results in an appropriate immune response scaling between tolerance and anti-pathogen inflammation. Among the different factors released by IECs, antimicrobial peptides (AMPs) play a role in the establishment and maintenance of epithelial homeostasis by keeping the resident and transient bacterial population in check [41]. AMP expression upon bacterial challenge is specifically regulated by epigenetic mechanisms. In the study by Fischer et al. [42], the authors demonstrate that HDAC inhibition selectively enhances the expression of antimicrobial peptide HBD2. An increased phosphorylation of H3S10 preferentially occurs at the HBD2 promoter, inducing its expression through activation of IκB. This cascade culminates with the activation of NFκB, which in turn is acetylated at its p65 subunit by histone acetyltransferase p300 [42]. This epigenetic control mediated by HDAC and p300 is selective for HBD2 and other genes involved in the antimicrobial defense, whereas the expression of pro-inflammatory cytokines is not affected. This suggests the existence of specific epigenetic regulation of AMPs among other genes involved in the innate immune response.

Interaction with pathogens leads to an alteration of host transcriptional programs to promote the immune defense against invading pathogens, but many viruses and bacteria have evolved strategies to counteract the immune response, including reprogramming of the host transcriptional programs through chromatin and histone modifications [43-46]. Interestingly, epigenomic modifications imposed by infection can be imposed via both histone modifications and chromatin remodeling. For instance, during infection with the *Listeria monocytogenes*, the bacterial factor LntA is released and enters the nucleus where it interacts with the heterochromatin protein BAH Domain-Containing Protein 1 (BAHD1), part of a complex with other remodeling factors (such as HP1, HDACs and MBD1) to silence expression of interferon-stimulated genes (ISGs). By interacting with BAHD1, LntA prevents the recruitment of the remodeling complex at ISGs promoters, thereby upregulating their expression [47]. *Listeria* infection also causes the repression of a subset of immune-related genes through deacetylation on H3K18, a process mediated by the host deacetylase sirtuin 2 (SIRT2) [48]. As for *Listeria*, other bacteria were shown to promote infection by reprogramming the host transcriptional epigenome. In particular, *Shigella* delivers its effector protein OspF into the nuclei of epithelial cells, where it dephosphorylates HP1γS83 and MAPK, thereby inhibiting downstream phosphorylation of H3S10 at the promoter of a set of innate genes including IL-8 [49-51].

2.5. Trained immunity and endotoxin tolerance: two sides of the same coin

The precise control of the innate immune response is strictly required to avoid development of systemic infection, resulting from inadequate response, or autoimmune disorders characterized by chronic inflammation. Although immune memory has been so far ascribed exclusively to adaptive immune cells, this dogma has been recently challenged by evidence showing that innate immune cells such as monocytes, macrophages, and NK cells exhibit memory characteristics (Table 1) (for review see [16,17]). The phenomena of "trained immunity" and endotoxin tolerance are examples of innate memory. Depending on the type and concentration of ligand encountered, the cell responds with an enhanced (trained immunity) or decreased (tolerized) response to subsequent PRR stimulation. Microbial ligands, including components of peptidoglycans such as β-1,3-glucan and

muromyl dipeptide (MDP), induce trained immunity through the engagement of dectin-1 and nucleotide-binding oligomerization domain 2 (NOD2), respectively. This results in increased production of pro-inflammatory mediators such as IL-1β and tumor necrosis factor alpha (TNFα) upon secondary stimulation with TLR2/4 agonists. By contrast, monocytes and macrophages pre-exposed to LPS challenge enter a refractory state known as endotoxin tolerance (ET), characterized by decreased production of pro-inflammatory mediators after subsequent stimulations with LPS [52]. In an interesting study by Saeed and colleagues, the authors documented the importance of epigenetic programming in trained immunity and ET, identifying about 3000 distal regulatory elements specifically induced by β-glucan priming, and induction of H3K27ac at about 500 distal regulatory regions in ET. The characterization of the epigenomes and transcriptomes associated with differential priming of macrophages led to the identification of biological pathways and transcription factor repertoires differentially regulated in these two conditions, revealing an exclusive epigenetic signature elicited by β-glucan training.

Endotoxin tolerance. One of the first works describing the impact of epigenetic changes on ET was published by Medzhitov and colleagues in 2007 [52], describing ET as a highly dynamic and coordinated gene expression program, characterized by two distinct sets of differentially responsive genes following a second challenge with the same stimulus. Pro-inflammatory "tolerizable" (class T) genes show decreased or abolished expression; alternatively, non-tolerizable (class NT) genes display increased or unchanged expression upon further LPS challenge, including antimicrobial genes. These differential gene expression patterns under ET result from distinct TLR-induced epigenetic modifications. After initial stimulation with LPS, both classes of genes are actively transcribed and their promoters are acetylated at histone H4 and imprinted by the H3K4me3 mark [52]. Following a second LPS challenge, class NT genes maintain H3K4me3 and their promoters are re-acetylated in tolerant macrophages. Conversely, class T genes appear to maintain their basal promoter state, do not regain H3K4me3 or H4 acetylation, and remain silent and refractory to stimulation (Figure 1B). During restimulation of tolerant macrophages, chromatin remodeling is stably and inducibly maintained only in class NT genes, where recruitment of chromatin regulators like Mi-2β and BRG1 (the catalytic subunit of the SWI/SNF complex) facilitate initiation and elongation of transcription by recruiting histone acetyltransferases (HATs) to promoter sites [52]. Transcriptional silencing of class T genes during ET is generated through formation of facultative heterochromatin, a process controlled by transcriptional repressors such as RELB. RELB is required to direct the assembly of a multi competent repressor complex that induces facultative heterochromatin formation on the promoter of pro-inflammatory genes, such as *il1β* and *tnfα* [53]. This occurs through interaction with the G9a HMT, which dimethylates histone H3K9. This histone modification enables binding of the repressor HP1 as well as recruitment of DNA methyltransferase DNMT3A/B, leading to increased methylation of the CpG promoter region [53]. A more recent study performed in the context of the International Human Epigenome Consortium (IHEC) provided a detailed epigenetic and transcriptional characterization of human macrophage tolerance, by showing that transcriptional response of macrophages to LPS re-exposure is a gradient, with genes showing complete tolerance, partial response or a full response [12]. Dynamic H3K27ac changes at promoters and distal enhancers occur in tolerized macrophages with distinct pattern between the different groups of genes In particular, tolerized genes and partially tolerized genes showed no or impaired accumulation of H3K27ac respectively after LPS rechallenge, while responsive genes were equally acetylated compared to naïve cells (Figure1B) [12]. The most affected genes spread across that

