The intersection of epigenetics and metabolism in trained immunity

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Summary

In the last few years we have witnessed an increasing body of evidence that challenges the traditional view that immunological memory is an exclusive trait of the adaptive immune system. Myeloid cells show increased responsiveness upon subsequent stimulation with the same or a different stimulus, well after the initial challenge. This *de facto* innate immune memory has been termed 'trained immunity,' and is involved in infections, vaccination and inflammatory diseases. Trained immunity is based on two main pillars: the epigenetic and metabolic reprogramming of cells. In this review we discuss the latest insights into the epigenetic mechanisms behind the induction of trained immunity, as well as the role of different cellular metabolites and metabolic networks in the induction, regulation and maintenance of trained immunity.

Introduction

Traditionally, the vertebrate immune system has been divided into two main branches: the adaptive immune system and the innate immune system. According to this classification, the innate immune system constitutes a first line of defense that recognizes stimuli of a different nature, eliciting a non-specific, rapid, protective response against pathogens (Medzhitov and Janeway, 2000). The adaptive immune system forms a more specialized line of defense, developing specific long-lasting memory that protects the organism against later encounters with the same pathogen (Bonilla and Oettgen, 2010). However, this dogma has been challenged in recent years by the discovery that cells from the innate immune system can also acquire memory-like capacities after pathogenic challenge. Myeloid cells of the innate immune system show increased responsiveness upon subsequent stimulation with the same or a different stimulus (Netea et al., 2020). This phenomenon has been termed innate immune memory, or “trained immunity”, and plays a fundamental role in protection against infections, the induction of non-specific protective effects of vaccines and the pathogenesis of several inflammatory diseases (Dominguez-Andres and Netea, 2018; O'Neill, L., & Netea, 2020; Netea et al., 2020).

The induction of trained immunity is founded on two main pillars: epigenetic and metabolic reprogramming of innate immune cells. After stimulation with certain ligands of microbial origin, such as β-glucan (present on the cell wall of different fungi) or Bacille Calmette-Guérin (BCG), (the main vaccine against tuberculosis worldwide) these immune cells experience strong functional reprogramming changes that allow enhanced responsiveness under subsequent stimulation (Arts et al., 2018; Quintin et al., 2012). Of note, the capacity to induce trained immunity is not an exclusive property of microbial stimuli, hence endogenous ligands such as uric acid or oxidized

low-density lipoproteins (oxLDL) can also trigger these mechanisms, causing the development of diseases such as gout or atherosclerosis (Bekkering et al., 2014; Crişan et al., 2017). Notably, there are a group of ligands that do not induce trained immunity, but cause the development of immunological tolerance, which is the opposite program of trained immunity. Such ligands, like lipopolysaccharide (LPS) found in the cell envelope of Gram-negative bacteria induce high levels of gene transcription and the production of inflammatory factors during the acute phase. This is triggered by the interaction between the microbial ligand and its specific receptor, TLR4. However, in contrast to trained immunity, when the stimulation ceases, tolerized cells undergo a functional program characterized by the epigenetic silencing of inflammatory genes and demonstrate a lack of responsiveness to secondary stimulation (Novakovic et al., 2016).

The stimulation of innate immune cells involves the activation of diverse intracellular pathways that result in the upregulation of proinflammatory gene transcription, and the production of proinflammatory cytokines and chemokines (Liu et al., 2017). To facilitate this, the cellular machinery must be able to access regions of the genome that contain the regulatory elements of the genes involved in these processes. This is orchestrated through several stable and durable epigenetic modifications, which unfold chromatin and expose promoter and enhancer regions regulating immune-related genes, thus making them accessible to transcription factors (Figure 1; Klemm et al., 2019). The persistence of these epigenetic modifications permits cells to remain in a ‘trained’ state concurrent with an increased accessibility of proinflammatory genes, facilitating a faster and increased transcriptional responsiveness after rechallenge (Saeed et al., 2014). For this reason, understanding the mechanisms behind these epigenetic modifications and their persistence in hematopoietic and non-hematopoietic niches is fundamental to

define the nature, impact and consequences of trained immunity in health and disease. However, the mechanisms describing the dynamic organization of epigenetic marks at discrete gene loci that regulate the immune response are still poorly understood. In this review we will detail the latest insights in the epigenetic mechanisms that govern the induction of innate immune memory and discuss their impact in the way we define and ultimately measure trained immunity.

Defining the cell populations that mediate trained immunity

Induction of trained immunity has been described in multiple innate immune cell populations including monocytes/macrophages, NK cells, innate lymphoid cells, with preliminary observations suggesting that similar characteristics may also be present in polymorphonuclear leukocytes (Domínguez-Andrés et al., 2019; Mitroulis et al., 2018; Sun et al., 2009). While innate immune cells do not express rearranging antigen receptor genes that are a mainstay of lymphocytes, they do express pattern recognition receptors (PRRs) and other receptors that allow them to recognize and respond to both pathogen-associated molecular patterns (PAMPs) and endogenous danger signals (damage-associated molecular patterns, DAMPs) (Bianchi, 2007; Takeuchi et al., 2010). Although these responses are not as specific as those induced in T- and B-cell receptors, accumulating evidence over the last two decades has shown that distinct members of pattern recognition receptor families (e.g., Toll-like receptors, NOD-like receptors, C-type lectin receptors, RIG-I-like receptors) trigger different intracellular signaling pathways that lead to discrete innate immune responses that are tailored to the type of pathogen encountered (Mills, 2011). Among the most important PRRs involved in the induction of trained immunity are dectin-1, the receptor for β-glucan, and NOD2, the receptor for muramyl peptide, a molecule present on the cell envelope of BCG (Kleinnijenhuis et al., 2012).

Among the innate immune cell types described that are able to display the adaptive characteristics of a trained immunity phenotype, most research has been focused on monocytes/macrophages and NK cells. Probably the first evidence that macrophages can build adaptive features came from investigations of LPS tolerance at the molecular level (Foster et al., 2007). In a seminal study, the molecular mechanisms responsible for these processes were described by Medzhitov and colleagues, who showed that changes in chromatin architecture through chemical histone modifications were associated with the silencing of genes coding for inflammatory molecules (Foster et al., 2007). Interestingly, genes coding for antimicrobial molecules did not display a tolerized phenotype, but an increased expression, reminiscent of what would later be described as trained immunity. This observation was further expanded by studies demonstrating that exposure of monocytes/macrophages to *C. albicans* or β-glucan components from its [*Candida*] cell wall can enhance the subsequent response of monocytes/macrophages to stimulation with unrelated pathogens or PAMPs (Quintin et al., 2012). Subsequently, induction of trained immunity in monocytes was shown to be accompanied by changes in chromatin epigenetic marks, as shown by histone marks such as H3K4me1, H3K4me3 or H3K27Ac (Quintin et al., 2012; Saeed et al., 2014), as detailed further below. In addition to these effects induced by infections (caused by bacterial and fungal pathogens) other studies have shown that monocytes/macrophages can also acquire trained immune responses following infection with parasites (Chen et al., 2014) or viral pathogens (Barton et al., 2007).