gradient were cytokine genes, with *tnf* and *cxcl9* showing complete or partial tolerance and *il-6* and *il-8* showing normal responsiveness. Notably, in some cases, such as IL-6, there is normal induction of the corresponding transcript but IL6 protein is undetectable, suggests the existence of post-transcriptional regulation mechanisms involved in ET [12]. Therefore, tolerant macrophages display a phenotypic and functional reprogramming, switching from a pro-inflammatory to an immunosuppressive phenotype, and enhancing protective functions like phagocytosis and tissue repair [11,52,54]. This is observed in leukocytes rendered tolerant by exposure to LPS or after isolation from patients with sepsis and immunoparalysis, which have an apparently defective glycolysis and oxidative metabolism [55]. Interestingly, IFNγ was recently shown to partially recover metabolic functions in tolerized monocytes from sepsis patients These findings are also consistent with a previous report by Chen and Ivashkiv demonstrating that in human primary monocytes IFNγ restores expression of pro-inflammatory factors by inducing transcription factors that recruit BRG1 to their promoters in tolerized monocytes [56]. Finally, in a study by Novakovic et al. the authors adopted an experimental human endotoxemia model to induce tolerance *in vivo*, and reported that β-1,3-glucan reverses epigenetic tolerance and restores the ability of human macrophages to produce cytokines important for anti-pathogen response [12].

Trained Immunity. The notion of innate immune memory was introduced for the first time decades ago, when it was attributed to activated macrophages independent of T or B lymphocytes [57-60]. However, recently the molecular mechanisms underlying trained immunity were unveiled: three studies published between 2012 and 2014 identified monocytes and macrophages as mediators of trained immunity, associating H3K4 methylation as the molecular signature of innate immune memory [11,60,61]. This particular epigenetic mark was also confirmed in a study by Ostuni et al. [62] where they described the kinetic events during macrophage training and identified the epigenetic aspects thereof. The authors proposed the name “latent enhancers” to highlight that they are inactive, unbound and unmarked under basal conditions and selectively unveiled by activating modifications upon stimulation [62]. Latent enhancers are identified on the basis of two key characteristics: (i) lack of H3K4me1, H3K27ac and PU.1 binding in unstimulated cells; and (ii) presence of an LPS-induced stable H3K4me1 mark, usually but not always accompanied by the appearance of transient H3K27ac (Figure 1B). Interestingly, once unveiled, many of these latent enhancers do not return to a latent state after waning of the inflammatory stimulus; instead, they persist and can undergo a faster and enhanced response upon restimulation with the same stimulus or other novel stimuli [62]. Furthermore, H3K4me1 mark is selectively retained by latent enhancers after termination of stimulus, reflecting the molecular signature of this “short term” epigenetic memory [62]. This study identified latent enhancers as an epigenomic footprint left by the stimuli to which the cell had been exposed.

Immunological memory of NK cells. NK cells are innate lymphocytes acting at the interface between adaptive and innate immunity, expressing canonical T but also B and myeloid cell signaling proteins [63,64]. They are able to eradicate virus-infected or transformed cells through the release of cytolytic granules and inflammatory cytokines such as TNFα and IFNγ [65-67]. NK cells activation is dictated by a delicate equilibrium between activating signals triggered by receptors recognizing stress-induced ligands on infected cells or tumor cells such as the Natural Cytotoxicity Receptors (NCR) or the CD2 family of receptors [68,69]; and inhibitory signals, including those delivered by inhibitory Killer-cell Immunoglobulin-like Receptors (KIRs) [70]. Several lines of evidence indicate that NK cells can exert immunological memory after encountering viruses or haptens, generating antigen specific memory NK cells [71-73]. Moreover, immunological memory of NK cells has also

been described in the absence of a defined antigen. Cytokine-activated NK cells, while phenotypically similar to naïve cells, last for 7–22 days when transferred into naïve hosts and are able to produce higher levels of IFNγ when restimulated [72]. Long-lived memory-like NK cells have been identified in HCMV-infected individuals; they represent distinct subsets of NK cells, characterized by adaptive-like properties, including long-term persistence and enhanced responsiveness after pathogen infection or exposure to other stimuli [74]. These properties result from epigenetic changes that have been suggested to maintain the memory-like state. As reported in two independent studies, memory NK cells show a gene expression pattern distinct from that of conventional NK cells in having downregulated multiple signaling proteins and transcription factors [64,74]. Interestingly, evidence reported in both studies support the role of epigenetic modifications in shaping the molecular profile of memory-like NK cells. The lack of signaling protein expression that is observed in these NK cells is associated with a global hypermethylation of the corresponding promoter regions, as in the case of SYK tyrosine kinase, FCER1G, and SH2D1B genes [74]. Comparisons of conventional and memory-like NK cells also revealed the deregulation of transcription factors PLZF and ZBTB38 of the BTB-ZF family [64]. Silencing of PLZF, correlates with the acquisition of "adaptive" NK cell function and marks memory-like NK cells. Moreover ZBTB38, which binds methylated CpG sites and negatively regulates apoptosis, is hypomethylated and transcriptionally upregulated in memory-like NK cells; thus suggesting that BTB-ZF protein might be involved in the regulation of epigenetic responses of NK cells [64].

Future studies will be needed to further define the possible contribution of other epigenetic mechanisms, such as chromatin remodeling or changes in the repertoire of transcription factors in the formation of immunological memory of NK cells.

The evidence and the molecular mechanisms discussed thus far support the description of innate immune memory as an epigenetically regulated process. The tuning of innate immune memory in the form of trained immunity or ET, depending on the quality and the concentration of the stimuli perceived, further highlights the dynamism of this system.

3. The panorama of non-coding RNAs-mediated innate immune response

One of the most important advances in the field of contemporary molecular biology has been the discovery of the biological roles of non-coding RNAs (ncRNAs). The abundance of protein-coding genes decreases as the complexity of organisms increases, with a concomitant rise in the number of non-coding intergenic and intronic sequences, most of which are in fact transcribed. Therefore, a progressive shift in transcriptional output from mainly protein-coding messenger RNAs (mRNAs) to mainly ncRNAs can be considered an essential reorganization in the evolution of multicellular organisms. Indeed, what was previously and still occasionally referred to as "junk DNA", corresponding to 98% of the human genome, originates thousands of RNA transcripts that will not be translated. Three major classes of ncRNAs have been differentiated on the basis of their size: small ncRNAs (~18–31 nucleotides in length), medium ncRNAs (from 31 to 200 nucleotides) and long ncRNAs (from 200 nucleotides up to several hundred kilobases) (for review see [75-78]). ncRNAs can be further divided based on their function. Housekeeping ncRNAs, which include ribosomal RNAs, transfer RNAs, small nuclear RNAs and small nucleolar RNAs have crucial roles in many cellular processes. Regulatory ncRNAs such as microRNAs, small interfering RNAs, piwi-interacting RNAs, long ncRNAs and enhancer RNAs play an important role as molecular regulators of gene expression (for review see [77-81]).

This section focuses on what is currently known regarding the most studied classes of regulatory ncRNAs involved in modulating the innate immune response: microRNAs (miRNAs), long ncRNAs (lncRNAs) and enhancer RNAs (eRNAs) (Figure 1A).

3.1. miRNA-based translational regulation of the innate immune response

miRNAs are highly conserved single-stranded RNAs of 18–22 nucleotides in length that modulate translation through nucleotide base-pairing, generally at the 3' untranslated region (3'UTR) of mRNAs [82,83]. The level of complementarity between the mRNA 3'UTR and miRNA 5' nucleotides 2 through 8, called the "seed sequence" [82-84], determines whether the mRNA will be degraded (perfect base-pair complementarity) or translationally impaired (partial complementarity) [83,84]. Following nuclear and cytoplasmic multi-step processing, single stranded miRNAs are incorporated into a ribonucleoprotein (RNP) complex called the miRNA-induced silencing complex (miRISC), which can interact with mRNA targets [85].

Victor Ambros and colleagues first reported the discovery of miRNAs in 1993 by identifying the *lin-4* gene in *Caenorhabditis elegans* [86,87]. Since their discovery, it has been recognized that miRNAs play a versatile role in regulating gene expression. A single miRNA can regulate many mRNAs with similar recognition sites, with consequences for numerous cellular pathways. It has been extensively shown that miRNAs participate in essential processes such as cellular homeostasis, development, tissue differentiation, cell proliferation, apoptosis, stress response, and immune response. Specific to the focus of this review, hundreds of miRNAs modulating different aspects of both innate and adaptive immunity have been identified [88].

One of the pioneering works interrogating miRNA regulation of the innate immune response was published almost a decade ago by David Baltimore and colleagues [89]. They showed for the first time that miR-146a, induced by LPS in an NFκB-dependent manner, is able to operate a negative feedback regulatory circuit after LPS triggering by direct targeting of IL-1 receptor-associated kinase (IRAK1), and TNF receptor-associated factor 6 (TRAF6) [89]. Since then, many others have demonstrated the ability of miRNAs to regulate TLR signaling pathway at multiple levels, including by direct targeting of receptors, signal transducers and effector molecules (for review see [90]). In this setting, the functional role of miRNAs in promoting or inhibiting inflammation has been reported. Of note, the expression of a subset of miRNAs, including miR-146a, miR-155 and miR-9 are induced early during TLR signaling, suggesting that they contribute to regulation of the acute phase of inflammation (Figure 1A). Indeed, miR-155 and miR-9 promote the inflammatory signal by targeting negative regulators of inflammation, such as SOCS1 and NFκBp50 (p50), respectively [91,92]. Another subset of miRNA genes is induced later after TLR activation, mainly acting as repressors of the inflammatory signal [90]. Among those, expression of miR-181c and let-7e is promoted by LPS, whereas miR-125b is suppressed. It was demonstrated that these AKT1-dependent miRNAs regulate the response of macrophages to LPS through the direct and indirect targeting of components of TLR4 signaling (Figure 2B) [93].

miRNA activity is also tightly connected with other anti-inflammatory mediators, as exemplified by the inhibitory effect of IL-10 on the expression of the pro-inflammatory miR-155, and, conversely, the characterization of miR-146b and miR-187 as intracellular effectors of IL-10 as well as other pro-resolving mediators [94-96]. miR-146b negatively modulates the inflammatory response by directly targeting multiple TLR components (i.e. TLR4, IRAK1, TRAF6, MYD88) [94];

miR-187 exerts a post-transcriptional regulation of the anti-inflammatory responses by directly targeting NFκBIZ and TNFα mRNA as well as indirectly inhibiting IL-6 and IL-12p40 translation (Figure 2A) [96]. Moreover, miR-21 promotes an anti-inflammatory response by indirectly increasing IL-10 expression as a consequence of its targeted repression of PDCD4 (Figure 2B) [97]. Given the importance of IL-10 and other anti-inflammatory mediators (such as TGFβ and glucocorticoids) in mediating the process of ET, the evidence strongly supports miRNA involvement in these inflammation-dependent negative feedback circuits. The first evidence to this effect was provided in two studies by El Gazzar and colleagues [98,99]. They demonstrated that upregulated miR-146a in tolerant monocytes was responsible for promoting the binding of the transcriptional repressor RelB to the TNFα promoter, leading to the silencing of pro-inflammatory genes via the formation of facultative heterochromatin. miR-146a also facilitates the assembly of a translational repressor complex involving Argonaute 2 (Ago2) and the RNA-binding motif protein RBM4. In tolerized cells, miR-146a indirectly leads to dephosphorylation of RBM4 (due to inactivation of p38 MAPK) and its subsequent retention to the cytoplasm, where it interacts with Ago2. The Ago2-RBM4 repressor complex is then directed to the P-bodies where it disrupts cytokine expression. This further level of RBM4-mediated regulation creates a negative loop with miR-146a to sustain ET. Other miRNAs, namely miR-146a, miR-221, miR-579 and miR-125b also recruit RBPs into the miRISC and target TNFα 3' UTRs, thus contributing to its translational repression in LPS-tolerant cells [98,100]. Furthermore, in our recent study we reported miR-146b induction in response to different anti-inflammatory stimuli in monocytes tolerized by LPS, IL-10 or TGFβ, and demonstrated that tuning miR-146b expression results in the induction or reversion of ET in monocytes [101].