An important aspect to be considered regarding trained immunity induced in monocytes is their lifespan. Monocytes have a short half-life in circulation, and the observation that trained monocytes have been identified in the circulation of BCG-vaccinated individuals

for at least three months after vaccination (Kleinnijenhuis et al., 2012) suggests that reprogramming must take place at the level of progenitor cells in the bone marrow as well. Indeed, evidence has demonstrated that this is the case in murine experimental models after administration of beta-glucan (Mitroulis et al., 2018) or BCG (Kaufmann et al., 2018), and that innate immune memory can be transferred via hematopoietic stem and progenitor cells. Interestingly, other studies have also suggested that microbiota can induce long-term functional reprogramming of bone marrow progenitors, and subsequently dendritic cells, to induce protection against *Entamoeba histolytica* (Burgess et al., 2019), suggesting that these processes can also happen during homeostasis. Vaccines known to induce trained immunity, such as BCG, can also induce similar effects at the level of bone marrow progenitor cells in humans, as has been demonstrated by a very recent study (Cirovic et al., 2020).

Emerging evidence suggests that NK cells also build immunological memory after a previous insult. NK cell memory has been documented following exposure to cytokine combinations (e.g., IL-12, IL-15, and IL-18) (Burgess et al., 2014) or hapten sensitization (O'Leary et al., 2006). Even more importantly, recent studies have shown that NK cells undergo expansion during and after viral infection: in seminal studies it has been shown that CMV infection can activate NK cells, which in turn can induce long-term T cell-independent protection against reinfection by rapidly degranulating and producing cytokines (Sun et al., 2009). These data were supported by studies showing that adoptive transfer experiments with activated NK cells can protect naïve recipient mice against viral infection. Consistent with these data, BCG vaccination has also been shown to induce long-term hyper-responsiveness of NK cells, with BCG conferring nonspecific protection against *C. albicans,* at least partially through NK-cells (Kleinnijenhuis et al., 2014). Other studies have also revealed NK memory in primates:

in this instance, splenic and hepatic NK cells from Ad26-vaccinated macaques efficiently lysed antigen-matched but not antigen-mismatched targets, up to five years after vaccination (Reeves et al., 2015). In addition to these studies demonstrating antigen-specific mechanisms of NK cell immune memory, recent studies found that liver-resident type 1 ILCs (ILC1s) expand and persist after the resolution of infection with mouse cytomegalovirus. The presence of stable epigenetic, transcriptional, and phenotypic changes one month after the resolution of the infection, strongly suggests an innate immune memory response (Weizman et al., 2019).

Importantly, studies performed in the last couple of years have introduced a concept of 'extended trained immunity' as proposed by the work of Cassone (Cassone, 2018). Recent studies have demonstrated that quiescent epidermal stem cells are mobilized into action when tissue is injured. Interestingly, wound healing is further improved if an earlier injury was present, a process called inflammatory memory (Naik et al., 2017), which is in fact similar to trained immunity induced in innate immune cells. The discovery of "memory" in epidermal stem cells extends the concept of inflammatory memory beyond the confines of the immune system and opens up the possibility that many non-immune cell types within tissues may possess an epigenetic memory of their encounters with stressful environments. In line with this concept, respiratory epithelial progenitors become more stem-like during human allergic inflammatory disease, and the associated accessible chromatin changes differ in their ability to return to normal when the stimulus is withdrawn (Ordovas-Montanes et al., 2018). Despite the growing number of studies describing the abilities of these different cell subsets to undergo trained immunity functional programs, the functional consequences of these processes on different cell types and tissues remains to be fully elucidated in the years to come.

Epigenetic reprogramming underpins trained immunity

A successful innate immune response relies on the rapid and coordinated transcription of hundreds of genes that encode for cytokines and signalling molecules. This response needs to be highly calibrated, with a poor or uncoordinated transcriptional response having deleterious consequences on the ability of the host to resolve inflammation. Ultimately, the strength of the immune transcriptional response is determined by the epigenetic state of innate immune genes and the surrounding genomic neighbourhood.

Both DNA methylation and histone modifications are involved in the regulation of patterns of gene expression. DNA methyltransferases (DNMTs) recognise CpG-rich sequences to methylate cytosines (m5C) which results in transcriptional repression. The tails that extend out of histone octamers can be recognised by proteins that harbour histone-binding domains that may either 'read', 'write' or 'erase' histone marks. These enzymes can catalyse the addition or removal of a highly vast and diverse array of different histone modifications, such as methylation, acetylation, phosphorylation and ubiquitination. Several histone modifying enzymes and their accompanying histone modification have been well studied. For example, histone 3 lysine 4 trimethylation (H3K4me3) is an active promoter chromatin mark that is catalysed by the Trithorax (Trx) complex. The Mixed lineage Leukemia (MLL) gene is the human homolog of Trx. Histone 3 lysine 4 trimethylation (H3K4me1) chromatin marks are enriched at active and poised enhancers, and are written by enzymes including the Set7 lysine methyltransferase (encoded by *SETD7*). In contrast, histone 3 lysine 27 (H3K27) methylation, which induces gene silencing, is catalysed by the Polycomb Group (PcG) proteins (Geisler and Paro, 2015).

Numerous lines of evidence have revealed that different combinations of DNA and histone modifications will determine whether DNA is maintained in an accessible or 'open' state, versus an inaccessible or 'closed' state. Highly accessible DNA is readily bound by the transcriptional machinery and transcription factors, to facilitate rapid and robust transcriptional activation. In this way the 'openness' of DNA is directly linked to the transcriptional status of protein coding genes. Furthermore, by adjusting chromatin accessibility, cells are able to transmit information from external stimuli (such as training stimuli) into durable changes in gene regulatory patterns. In support of this, ATAC sequencing (ATAC-seq) experiments have revealed distinct accessibility patterns at transcription factor binding sites in trained macrophages *in vivo* (Novakovic et al., 2016).

During a trained immune response, the epigenetic reprogramming of a large number of immune genes and their associated enhancers in several types of immune cells, such as macrophages and monocytes occurs. Specifically, the H3K4me3 promoter mark accumulates on immune gene promoters. In addition, there is the modulation of H3K4me1 and H3K27Ac epigenetic marks on enhancers (Novakovic et al., 2016; Quintin et al., 2012), with the persistence of H3K4me1 at decommissioned enhancers (Saeed et al., 2014). Interestingly, mice lacking *Setd7* are unable to successfully mount β-glucan-induced trained immune responses (Keating et al., 2020). This indicates that by writing a persistent H3K4me1 signature at a subset of enhancers, Set7 is a key regulator of trained immunity. However, the gene regulatory mechanisms describing how these epigenetic changes only occur at discrete locations in the genome are only beginning to emerge and are critical to advance our understanding of epigenetic transcriptional memory.