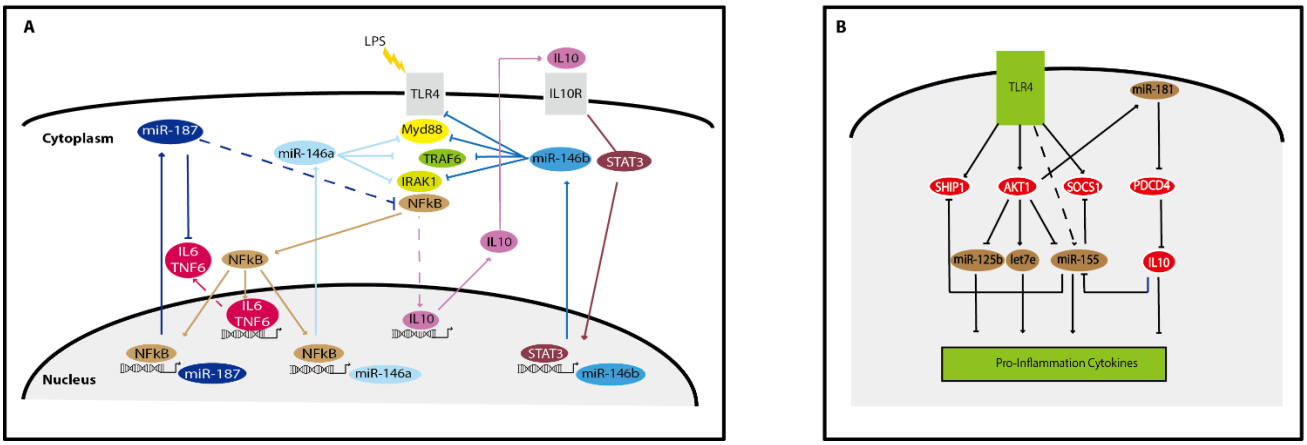

Figure 2. Regulation of Innate Immunity by micro-RNAs. (A) Regulation of TLR4 signaling pathway by miR-146a, miR-146b, and miR-187. (B) Regulation of TLR4 signaling pathway by miR-125b, let7e miR-181, and miR-155. Dashed lines represent indirect induction/ repression and solid lines represent direct induction/repression.

Together, these studies identify miRNAs as important immunomodulators, contributing to keep the innate immune response in check through the reinforcement of positive or negative feedback circuits induced by inflammatory and anti-inflammatory signals.

3.2. lncRNA-based regulation of inflammatory responses

Long non-coding RNAs (lncRNAs) comprise a heterogeneous group of ncRNAs that are transcribed by RNA Pol II from different genomic regions, and are usually more than 200 nucleotides long. Like mRNAs, they undergo capping, splicing and polyadenylation. This class of ncRNAs includes thousands of RNAs of different origin: they can be transcribed as natural antisense transcripts (NATs) of protein-coding genes or reside in introns or intergenic genomic regions. They control gene expression through a variety of modalities that have only recently begun to be elucidated. Like miRNA, they can interact with proteins to form ribonucleoprotein complexes, as well as with DNA and RNA targets. However, their size allows them to fold into secondary structures, giving them great versatility in mediating cellular processes (for review see [102,103]). lncRNAs have distinct modes of action based on their nuclear or cytoplasmic localization. Most lncRNAs have been reported in the nucleus, where they function by guiding chromatin modifier complexes to specific chromosomal loci. They can act as either repressors or activators of transcription depending on the histone modifiers they recruit. For instance, lncRNAs can recruit DNA methyltransferase 3 (DNMT3), Polycomb repressive complex 2 (PRC2) and histone H3 lysine 9 (H3K9) methyltransferase, enacting heterochromatin formation and transcriptional repression [104]. Conversely, lncRNAs have been shown to elicit transcriptional activation through the recruitment of the histone H3K4 methyltransferase MLL1 complex [105]. Nuclear lncRNAs can be classified as *cis*-acting or *trans*-acting on the basis of their modality of action. The *cis*-acting lncRNAs regulate functions on chromosomal loci in proximity to their own transcription sites as well as distant sites on the same chromosome. Conversely *trans*-acting lncRNAs act on distant sites at independent loci. How lncRNAs recognize proximity is not yet known, though many mechanisms have been proposed [106]. Cytoplasmic lncRNAs, instead, can cause either stabilization of their base-paired mRNA target [107,108] or can interact with miRNAs as "competing endogenous RNAs" (ceRNAs) by binding and sequestering specific miRNAs to attenuate their regulatory function. In this way lncRNAs can serve as miRNA sponges, and evidence is emerging that this mechanism is relevant in several processes including tumorigenesis, cell differentiation and pluripotency.

Many studies have reported that lncRNAs act as additional regulators of inflammatory responses in the murine innate immune system, although their molecular functions are still poorly understood. Specifically, much evidence indicates that lncRNAs can regulate the expression of TLR-mediated inflammatory genes (Figure 1A). The long intergenic non-coding RNA Cox2 (lincRNA-Cox2), one of the first lncRNAs discovered, is highly expressed in both macrophages and dendritic cells exposed to TLR ligands. lincRNA-Cox2 regulates expression of distinct classes of immune genes, such as chemokines, chemokine receptors and interferon-stimulated genes, through interactions with heterogeneous nuclear ribonucleoprotein A/B and A2/B1 (hnRNP-A/B and A2/B1) to form a transcriptionally repressive complex [109]. Another recently discovered lncRNA pseudogene for the ribosomal protein S15a (Rsp15a) called *Lethe* is a negative regulator of NFκB transcriptional activity. *Lethe* interacts with the RelA subunit of NFκB, preventing its binding on the promoter of target genes IL-6, IL-8 and SOD2 (Figure 3A) [110]. As of now, scant experimental evidence supporting the regulatory role of lncRNAs in the human innate immune system has been reported. A recent study by Li and colleagues reports a panel of 159 lncRNAs highly modulated in stimulated THP-1 macrophages [111]. Among these lncRNAs, linc1992 was shown to be essential for TNFα induction. By forming a complex with the heterogeneous nuclear ribonucleoprotein L (hnRNPL), *linc1992* is able to regulate TNFα transcription by binding its promoter, and this complex formation is required to maintain basal transcription of TNFα. Considering this mode of action, *linc1992* has been

renamed TNFα and hnRNPL-related immunoregulatory lincRNA (THRIL). High levels of TNFα secretion initiate a negative feedback loop in which THRIL downregulation causes dysregulation of several cytokines during innate immune cell activation, suggesting that THRIL is able to regulate the expression of an array of inflammatory genes (Figure 3B) [111]. The nuclear antisense lncRNA PACER (P50-Associated COX-2 Extragenic RNA) has been found within the upstream promoter region of cyclooxygenase 2 (COX-2), a key enzyme in the modulation of inflammatory responses. PACER functions by directly sequestering the p50 repressive subunit of NFκB from the COX-2 promoter, leading to chromatin modifications enabling RNA pol II assembly and COX-2 transcription (Figure 3B) [112]. There are many reports of lncRNAs that regulate the transcription of specific cytokine-expressing genes. For example lncRNAs acting as natural antisense transcripts are able to regulate IL-1 family expression levels [13]. By using a ribosomal RNA (rRNA)-depleted RNA sequencing approach, Ilott and colleagues examined for the first time the differential expression of lncRNAs affecting TLR4 signaling through LPS stimulation in human monocytes. They found that 221 lncRNAs were differentially expressed: among these, 182 were up-regulated and 39 down-regulated following LPS stimulation. These lncRNAs were classified on the basis of their proximity and relative orientation to protein-coding genes and annotated as antisense, intergenic or mRNA-flanking [13]. Importantly, this work demonstrated for the first time that only 2 of the 221 differentially expressed lncRNAs overlapped with previously identified LPS-regulated lncRNAs from mouse bone marrow-derived dendritic cells, consistent with the poor conservation of lncRNAs across species. It was also reported that several lncRNAs were associated with H3K4me1 histone marks, thus suggesting transcription occurring from enhancer regions [13]. The NFκB Interacting lncRNA (*NKILA*) is able to form a complex with NFκB/IκBα that masks the phosphorylation sites on IκBα, preventing its phosphorylation and thus maintaining NFκB in its inactive state [113]. Importantly, NKILA has an NFκB binding motif in its promoter, indicating that its expression is induced by NFκB. Hence, NKILA establishes an essential regulatory loop to prevent an over-activation of the NFκB pathway.

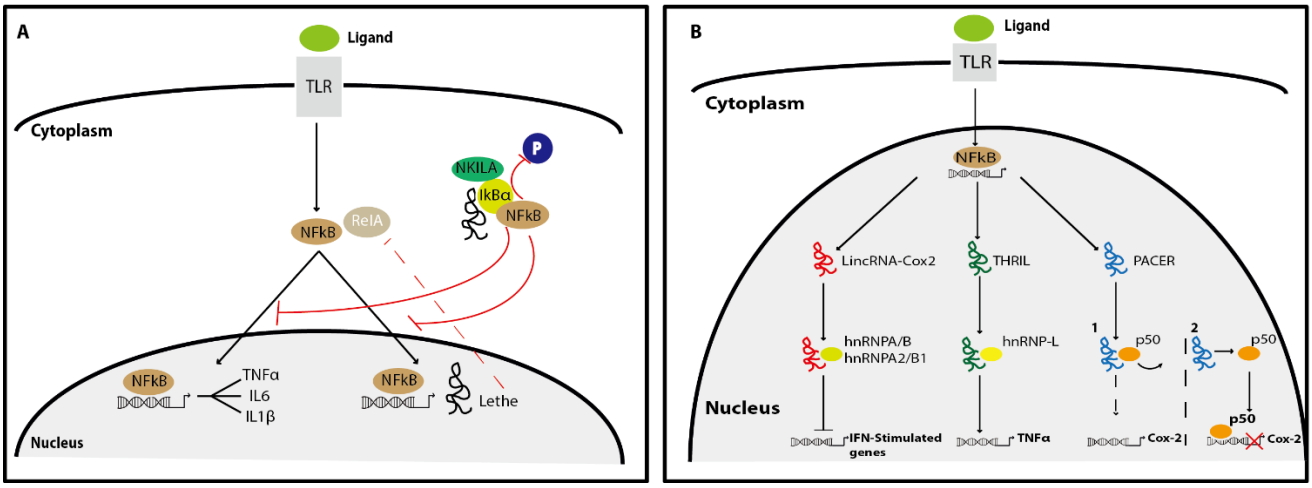

Figure 3. Regulation of TLR-mediated inflammatory genes. **(**A) Modulation of NFκB transcriptional activity by lncRNA Lethe and NKILA. (B) Modulation of NFκB transcriptional activity by LincRNA-Cox2, THRIL, and PACER. Dashed lines represent indirect induction/ repression and solid lines represent direct induction/ repression.

While there are several reports of lncRNAs involved in transcriptional regulation of inflammatory responses, there are few examples of lncRNAs contributing to TLR4 reprogramming occurring upon the establishment of ET. Indeed, just as miRNAs play a role in the development of this status, lncRNAs could impact TLR4 reprogramming as well. In a very recent work, Murphy and Medvedev reported that LPS challenge and TLR4 tolerization determine differential expression of several lncRNAs in human THP-1 macrophages [114].

This area of investigation is in its nascence and future studies will seek to uncover the contribution of lncRNAs to the regulation of inflammatory responses, especially in the establishment and maintenance of ET. Emerging evidence of their impact on different cellular processes will increase interest around lncRNAs as a further level of regulation to add to that of miRNAs.

3.3. eRNA-based regulation of inflammatory responses

Enhancer-RNAs (eRNAs) are a class of ncRNAs transcribed from enhancer regions of the genome. Even if there is not a consensus on their exact biological role, most evidence supports the hypothesis that eRNA transcription is an indicator of enhancer activity [115-117]. Relative to non-transcribed enhancer regions, eRNA-producing enhancers show the following specific features: higher binding of transcriptional co-activators such as p300 histone acetyltransferase (HAT) and CREB binding protein (CBP), greater chromatin accessibility and enrichment of activating histone marks such as H3K27ac, as well as protection from repressive chromatin marks, including DNA methylation [118-120]. Moreover, a chromatin signature characterized by high level of H3K4me1/H3K4me2 and low level of H3K4me3 is observed in active enhancers. In this view, enhancers themselves, by generating non-coding eRNAs, act as transcriptional units [13,118].

The most important question arising from the discovery of eRNAs is: do they have a functional effect on other genes (either in *cis* or in *trans*), or do they just represent transcriptional noise? As with other classes of ncRNAs, the field is beginning to prove that the former is true. One of the first reports of functional effects associated with eRNAs was reported by Kim and colleagues [121]. They demonstrated that, upon neuronal stimulation, a subset of enhancers was able to produce eRNAs. These enhancer regions were proximal to strongly induced mRNAs, suggesting that eRNAs can direct the expression of nearby genes.

Many recent reports demonstrate the involvement of eRNAs in regulating different cellular functions, including immune responses. Studies from the Lindsay group identified 76 eRNAs in primary human monocytes that are differentially expressed in response to LPS. The authors demonstrated that the expression of these eRNAs is dependent on the NFκB and MAP kinase pathways, and is highly coordinated with that of proximal inflammatory genes, suggesting that they act in *cis* to regulate the expression of their proximal coding gene. Indeed, the authors demonstrated that the expression of *il1β* gene is regulated by a downstream eRNA (eRNA-IL1β). Notably, the same eRNA was also described as being able to act in *trans* by affecting the expression of CXCL8 [13]. Further evidence also demonstrates transcriptional activities of eRNAs at super enhancer regions, so called SE-eRNA, whose expression is dynamically induced at most of the key genes regulating innate immunity and inflammation [120].

We can speculate that eRNA and SE-eRNA, together with other ncRNA classes, (in particular lncRNA, associated with an enhancer-like chromatine state) are important regulators of the innate immune response. Further studies are needed to elucidate their mechanism of action and their functional relevance.