The role of lncRNAs and 3D nuclear architecture in trained immunity

The central dogma of biology describes RNA as molecules that merely act as bridges that connect the flow of information from DNA to protein. However, recent advances in transcriptome sequencing technology have revealed that the genome is pervasively transcribed into RNA, with approximately only ~2% of these RNAs found to be "coding" or translated into protein (or mRNA) with the remainder being "noncoding" RNA. Long non-coding RNA (lncRNAs) and enhancer RNAs (eRNAs, discussed below), are a subset of these non-coding RNAs that are emerging as key regulators that are instructive of gene activity (Engreitz et al., 2017). As a class, lncRNA transcripts are highly diverse, ranging from approximately > 200 nucleotides (nt) to well over 10 Kbp in length, and may even be spliced and polyadenylated. Despite lacking full protein coding potential, aberrant regulation of several lncRNAs has been shown to underpin the development of disease states, including cancer and inflammation (Magagula et al., 2017).

Advances in chromosome conformation capture (3C)-based techniques have revealed that chromatin in the eukaryotic nucleus is divided into domains enriched in chromosomal loops, called topological associating domains (TADs). Within TADs, and at the interface between TADs, chromosomal loops bring DNA elements, such as enhancers and lncRNAs, proximal to protein coding genes to regulate their expression (Figure 2a) (Dixon et al., 2012, Fanucchi et al., 2013). In this way, 3D folding of the genome can significantly impact gene regulation. LncRNAs have been demonstrated to exploit three-dimensional (3D) folding of DNA to direct chromatin remodelers in *cis* or *trans* to regulate genes via diverse mechanisms that includes acting as recruiters of chromatin remodelers extensively reviewed in (Li and Fu, 2019; Magagula et al., 2017).

Few of the thousands of identified lncRNAs have an identified function. For some lncRNAs, this mystery is due to their low expression level (even 1 or 2 copies per cell), which severely hampers their detection and characterisation. Therefore, carefully designed studies are required to determine whether lncRNAs are functional transcripts and not simply transcriptional noise. Several studies that employ both loss- and gain-of-function approaches have been able to carefully dissect the function of several lncRNAs. For example, lncRNAs such as NeST and HOXA distal transcript antisense RNA (HOTTIP) have been convincingly shown to interact with WD repeat-containing protein 5 (WDR5) and direct MLL1 to target genes in *cis*, allowing the deposition of H3K4me3 at the promoters of Interferon Gamma (IFNG) and the HOXA genes respectively (Wang et al., 2011; Gomez et al., 2013). Thus a single lncRNA can regulate multiple co-regulated genes located within the same TAD. For example, TH2LCRR was demonstrated to regulate the deposition of H3K4me3 levels on the promoters of the Th2 cytokines, IL-4, IL-5 and IL-13 (Spurlock et al., 2015).

Motivated by the instructive role 3D chromatin architecture and lncRNAs play in transcriptional regulation, we hypothesized that lncRNA-dependent regulation could greatly influence the epigenetic reprogramming of immune genes during trained immunity. We devised a bioinformatic pipeline that incorporated 3D nuclear architecture, lncRNA and enhancer expression data and the epigenetic status of immune genes at the genomic scale. This approach enabled us to harvest dozens of lncRNAs which we termed Immune-gene priming lncRNAs (IPLs) (Fanucchi et al., 2019). Careful analysis of a prototypical IPL, which we named UMLILO (Upstream Master LncRNA of the Inflammatory chemokine LOcus), revealed that UMLILO engaged in chromosomal contacts with the ELR+ CXCL chemokines (IL-8, CXCL1,

CXCL2, and CXCL3) to direct the WDR5/MLL1 complex across the CXCL chemokine promoters. In this way, H3K4me3 epigenetic priming of the CXCL promoters occurred prior to their transcriptional activation. Furthermore, UMLILO expression was positively correlated with the level of H3K4me3 accumulation on the CXCL promoters. Importantly, the IPL-mediated mechanism was shared with other key trained immune genes, such as IL-6 and IL1β and other lncRNAs harvested by the bioinformatic pipeline. At the transcriptional level, training of human monocytes resulted in an NFAT (Nuclear Factor of Activated T cells)-mediated increase in the expression of IPLs, which in turn resulted in the epigenetic reprogramming of the innate immune genes**.** This study provided the first evidence that lncRNA-mediated regulation is central to the mechanism of how H3K4me3 chromatin marks are established during trained immunity. In addition to NFAT binding sites, most IPL promoters contain multiple, highly conserved transcription factor binding sites, such as CCAAT-enhancer binding proteins (C/EBPB), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and Signal transducer and activator of transcription 1 (STAT1). Although this currently remains untested, this may explain how divergent training stimuli (e.g. MAMPs or endogenous molecules such as cell metabolites) that activate different signal transduction pathways may converge on the activation of the IPLs, enabling epigenetic reprogramming of immune genes and the “writing” of epigenetic memory.

The role of eRNAs in trained immunity

Enhancers are regulatory sequences that may be located at a significant genomic distance from their target genes in one-dimensional space. In 3-dimensional space chromatin looping brings enhancers close to their target gene promoters. eRNAs are expressed in a cell- and tissue context - specific manner. β-glucan has been shown to partially reverse LPS-induced tolerance in macrophages, by reprogramming the

enhancer landscape (Novakovic et al., 2016). These changes were observed for the active enhancer chromatin marks: monomethylation of H3 lysine 4 (H3K4me1) and acetylation of H3 lysine 27 (H3K27Ac). It remains unknown whether eRNAs that emerge from these enhancer regions are absolutely required for the regulation of trained immunity.

In separate studies, eRNAs have been shown to interact with components of the Mediator complex or Yin Yang 1 (YY1) to regulate chromosomal looping interactions between enhancers and target genes (Lai et al., 2013, Sigova et al., 2015). In addition, eRNAs can bind to p300 and CBP (cyclic adenosine monophosphate response element-binding protein (CREB)-binding protein) to stimulate catalytic HAT activity and as a consequence, transcriptional activation. We speculate that by directing histone acetylation and looping at key enhancers, eRNAs may play an integral role in trained immune responses.

Insulation of the transcriptional response of trained immune genes

Genes can exist in two different states: an ON state where the gene is expressed and an OFF state with no transcriptional activity. The conversion from an inactive to an active promoter is slow, and requires a multi-step process that includes the demethylation of CpG sites, the deposition of active histone methylation marks (e.g. H3K4me3) and the recruitment of the basal transcriptional machinery and Pol II (Kaern et al., 2005). Therefore, transcription may not always be a continuous smooth process, but rather only occur when gene promoters are in a permissive state. As a consequence, transcription can occur in ‘bursts’ or pulses in only a small subset of the population, which results in high variability in gene expression between different cells in isogenic populations (Larsson et al., 2019). One way in which eukaryotes have evolved

to circumvent such stochasticity in gene expression is to maintain the promoters of certain genes in a H3K4me3-primed state prior to gene expression. In these instances, RNA Pol II remains in a paused state on these gene promoters, which permits rapid transcription upon activation by signal-dependent transcription factors (Shao and Zeitlinger, 2017).