4. Role of innate immunity in the epigenetic plasticity of cellular reprogramming

As discussed in the previous sections, innate immune cells are able to detect viral- and microbial-associated molecular patterns through the engagement of specific TLRs. In particular, TLR3 plays an important role in the recognition of double-stranded RNA from retrovirus and activates specific signaling pathways that culminate in the activation of NFκB and IRF3 and the consequent induction of epigenetic changes [122]. A surprising observation reported in 2012 highlighted a previously unrecognized role for innate immunity in the generation of induced pluripotent stem cells (iPSCs) [123]. This was the first study demonstrating the active role of TLR3 signaling in facilitating nuclear reprogramming in the presence of the so called "Yamanaka factors" (i.e. a specific transcription factor cocktail consisting of Oct4, Sox2, Klf4 and c-Myc) [123-125]. The authors demonstrated that retroviral vectors used in nuclear reprogramming were actively involved in inducing the activity of epigenetic modifiers by activating innate immune signaling. Stimulation of TLR3 by viral double stranded RNA facilitates activation of the NFκB and IRF3 pathways and results in the induction of endogenous genes in the core pluripotency network. More precisely, upon TLR3 activation NFκB interacts with HAT proteins p300 and CBP HP1 inducing an open chromatin state on promoter regions of *Oct4* and *Sox2* genes, accompanied by increased H3K4me3 and reversal of H3K9me3 at the *Sox2* and *Oct4* promoters [123]. These changes in the methylation status of *Sox2* and *Oct4* promoters were also associated with the downregulation of multiple HDAC family members (i.e. HDAC 1, 5, 8, 9 and 10), as well as decreased expression of the H3K79 HMT Dot1L and H3K4 HMT Ash11 [123]. Several follow-up studies have been conducted after this striking discovery, further supporting that epigenetic plasticity is induced by activation of innate immunity during transdifferentiation of somatic cells. It has been shown that iNOS, a major effector of innate immunity, is required to facilitate transdifferentiation of fibroblasts to endothelial cells. TLR3 agonist polyinosinic:polycytidylic acid (poly I:C) induces nuclear localization of iNOS and its binding to RING1A, a member of polycomb repressive complex. The result of this interaction is the nitrosylation of RING1A by iNOS effecting reduced binding of RING1A to chromatin, reducing levels of the repressive histone marker H3K27me3. Therefore, the release of epigenetic repression by nitrosylation of RING1A is critical for effective transdifferentiation [126].

These recent findings not only provide new therapeutic avenues in the field of regenerative medicine but might also have wider implications with respect to the immune response to pathogens or cellular damage. From these studies emerged the induction of epigenetic modifiers by innate immune activation affecting greater epigenetic plasticity and allowing the cell to adopt a fluidity of phenotypes to better respond to environmental cues.

5. From epigenomics to single cell immunology

The experimental evidence discussed so far provides a panoramic view of the complex dynamics adopted by immune cells to respond to environmental stimuli and challenges. Key hallmarks of this response are sensing and adaptability to environmental changes, generation of robust response, and capability to dynamically tune the intensity and duration of the response. Altogether, these features imply the existence of mechanisms that enhance diversification of individual immune cells while maintaining their ensemble integrity and coordination. Epigenetics is intrinsically related to the diversity of cellular processes and states because chromatin-based

mechanisms temporally and dynamically modulate graded adjustments of gene expression. Epigenomic studies have highlighted how combinations of histone modifications correlate more accurately with transcriptional states than individual histone marks. Until recently the power of these studies were inherently limited because most of them have been based on ensemble measurements. Due to the intrinsic heterogeneity of any cell population, ensemble behaviors do not accurately represent individual cells, thus limiting the relevance of data obtained by ensemble measurements. Therefore, it has become imperative to move from genome wide studies to single cell analyses to define the dynamicity and plasticity of epigenetic phenomena at a greater resolution. Single cell techniques discriminate intercellular differences among cell populations in a temporal and cell specific context, thereby eliminating the heterogeneity issue and leading to a more refined understanding compared with bulk analysis. Interestingly, the further implementation of single cell approaches allows novel interpretations of previous ensemble studies, facilitating new advances in epigenomics (Figure 4). Here we briefly describe the advance in methodologies that are currently driving the field of epigenetics and report recent studies where differential single-cell techniques were applied to unravel some key aspects of epigenetic regulation of the innate immune response.

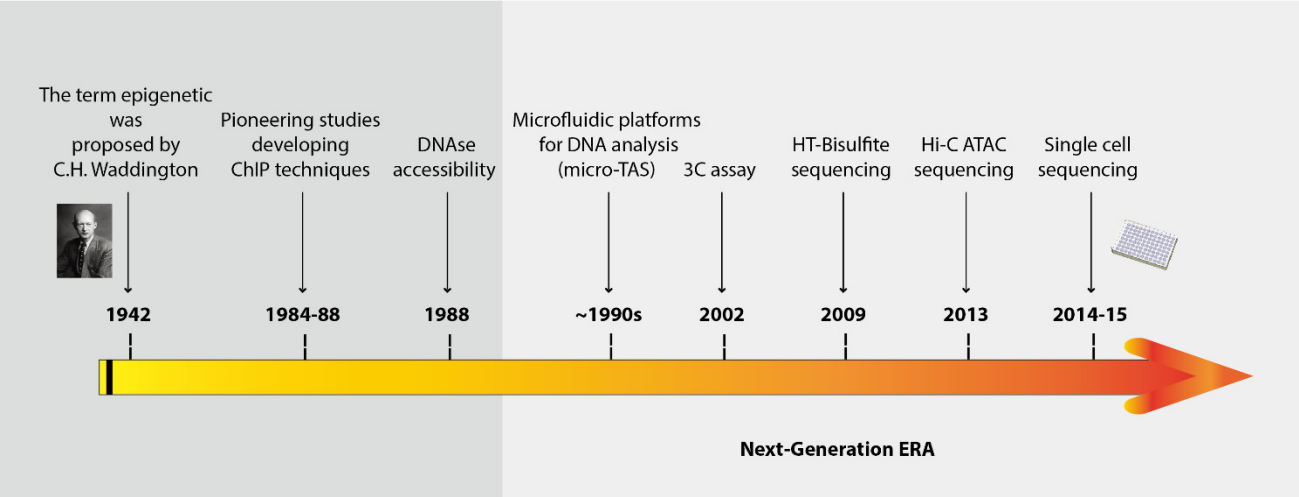

Figure 4. Technological Evolution in Epigenetics research. Time line of the major technological advances of the epigenetic field ranging from the pioneer studies on chromatin to the Next Generation Era with the development of high throughput sequencing systems.

5.1. Advances in epigenetic techniques

Numerous techniques have been adopted to analyze epigenetic processes at the level of specific genes or to analyze epigenetic changes that occur in defined regions of the genome. Conventional methods developed since the late 1980s mainly addressed two broad areas of epigenetics: DNA methylation (e.g. bisulfite-based techniques), histone modifications (e.g. chromatin immunoprecipitation, or ChIP), and mapping chromatin conformation studies (ATAC-seq or DNAse-based sequencing approaches to detect open chromatin regions or high-conformation capture, HI-C, to assess chromosomal conformation) [127-129]. Common drawbacks of these conventional methods are the

requirement of large samples (about millions of cells) and long experimental timescales. The introduction of microfluidic systems revolutionized the field, offering the advantage of performing thousands of reactions in nanoliter volumes on a single device, thus making possible to detect multiple genomic loci. The first devices introduced were called "lab-on-a-chip" or micro total analysis systems (microTAS). They offer the advantage of an automated system, capable of integrating all sample-handling steps in microfluidic channels on a PDMS chip. The expanded implementation of microfluidic systems is currently driving the field of epigenetics and represents the ideal devices for the development of single cell-based epigenetic studies (Figure 4).

5.2. Lineage specification and myeloid cell differentiation

In many mammalian tissues, mature differentiated cells are replaced by self-renewing stem cells, either continuously during homeostasis or in response to challenge and injury. Studies performed on embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs) demonstrate heterogeneity in the expression of key pluripotency-related transcription factors, especially Nanog, Oct 4, Sox2, Gata2, PU.1 and C/EBPα [130,131]. However, only with the advent of single-cell methods has it become clear that a radical change in our perspective on pluripotency and cell differentiation is needed. For instance, temporal fluctuation in the levels of pluripotency-associated genes may allow cell progenitors to respond to a wide range of environmental signals, dictating their ability to self-renew or differentiate. These early stochastic changes are then followed by a more hierarchical phase where a few key transcription factors drive the activation of specific transcriptional networks that ultimately determine cell fate [131,132]. At the single cell level, both hematopoietic multipotential progenitors and bipotential intermediates express a mixed-lineage pattern of genes, but they also exhibit promiscuous expression of lineage-associated genes with the potential of priming different cell fate paths [133,134]. In recent work from Leighton Grimes's group the authors performed a single-cell RNA sequencing (scRNA-seq) on stem/multipotent progenitors to delineate discrete genomic states and the relative transitional cellular intermediates. Each cellular intermediate is defined in terms of pattern-specific guide genes inferred computationally by correlating pairwise gene expression with cellular genomic states. Interestingly, IRF8 and GFI1 (required for monopoiesis and granulopoiesis, respectively) function as antagonistic determinants in the granulocyte monocyte progenitor (GMP) population and enforce a strong partitioning between monocyte-specified and granulocyte-specified cells. GFI1 induction in GMP cells increases granulocyte potential and antagonizes the specification of the monocytic-dendritic progenitors by repressing enhancers activated by IRF8-PU.1. Moreover, GFI1 recruits the histone demethylase LSD1 to remove the transcriptionally activating H3K4me2 mark. Loss of GFI1 results in increased H3K4me2 levels and enhanced monocytic potential. Loss of expression of either *Gfi1* or *Irf8* reduces the heterogeneity of GMP genomic states and reciprocally alters the expression of the gene regulatory networks underlying monocytic (*Zeb2*, *Klf4*, *Irf5*) or granulocytic (*Ets1*, *Per3*) regulatory states respectively. By using reporters for reciprocally expressed transcription factors (IRF8-GFP and GFI1-GFP reporters), the authors were able to distinguish between bipotential cells, their lineage-committed progeny, and rare intermediates poised for binary cell fate choice. These rare cell populations have collapsed the multipotential program and display a mixed-lineage transcriptional state, with low levels of expression of both *Gfi1* and *Irf8* [134]. The authors proposed a model of mixed-lineage transition states to describe the cell differentiation process as a hierarchical set of hematopoietic intermediates characterized by the expression of

counteracting regulatory gene network components (such as *Irf8* and *Gfi1*), that generate oscillations of these multi- and bi-potential genomic states. The consequent dynamic instability results in bursts of alternative-lineage gene expression, which ultimately might culminate with the acquisition of a developmental metastable intermediate that could be "trapped" by eliminating opposing lineage determinants, finally leading to either monocytic or granulocytic lineages.

Another interesting development is the recent challenge to the general concept that cell regeneration is restricted to differentiation from tissue-specific stem cells. Recently, independent studies demonstrated that tissue macrophages, together with a few other cell types, represent a rare exception to this pathway. Significantly, it was demonstrated that monocytes are not direct precursors for most tissue-resident macrophages at steady state [135]. Few tissue macrophages derive from monocytes during homeostasis; rather, monocytes give rise to short-lived, non-proliferating inflammatory macrophages that infiltrate tissues during inflammation. The majority of tissue-resident macrophages were found to be derived from ESCs and renew independently of HSCs [136]. Therefore, this process may not require progenitors, as mature macrophages can proliferate in response to specific stimuli indefinitely and without transformation or loss of functional differentiation.

Recently, Soucie and colleagues dissected the self-renewal potency of tissue resident macrophages at the single cell level through the characterization of the epigenetic traits specifically associated with self-renewal and macrophage differentiation [131]. Self-renewing macrophages activate a network of genes that is also required for ESC self-renewal (such as *c-Myc* and *klf2*) and do not require the acquisition of dedicated, self-renewal-specific enhancers as quiescent macrophages do [131]. Interestingly, the regulatory mechanisms by which activation of these regulatory gene networks is accomplished involves a cell-specific set of enhancers in self-renewing macrophages.

These self-renewal-associated enhancers are already present in quiescent cells in the poised state, as demonstrated by the presence of a typical H3K4me1/PU.1+ enhancer signature. One of the triggering events leading to the activation of self-renewal-related enhancers is the relaxation of repression of *c*-MAF and MAFb, two key macrophage transcription factors. Indeed $Maf^{-/-}$ macrophages retain their differentiated state but can be expanded and maintained in long-term culture [131].

These examples illustrate the great potential of single cell-based technologies to deconstruct heterogeneous populations, lineage specifications and determinants of cell fate as well as to elucidate key molecular processes during development and differentiation of immune cells.