Stochastic or 'noisy' transcription may be undesirable when immune cells are required to respond robustly and uniformly across the cell population in response to external stimuli. This is particularly important for 'H3K4me3-primed' innate immune genes, such as the CXCL chemokines (IL-8, CXCL1, CXCL2 and CXCL3), which need to respond immediately to external stimuli, and therefore, exhibit a robust transcriptional response upon activation (Figure 2b). For this reason, we speculate that genes displaying fast, nonstochastic gene expression exhibit a distinct TAD nuclear architecture and may also be assisted in transcriptional regulation by IPLs, such as UMLILO. The CXCL TAD is a preformed TAD that contains both a super enhancer and UMLILO (Fanucchi et al., 2019). Therefore, it is possible that this preformation of nuclear architecture around the super enhancer may create a domain of IPLs, chromatin remodelling proteins and other transcriptional regulators close to target genes (Quinodoz and Guttman, 2014). Consequently, target gene promoters would overcome noise associated with stochastic promoter activation, and therefore, upon the arrival of signal-dependent transcription factors would be able to achieve rapid and robust transcriptional activation. Contrastingly, genes that are not located within the same TAD and not engaged in pre-formed contact, may lack domains of IPLs and other transcriptional regulators. In this way these genes may exhibit more stochastic gene expression. This is exemplified during trained immune responses, when training increases IPL expression and, as a result, H3K4me3 levels, leading to robust

transcription of trained immune genes. It is important to note that insulation from stochastic transcription may be undesirable in certain immune responses, whereby the lack of a uniform response may generate a heterogeneous set of cell states. In these instances, the lack of a uniform immune response may be advantageous to resolve changes in pathogen exposure. Therefore, the calibration of transcriptional responses is likely to be cell- and context-specific.

Despite being widely used to study immune gene regulation processes, it is reported that mice are more resistant to inflammatory stimuli than humans (Asfaha et al., 2013). This may be partly explained by the observation that a large portion of non coding elements display very poor evolutionary conservation. Indeed, mice lack both UMLILO and IL-8, and H3K4me3 does not accumulate on the promoters of *Cxcl1*, *Cxcl2* and *Cxcl3* after β-glucan-induced training in murine macrophages (Fanucchi et al., 2019; Figure 3). Single cell RNA FISH imaging assays in TNF-stimulated murine cells reveal that very few cells across the population express the Cxcl chemokines. This is in strong contrast to human cells, which robustly express the chemokines in almost every cell under the same conditions. Remarkably, the insertion of the UMLILO genomic sequence by CRISPR-Cas9 homology-directed repair into the Cxcl murine TAD resulted in the increase of H3K4me3 levels on the murine Cxcl promoters and robust transcription of the murine Cxcl chemokines, uniformly across a population of murine cells. Importantly, these data suggest that IPLs are not an absolute requirement for gene expression, but rather increase the H3K4me3 levels on target genes, and thus insulate key immune genes from stochastic gene transcription.

Numerous studies have proposed models that may explain the robust transcriptional responses observed at trained immune genes. The decades old “two state” model

which is predominantly applied to bacterial models of transcriptional bursting, models only the ON/OFF status of the promoter, and thus is inadequate. Indeed, though this model has been used widely for almost three decades to describe cellular heterogeneity, it fails to adequately describe the robust transcription observed in trained immunity. Especially when considering other factors, such as RNA Pol II dynamics, nuclear architecture and lncRNA-dependent regulation. Recent studies have evolved this model to a multiscale model, which incorporates the dynamics of RNA Pol II recruitment and release at promoters as well as enhancer-promoter interactions (Larsson et al., 2019; Tantale et al., 2016). This is necessary to help explain the presence of multiple timescales in the bursty expression of eukaryotic genes. DNA methylation and H3K4me3 levels are critical determinants in the ability of RNA Pol II to access promoters. As IPL-mediated epigenetic rewiring of immune genes and robust immune transcription appear to underpin a successful immune response, we posit that this model may better describe the transcriptional regulation of trained immune genes.

The role of metabolo-epigenomics

The mechanisms underpinning the induction, maintenance and regulation of trained immunity rely on the complex interplay between many different metabolic pathways and the epigenetic machinery of the cell. Therefore, to decipher the epigenetic mechanisms behind the induction of trained immunity, one also needs to understand the role of specific metabolites and metabolic networks in the process. Metabolism and epigenetics are two pillars that support trained immunity, and there is a continuous interplay between them. Different metabolic pathways act as a continuous source of energy and building blocks to fuel the active remodeling of the epigenetic landscape of cells, but also provide the necessary substrates to modify the structure of the corresponding regions of the chromatin and the genome. The integrated analysis of

metabolism and epigenetics in this context can be considered as a field unto itself: metabolo-epigenomics.

When cells are in a resting state, they generally exhibit low biosynthetic demands. In this state, they predominantly rely on the highly efficient (though relatively slow) metabolic pathways, such as oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO). For example, through the sequential steps of glycolysis, the TCA cycle and the electron transport chain, mitochondrial oxidative phosphorylation enables cells to produce 38 molecules of ATP from a single molecule of glucose. FAO breaks down fatty acids into acetyl-CoA, which enters the TCA cycle to fuel the synthesis of ATP. However, upon activation, immune cells need to rapidly access a supply of substrates to replenish the many biological processes needed to trigger the immune response. In this regard, the induction and maintenance of trained immunity involves the participation of diverse metabolic pathways, including aerobic glycolysis, glutaminolysis, cholesterol metabolism and fatty acid synthesis. This fulfills the high energetic and nutritional requirements of activated cells, including the dynamic regulation of the epigenetic landscape (Figure 4).

The role of metabolic rewiring in the activation and maintenance of the epigenetic mechanisms behind trained immunity is not only related to the level of ATP production. Several metabolites exert direct regulatory roles on epigenetic enzymes including acting as cofactors, and donor or acceptor groups for epigenetic modifications:

Acetyl-CoA

In the context of metabolo-epigenomics, acetyl-CoA acts as a substrate for histone acetylation. The presence of acetyl-CoA in the cell is fundamental for the activity of Histone Acetyl Transferases (HATs). Therefore, the increased activity of metabolic

pathways leading to acetyl-CoA production results in the increased deposition of acetylation marks on histone tails. Histone acetylation in mammalian cells is dependent on adenosine triphosphate (ATP)–citrate lyase (ACLY), the enzyme that converts citrate into acetyl-CoA. Therefore, the availability of substrates that can be converted into citrate, such as glucose, fatty acids or glutamine, can affect histone acetylation in an ACLY-dependent manner (Wellen et al., 2009). A recent study using metabolic tracing studies showed that TLR signaling in murine and human macrophages redirects metabolic fluxes to increase acetyl-CoA production from glucose, resulting in augmented histone acetylation (Christ and Latz, 2019).

α-ketoglutarate, 2-hydroxyglutarate, succinate and fumarate

The deamination of glutamate to form α-KG is the last step in the glutaminolysis pathway, which allows this amino acid to fuel the TCA cycle and as a result is crucial for the induction of trained immunity. Besides this, α-KG is a fundamental cofactor for the activity of a group of enzymes known as α-KG-dependent dioxygenases, such as ten-eleven translocation (TET) enzymes, lysine-specific protein demethylases (KDM) or Jumanji C-domain-containing (JMJD) enzymes, which use dioxygen as an oxidant to catalyze various reactions via CH bond activation. The first group, TET proteins, are responsible for DNA demethylation (Tahiliani et al., 2009). TET proteins are capable of successively oxidizing the methyl group of methylated cytosines so that it is eliminated. This family of dioxygenases is composed of 3 members: TET1, TET2 and TET3. Demethylation of marks that accumulate in histone lysine residues are catalyzed by lysine-specific protein demethylases (KDM1) and Jumanji C-domain-containing (JMJD) enzymes (Xiao et al., 2012). Other α-KG-dependent dioxygenases, such as the EglN prolyl hydroxylases, mark the transcription factor HIF1α, whose activation is fundamental for the activation of the mTOR-Akt-HIF1α axis. This axis is necessary for

the induction of trained immunity in human monocytes (Cheng et al., 2014), and facilitates HIF1α degradation (Sowter et al., 2003). 2- hydroxyglutarate (2-HG), succinate and fumarate, all derivatives of α-KG, act as competitive inhibitors of these α-KG-dependent deoxygenases. Consequently, human monocytes trained with β-glucan present higher levels of these α-KG-derived metabolites and lower activity of KDM5 lysine demethylase, which is related to less DNA methylation, H3K4me3 demethylation and higher gene expression (Figure 4). In line with this, treatment of human monocytes with methylfumarate decreases the activity of KDM5 and promotes the methylation of histone lysine 4 residues (H3K4me3) at the promoters of the proinflammatory genes *IL6* and *TNFA*. This leads to enhanced responsiveness after heterologous secondary stimulation with LPS, a hallmark of trained immunity.

S-adenosylmethionine

DNA methylation occurs when a methyl group forms a stable, covalent bond with a cytosine group of DNA, generating the modified base 5-methylcytosine. In most of the cases described, this methyl group comes from S-adenosylmethionine (SAM) the most important methyl donor molecule described to date. In the worm *C. elegans*, low SAM levels restrict H3K4me3 accumulation at immune-responsive promoters, limiting the expression of genes necessary for the innate immune response against bacterial infection (Ding et al., 2015). Metabolites can interact with each other and influence several pathways. For example, itaconate, a derivative from the TCA cycle intermediate cis-aconitate, acts as a node between trained immunity and tolerance and sequesters glutathione. This in turn may influence the levels of SAM. When SAM transfers a methyl group to DNA, it is converted into S-adenosylhomocysteine (SAH), which can then be converted into methionine through the action of vitamin B12. Itaconyl-CoA, an intermediate of itaconate catabolism, reduces the levels of vitamin

B12 (Shen et al., 2017), which may potentially impact SAM levels and histone and DNA methylation, although this remains to be formally demonstrated. However, it was proven that the ratio between SAM and SAH determines the levels of histone methylation by modulating histone methylation (H3K4me3) in the liver in humans, thereby altering gene transcription (Mentch et al., 2015). In line with this, the incubation of RAW 264.7 macrophages with methionine prior to lipopolysaccharide (LPS) stimulation, attenuates the production of proinflammatory cytokines and enhances the levels of DNA methylation after LPS challenge, while treatment of cells with SAM inhibited the inflammatory responses (Ji et al., 2019).

Lactate

When a molecule of glucose is converted into two molecules of pyruvate through glycolysis, this pyruvate can have two different destinations. When the cells are in a resting state, and rely mostly on the TCA cycle to obtain ATP, these molecules of pyruvate will be converted into acetyl-CoA and enter the TCA cycle. However, in the context of trained immunity, cells have increased nutritional and energetic requirements. This increases their glucose consumption, which in turn upregulates the activity of the TCA cycle. As a consequence, a significant amount of pyruvate is transformed into lactate through aerobic glycolysis. For a long time, lactate was regarded as an inactive by-product of energy metabolism. However, in recent years, a growing number of studies have shown that this metabolite plays important roles in the regulation of immune responses, including the modification of the epigenetic landscape. Tumour-derived lactate alters the phenotype of tumour-associated macrophages (Colegio et al., 2014), while ex vivo treatment of human monocytes with lactate, modulates cytokine production with predominantly anti-inflammatory effects (Ratter et al., 2018). However, the mechanism describing the immunomodulatory

effects of lactate has been lacking. Recently, Zhang et al. described the existence of a previously unknown histone modification derived from lactate, which they refer to as histone lactylation (Zhang et al., 2019). They demonstrated that LPS stimulation of murine macrophages increased the accumulation of lactate, which can then bind to lysine residues on histones. Interestingly, after inflammatory stimulation of mouse bone marrow derived macrophages (BMDM), the appearance of lactylated histones correlated with increased expression of genes involved in maintaining a homeostatic state of the cell, and decreased expression of proinflammatory genes. Of note, histone lactylation appears after, and is inversely correlated with histone acetylation. Therefore, histone lactylation appears to act as a regulatory counterbalance of inflammatory gene expression at the epigenomic level, in the late phase of inflammation. How lactate is directed to specific histones at discrete genomic locations remains unknown.

NAD^+ and sirtuins

Together with high rates of glucose consumption and lactate production, another consequence of the increased glycolysis observed in β-glucan and BCG-trained cells is a high ratio of nicotinamide adenine dinucleotide (NAD+) to its reduced form (NADH). NAD^+ is an obligatory cofactor for the activity of all seven mammalian sirtuins (SIRTs), a group of enzymes which can remove lysine acetyl-groups from protein substrates, including histones. Therefore, the levels of NAD^+ enable SIRTs to modulate genome stability, gene transcription, and energy metabolism in response to the energy status of the cell. The activity of SIRTs was first linked to immunity 16 years ago, when Yeung et al. described how SIRT1 physically interacts with the RelA/p65 subunit of the immunomodulatory factor NF-κB and increases apoptosis in response to TNFα by deacetylating RelA/p65 at lysine 310 (Yeung et al., 2004). In the case of trained

immunity, a higher ratio of NAD+/NADH would facilitate the activity of SIRTs and in this way influence inflammatory responses. In line with this, LPS, which induces immune tolerance and opposes trained immunity, downregulates the expression of SIRT1 in macrophages, increasing the expression of suppression-related transcription factors such as p65, IRF8 and LXR (Jia et al., 2018). $Sirt2^{-/-}$ mice have enhanced proinflammatory responses after DSS colitis compared with wild-type littermates. This effect is attributed to increased polarization of macrophages towards a proinflammatory phenotype (Lo Sasso et al., 2014). In addition, SIRT6 regulates the expression of multiple genes involved in glycolytic metabolism, including the transcriptional regulator HIF1α, fundamental for the induction of trained immunity, through the deacetylation of histone H3K9 (Zhong et al., 2010).