5.3. Epigenomic approaches to identify biomarkers associated with inflammatory disease

Genomic mutations and dysregulation of gene expression are at the basis of a broad range of syndromes and diseases [137-140]. Potentially, detection of some of these alterations could be used in the clinic as biomarkers for early diagnosis to predict treatment response or drug toxicity [141,142]. Genetic biomarkers are valuable indicators of susceptibility to the development of disease. But in contrast to oncology, where traditional genetic biomarkers are extensively used in the diagnosis and prognosis of cancer, the use of biomarkers in inflammatory diseases is limited, due to the low correlation between the development of disease and specific genomic mutations. Auto-antibodies such as the rheumatoid factor (RF) in rheumatoid arthritis (RA) or specific HLA subtypes (e.g. HLA-B27 in spondyloarthritis) are prognostic biomarkers currently used, but they are not

disease-specific. Other potential biomarkers tested, such as cytokines and chemokines, which have been demonstrated to play a major role in promoting joint damage, failed in clinical studies to provide consistent and clear prognostic values [143-147]. The complexity and heterogeneity of these pathologies are related to the observation that onset and disease course are dependent on both genetic and environmental factors; epigenetic alterations may represent the link between these two important components. Recent developments in epigenetic profiling technologies, have allowed for large-scale discovery of molecular epigenetic biomarkers that could represent a very attractive tool in the clinic. Many new genomic regions have been found to exhibit disease-specific epigenetic alterations, including those that are located apart from well-known candidate regions such as CpG-islands and gene promoters [148]. Moreover, epigenetic biomarkers present some ideal requirements for an efficient biomarker: stability, availability of sample (epigenetic modifications can be measured in cell-free DNA from bodily fluids) and reproducibility with different methods and settings (Table 2) [149].

DNA methylation is the most widely characterized epigenetic modification and disruption of methylation patterns represents a characteristic feature in many pathological conditions, including cancer, neurodegenerative disorders and inflammatory diseases (for review see [150,151]). In the early 1990s a loss of DNA methylation was described in blood cells and synovial tissues of RA and systemic lupus erythematosus (SLE) patients [152]. Since then, several differentially methylated regions associated with rheumatic disease have been reported at specific genomic sites across different cell types [153-155]. Methylated DNA represents a robust epigenetic biomarker, driving its use in oncology studies. For instance, in the case of colorectal cancer, the abundance of methylated DNA in the extracellular environment facilitates its easy collection and measurement [156]. Moreover, methylated DNA samples are stable under most storage conditions and almost any biological tissue sample or body fluid can be used for analysis (Table 2). In the cancer field, DNA methylation biomarkers with diagnostic, prognostic and predictive power are already being investigated in clinical trials [157-160]. Histone modifications and histone-modifying enzymes are also altered in different inflammatory diseases. However, relative to DNA methylation, histone modifications present technical problems in terms of stability of the biological samples and consistency during different disease stages. Moreover, analysis of one or more genomic loci for the detection of individual histone modifications tends to be nonspecific. Because of these inherent limitations, most of the efforts have been directed mainly towards the development of epigenetic biomarkers able to detect DNA methylation patterns. However, whereas several studies support the notion that epigenetic changes can be drivers of cancer [161,162], it has been more difficult to demonstrate the role of epigenetic modifications in the etiology and progression of inflammatory diseases. More specifically, some current limitations are cellular heterogeneity of the sample material as well as the potential for methylation changes to be consequences of disease rather than part of its etiology. The integrated search of genetic and epigenetic risk associated marks will be helpful to overcome some of these limitations. Thanks to the capability to assess large numbers of loci for the presence or absence of certain epigenomic features, the parallel evaluation of many candidate biomarkers is possible. The implementation of cutting edge technologies has already made it possible to merge genome-wide epigenomics with recent advances in single cell approaches in a way that will facilitate the discovery of new reliable epigenomic biomarkers. Such single cell variability, as was previously discussed, may provide more precise information about the molecular mechanisms of disease. However further efforts are still needed to overcome some current limitations inherent to the methodologies currently adopted, including establishing standards for molecular assays to elude

discrepancies in separate trials and to filter out the noise coming from the high heterogeneity of biological samples.

The upcoming years will be critical in defining the potential of high-throughput techniques in the development of disease-specific epigenetic biomarkers with clinical relevance.

Table 2. Traditional vs epigenetic biomarkers.

Characteristics of ideal biomarker		
- Specific - Robust and reliably measurable with different methods and in different settings - Early detection, diagnosis and prognosis - Therapeutic stratifications - Post-therapeutic monitoring - Contained cost		
	Traditional biomarker	Epigenetic biomarkers
Description	Genes and protein-based	Associated with changes in the epigenetic landscape (DNA-methylation, Histone PTMs, ncRNAs)
Advantages	Easy availability of the sample; Clinical validity and utility demonstrated; Easier way of analysis with less advanced technologies in clinical practice	Easy availability of the sample; Higher stability in multiple biospecimens (blood, urine, plasma, FFPE tissues); Provide information about gene function in individual cell-types and incorporate information from the environment; Contribute to the improvement of precision/personalized medicine
Limitations	Static biomarker (gene sequence generally does not change); Less stability in biological samples	Compatibility with high throughput techniques (difficulties introducing new technologies into clinical laboratories); Avoids the use of a mixed cell population (a mixed cell population can result in cell fluctuation); Overcome regulatory impediments in different countries to achieve the implementation of epigenetic diagnosis in clinical routine

Summary of advantages and limitations of traditional and epigenetic biomarkers. For clinical applications a biomarker requires specific traits, summarized on the upper part of the table. Traditional biomarkers compared with epigenetic ones benefit from extended research and clinical application. For more robust analyses and results, an integrative approach of both biomarker types is desirable.

6. Conclusion and future perspectives

The molecular determinants precisely controlling the inflammatory response and differentiation of innate immune cells have begun to be elucidated. Recent advances in the field of epigenetics have revealed a new perspective that upends previous dogma concerning immune memory, unveiling the complexity and dynamicity of this biological process. The response to a specific pro- or anti-inflammatory stimulus derives not simply from the activation or inhibition of different gene networks, but involves many molecular actors that contribute to determine a profound reprogramming at the single cell level. Therefore, lineage-specific transcription factors, *de novo* enhancers, non-coding RNAs and chromatin regulators participate in a collaborative manner during the dramatic and rapid remodeling of the genomic landscape during the innate immune response. The crosstalk between all these molecular players is very intricate and the numerous efforts to dissect such complexity described in this review attest to the challenge of characterizing the regulation of innate immunity. However, the continuing development and implementation of cutting edge technologies, especially genome-wide epigenomics and single cell biology, will continue to enable higher-resolution understanding of the role of epigenetics in this paradigm.

Conflict of interest

The authors declare no conflict of interest.

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