Butyrate

Butyrate is a short chain fatty acid produced by the gut microbiota from the fermentation of dietary fibers in the colon. Recently, this metabolite has been attracting a lot of attention as a powerful immune modulatory metabolite in vitro and in vivo. This has been linked to its potent activity as an HDAC inhibitor. In this regard, butyrate preconditioning of macrophages enhances their anti-bacterial properties through HDAC3 inhibition, which enhances glycolysis and mTOR functions, facilitating an inflammatory response (Schulthess et al., 2019). In the context of innate immune memory, the HDAC inhibitory activities of butyrate have been related to suppression of trained immunity. The inhibition of class I HDACs by butyrate reduced the production of proinflammatory cytokines in PBMCs from gout patients and in cells trained ex vivo with monosodium urate crystals (Cleophas et al., 2016). Furthermore, dietary supplementation with sodium butyrate decreased the production of IL-6 and TNFα during oxLDL-induced trained immunity in individuals with metabolic syndrome

(Cleophas et al., 2019). The heightened interest in the study of microbiota and microbiota-derived metabolites warrants further investigation into the effects of butyrate in the regulation of trained immunity.

Translation, relevance and future perspectives

Here we have discussed how recently revealed epigenetic mechanisms and metabolic processes form the molecular basis of trained immunity. It is clear that there is a close relationship between the metabolic reprogramming of cells and physical modifications of their epigenome. Numerous studies reveal that this is mediated by multiple metabolites which exert central roles in the induction, maintenance and regulation of these modifications. However, many questions in the field remain unanswered. Once these modifications are established, for how long are they maintained? Are these epigenetic changes transmitted through cell division to daughter cells? And, can this epigenetic state be passed from parent to progeny in mammals, as already demonstrated in plants (Kachroo and Robin, 2013)?

Recent studies in mice have revealed that bone marrow and specifically HSCs are integral to propagation of long term immune memory induced by BCG and other training stimuli (Kaufmann et al., 2018; Mitroulis et al., 2018). However, it remains poorly understood for how long the reprogrammed state in the bone marrow persists, and how this state contributes to the generation of long term epigenetic memory (Fanucchi and Mhlanga, 2019).

One of the major causes of variation in trained immune responses, such as observed among vaccine recipients, is host genetic variability (Li et al., 2016). Indeed, in the past decade, hundreds of risk alleles for multiple inflammatory disorders have been identified by genome wide association studies (GWAS). Yet the progress towards

understanding the underlying mechanisms of these disease variants is seriously lacking. This is mainly due to the fact that approximately 90% of this genetic variation falls within the non coding portion of the genome (Farh et al., 2015). Importantly, polymorphisms may fall within transcription factor binding sites, the promoter region of lncRNAs, or within the lncRNA or enhancer elements themselves. This variation may significantly impact gene regulation by altering noncoding RNA expression or secondary structure, or even interfere with the ability for chromatin to engage in long range looping interactions. This reveals that a comprehensive catalog of functional non coding elements influential to this process is critical to enable predictions of how these numerous disease elements will influence inflammatory processes, such as innate immune memory and vaccination.

Taken together, there is a growing body of evidence which demonstrates the central role of epigenetic mechanisms in the regulation of trained immunity. The variety of epigenetic pathways presented here include DNA methylation, histone acetylation, lncRNAs, 3D nuclear architecture, enhancer RNAs and all the metabolo-epigenetic networks. These offer a multitude of new potential targets to modulate, regulate, potentiate or weaken innate immune memory responses. The potentiation of epigenetic mechanisms of trained immunity could be employed to enhance immune responses in immune compromised individuals or to increase the protective, non-specific effects of vaccines against infections. On the other hand, modulating these mechanisms through fine-tuning the epigenetic effects could offer new alternatives to the treatment of inflammatory diseases which have been related to trained immunity, such as atherosclerosis or diabetes.

Box 1: Timescales of trained immunity

Steady state: Unstimulated cells have low biosynthetic demands and reduced gene transcription. As a consequence, they have reduced basal metabolic activities and more condensed chromatin.

Acute stimulation: The interaction of a ligand with its specific PRR triggers a cellular response. In the case of molecules that induce trained immunity, the ligands can be of microbial origin, either bacterial or fungal (Quintin et al., 2012; Kleinnijenhuis et al., 2012), or endogenous molecules, such as cell metabolites, oxLDL or even glucose in high concentrations (Bekkering et al., 2014; Christ and Latz, 2019). To produce chemokines and cytokines, cells need to reprogram their metabolic and epigenetic landscape. Enhanced metabolic activity provides cells with large amounts of energy as well as the building blocks required to supply the needs of the activated cell. Rewiring the epigenetic landscape of the cell is required to unpack the chromatin around regions of genes involved in the immune response and allow the transcription of pro-inflammatory genes. Opening discrete chromatin regions increases the ability of transcription factors to access the promoter and regulatory regions of immune-related genes and initiates the transcriptional programs that will facilitate an immune response.

Resting phase: After the stimulus ceases, stimulated cells no longer are required to produce factors involved in the immune response. Several epigenetic modifications incorporated during the acute phase are maintained. As a consequence, chromatin surrounding the promoters of pro-inflammatory and metabolic genes remains accessible which improves the ability of transcriptional complexes to access these sites after secondary stimulation. Additionally, cellular metabolism does not return to baseline levels. Therefore, the cell does not mount an active response in this phase, but remains in a prepared (or trained) state, to facilitate the ability to mount a quick and robust response. This trained status can last from weeks to at least several months,

and can be transmitted to daughter cells, albeit as demonstrated in plants (Kachroo and Robin, 2013).

Restimulation: The rechallenge of cells with the same or a different stimulus induces a new response, which is facilitated by the higher metabolic activity of the cells and the increased accessibility to the pro-inflammatory regions of the genome. This allows the faster and stronger responsiveness characteristic of innate immune memory.

Figure legends:

Figure 1: Epigenetic rewiring of immune genes underpins trained immunity. Innate immune cells exposed to stimuli (including β-glucan, BCG and oxLDL) are epigenetically reprogrammed. This initial gene activation is accompanied by the accumulation of specific epigenetic marks (e.g. H3K4me3 on gene promoters). Some of these epigenetic marks persist in the absence of the initial stimulus (e.g. H3K4me1). As a consequence of epigenetic reprogramming, upon rechallenged with a secondary stimulus, immune genes are more robustly transcribed. This process is regulated by a novel class of lncRNAs, called immune priming lncRNAs (IPL), which are upregulated by the initial stimulus. IPLs directly interact with WDR5, to direct MLL1 proximal to immune genes, facilitating the deposition of H3K4me3 on the immune gene promoters.

Figure 2: The role of 3D nuclear architecture and lncRNAs in the regulation of trained immune gene expression. a) Within TADs, chromosomal loops bring DNA elements, such as enhancers and lncRNAs, proximal to protein coding genes to regulate their expression. This permits IPLs to direct the WDR5/MLL1 complex across immune gene promoters located within the same TAD, while being insulated from accessing genes in neighbouring TADs. **b)** By maintaining immune gene promoters in an accessible state, IPLs may insulate immune genes from stochastic promoter activity. This may facilitate rapid and robust immune gene transcriptional responses, which could translate into a stronger immune response.

Figure 3: The murine *Cxcl* genes lack IPL-dependent regulation and are not robustly transcribed. Upon induction with a stimulus that induces trained immunity, UMLILO facilitates the accumulation of H3K4me3 on the promoters of the human CXCL chemokines (IL-8, CXCL1, CXCL2 and CXCL3). UMLILO is highly conserved in higher vertebrates, but no homolog of UMLILO exists in mice. Upon induction with a

stimulus that induces trained immunity, the promoters of the murine chemokines (*Cxcl1*, *Cxcl2* and *Cxcl3*) are not strongly primed with H3K4me3, and the chemokines are not robustly transcribed (Fanucchi et al., 2019).

Figure 4: An overview of the interplay between lncRNA-dependent regulation, metabolism and epigenetics during trained immunity. β-glucan/dectin-1 signaling activates calcium-dependent NF-AT signaling, to induce the transcription of the IPLs resulting in the H3K4me3 epigenetic reprogramming of immune gene promoters. Together with enhancer elements, IPLs are able to access target genes via 3D chromosomal looping. Concurrently, there is the activation of mTOR-HIF1α signaling, which alters the activity of different intracellular pathways. As a consequence, there is an increase in the supply of metabolites and co-factors for epigenetic enzymes, which alter chromatin and DNA epigenetic state to induce transcriptional changes that are causal to trained immunity. CoA, coenzyme A; NAD+, nicotinamide adenine dinucleotide; Me, methyl.

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References

Arts, R.J.W., Moorlag, S.J.C.F.M., Novakovic, B., Li, Y., Wang, S.-Y., Oosting, M., Kumar, V., Xavier, R.J., Wijmenga, C., Joosten, L.A.B., et al. (2018). BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. Cell Host Microbe *23*, 89-100.e5.

Asfaha, S., Dubeykovskiy, A.N., Tomita, H., Yang, X., Stokes, S., Shibata, W., Friedman, R.A., Ariyama, H., Dubeykovskaya, Z.A., Muthupalani, S., et al. (2013). Mice that express human interleukin-8 have increased mobilization of immature myeloid cells, which exacerbates inflammation and accelerates colon carcinogenesis. Gastroenterology *144*, 155–166.

Barton, E.S., White, D.W., Cathelyn, J.S., Brett-McClellan, K.A., Engle, M., Diamond, M.S., Miller, V.L., and Virgin, H.W. (2007). Herpesvirus latency confers symbiotic protection from bacterial infection. Nature *447*, 326–329.

Bekkering, S., Quintin, J., Joosten, L.A.B., van der Meer, J.W.M., Netea, M.G., and Riksen, N.P. (2014). Oxidized Low-Density Lipoprotein Induces Long-Term Proinflammatory Cytokine Production and Foam Cell Formation via Epigenetic Reprogramming of Monocytes. Arterioscler. Thromb. Vasc. Biol. *34*, 1731–1738.

Bianchi, M.E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukoc. Biol. *81*, 1–5.

Bonilla, F.A., and Oettgen, H.C. (2010). Adaptive immunity. J. Allergy Clin. Immunol. *125*, S33–S40.

Burgess, S.L., Buonomo, E., Carey, M., Cowardin, C., Naylor, C., Noor, Z., Wills-Karp, M., and Petri, W.A. (2014). Bone Marrow Dendritic Cells from Mice with an Altered Microbiota Provide Interleukin 17A-Dependent Protection against *Entamoeba*

histolytica Colitis. MBio *5*, e01817-14.

Burgess, S.L., Leslie, J.L., Uddin, M.J., Oakland, N., Gilchrist, C., Moreau, G.B., Watanabe, K., Saleh, M., Simpson, M., Thompson, B.A., et al. (2019). Gut microbiome communication with bone marrow regulates susceptibility to amebiasis. BioRxiv 487652.

Cassone, A. (2018). The case for an expanded concept of trained immunity. MBio *9*.

Chen, F., Wu, W., Millman, A., Craft, J.F., Chen, E., Patel, N., Boucher, J.L., Urban, J.F., Kim, C.C., and Gause, W.C. (2014). Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion. Nat. Immunol. *15*, 938–946.

Cheng, S.-C., Quintin, J., Cramer, R. a., Shepardson, K.M., Saeed, S., Kumar, V., Giamarellos-Bourboulis, E.J., Martens, J.H. a., Rao, N. a., Aghajanirefah, a., et al. (2014). mTOR- and HIF-1 -mediated aerobic glycolysis as metabolic basis for trained immunity. Science (80-.). *345*, 1250684–1250684.

Christ, A., and Latz, E. (2019). The Western lifestyle has lasting effects on metaflammation. Nat. Rev. Immunol. *19*, 267–268.

Cirovic, B., de Bree, L.C.J., Groh, L., et al. (2020). BCG Vaccination in Humans Elicits Trained Immunity via the Hematopoietic Progenitor Compartment. *Cell Host Microbe*. S1931-3128(20)30296-1.

Cleophas, M.C.P., Ratter, J.M., Bekkering, S., Quintin, J., Schraa, K., Stroes, E.S., Netea, M.G., and Joosten, L.A.B. (2019). Effects of oral butyrate supplementation on inflammatory potential of circulating peripheral blood mononuclear cells in healthy and obese males. Sci. Rep. *9*, 775.

Colegio, O.R., Chu, N.Q., Szabo, A.L., Chu, T., Rhebergen, A.M., Jairam, V., Cyrus,

N., Brokowski, C.E., Eisenbarth, S.C., Phillips, G.M., et al. (2014). Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. Nature *513*, 559–563.

Crişan, T.O., Cleophas, M.C.P., Novakovic, B., Erler, K., van de Veerdonk, F.L., Stunnenberg, H.G., Netea, M.G., Dinarello, C.A., and Joosten, L.A.B. (2017). Uric acid priming in human monocytes is driven by the AKT–PRAS40 autophagy pathway. Proc. Natl. Acad. Sci. *114*, 5485–5490.

Ding, W., Smulan, L.J., Hou, N.S., Taubert, S., Watts, J.L., and Walker, A.K. (2015). S-adenosylmethionine levels govern innate immunity through distinct methylation-dependent pathways. Cell Metab. *22*, 633–645.

Dixon, J. R., Selvaraj, S., Yue, F., Kim, A., Li, Y., Shen, Y., et al. (2012). Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature 485, 376–380.

Dominguez-Andres, J., and Netea, M.G. (2018). Long-term reprogramming of the innate immune system. J. Leukoc. Biol.

Domínguez-Andrés, J., Ferreira, A. V, Jansen, T., Smithers, N., Prinjha, R.K., Furze, R.C., and Netea, M.G. (2019). Bromodomain inhibitor I-BET151 suppresses immune responses during fungal-immune interaction. Eur. J. Immunol *00*, 1–7.

Engreitz, J. M., Haines, J. E., Perez, E. M., Munson, G., Chen, J., Kane, M., et al. (2017). Local regulation of gene expression by lncRNA promoters,transcription and splicing. Nature 539, 452–455.

Fanucchi, S., Shibayama, Y., Burd, S., Weinberg, M. S., and Mhlanga, M. M. (2013). Chromosomal contact permits transcription between coregulated genes. Cell 155, 606–620.

Fanucchi, S., Mhlanga, M.M. Lnc-ing Trained Immunity to Chromatin Architecture. Front Cell Dev Biol. 2019 Jan 24;7:2.

Fanucchi, S., Fok, E.T., Dalla, E., Shibayama, Y., Börner, K., Chang, E.Y., Stoychev, S., Imakaev, M., Grimm, D., Wang, K.C., et al. (2019). Immune genes are primed for robust transcription by proximal long noncoding RNAs located in nuclear compartments. Nat. Genet. *51*, 138–150.

Farh, K.K.-H., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W.J., Beik, S., Shoresh, N., Whitton, H., Ryan, R.J.H., Shishkin, A.A., et al. (2015). Genetic and epigenetic fine mapping of causal autoimmune disease variants. Nature *518*, 337–343.

Foster, S.L., Hargreaves, D.C., and Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature *447*, 972–978.

Geisler, S. J., and Paro, R. (2015). Trithorax and polycomb group-dependent regulation: a tale of opposing activities. Development 142, 2876–2887.

Gomez, J. A., Wapinski, O. L., Yang, Y. W., Bureau, J. F., Gopinath, S., Monack, D. M., et al. (2013). The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon-g locus. Cell 152, 743–754.

Ji, J., Xu, Y., Zheng, M., Luo, C., Lei, H., Qu, H., and Shu, D. (2019). Methionine Attenuates Lipopolysaccharide-Induced Inflammatory Responses via DNA Methylation in Macrophages. ACS Omega *4*, 2331–2336.

Jia, Y., Li, Z., Cai, W., Xiao, D., Han, S., Han, F., Bai, X., Wang, K., Liu, Y., Li, X., et al. (2018). SIRT1 regulates inflammation response of macrophages in sepsis mediated by long noncoding RNA. Biochim. Biophys. Acta - Mol. Basis Dis. *1864*, 784–792.

Kachroo, A., and Robin, G.P. (2013). Systemic signaling during plant defense. Curr. Opin. Plant Biol. *16*, 527–533.

Kaern, M., Elston, T.C., Blake, W.J., and Collins, J.J. (2005). Stochasticity in gene expression: from theories to phenotypes. Nat. Rev. Genet. *6*, 451–464.

Kaufmann, E., Sanz, J., Dunn, J.L., Khan, N., Mendonça, L.E., Pacis, A., Tzelepis, F., Pernet, E., Dumaine, A., Grenier, J.-C., et al. (2018). BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell *172*, 176-190.e19.

Keating, S.T., Groh, L., van der Heijden, C.D.C.C., Rodriguez, H., Dos Santos, J.C., Fanucchi, S., Okabe, J., Kaipananickal, H., van Puffelen, J.H., Helder, L. et al. (2020). The Set7 Lysine Methyltransferase Regulates Plasticity in Oxidative Phosphorylation Necessary for Trained Immunity Induced by β-Glucan. *Cell Rep. 31*, 107548.

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L.A.B., Ifrim, D.C., Saeed, S., Jacobs, C., van Loenhout, J., de Jong, D., Stunnenberg, H.G., et al. (2012). Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc. Natl. Acad. Sci. *109*, 17537–17542.

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L.A.B., Jacobs, C., Xavier, R.J., van der Meer, J.W.M., van Crevel, R., and Netea, M.G. (2014). BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clin. Immunol. *155*, 213–219.

Klemm, S.L., Shipony, Z., and Greenleaf, W.J. (2019). Chromatin accessibility and the regulatory epigenome. Nat. Rev. Genet. *20*, 207–220.

Lai, F., Orom, U. A., Cesaroni, M., Beringer, M., Taatjes, D. J., Blobel, G. A., et al. (2013). Activating RNAs associate with mediator to enhance chromatin architecture and transcription. Nature 494, 497–501.

Larsson, A.J.M., Johnsson, P., Hagemann-Jensen, M., Hartmanis, L., Faridani, O.R.,

Reinius, B., Segerstolpe, Å., Rivera, C.M., Ren, B., and Sandberg, R. (2019). Genomic encoding of transcriptional burst kinetics. Nature *565*, 251–254.

Li, X., and Fu, X.-D. (2019). Chromatin-associated RNAs as facilitators of functional genomic interactions. *Nat Rev Genet*. 2019 20(9): 503-519.

Li, Y., Oosting, M., Deelen, P., Ricaño-Ponce, I., Smeekens, S., Jaeger, M., Matzaraki, V., Swertz, M.A., Xavier, R.J., Franke, L., et al. (2016). Inter-individual variability and genetic influences on cytokine responses to bacteria and fungi. Nat. Med. *22*, 952–960.

Liu, T., Zhang, L., Joo, D., and Sun, S.C. (2017). NF-κB signaling in inflammation. Signal Transduct. Target. Ther. *2*, 17023.

Magagula, L., Gagliardi, M., Naidoo, J., and Mhlanga, M. (2017). Lnc-ing inflammation to disease. Biochem. Soc. Trans. *45*, 953–962.

Medzhitov, R., and Janeway, C. (2000). Innate immune recognition: mechanisms and pathways. Immunol. Rev. *173*, 89–97.

Mentch, S.J., Mehrmohamadi, M., Huang, L., Liu, X., Gupta, D., Mattocks, D., Gómez Padilla, P., Ables, G., Bamman, M.M., Thalacker-Mercer, A.E., et al. (2015). Histone Methylation Dynamics and Gene Regulation Occur through the Sensing of One-Carbon Metabolism. Cell Metab. *22*, 861–873.

Mills, K.H.G. (2011). TLR-dependent T cell activation in autoimmunity. Nat. Rev. Immunol. *11*, 807–822.

Mitroulis, I., Ruppova, K., Wang, B., Chen, L.-S., Grzybek, M., Grinenko, T., Eugster, A., Troullinaki, M., Palladini, A., Kourtzelis, I., et al. (2018). Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. Cell *172*, 147-161.e12.

Naik, S., Larsen, S.B., Gomez, N.C., Alaverdyan, K., Sendoel, A., Yuan, S., Polak, L.,

Kulukian, A., Chai, S., and Fuchs, E. (2017). Inflammatory memory sensitizes skin epithelial stem cells to tissue damage. Nature *550*, 475–480.

Netea, M.G., Domínguez-Andrés, J., Barreiro, L.B., Chavakis, T., Divangahi, M., Fuchs, E., Joosten, L.A.B., van der Meer, J.W.M., Mhlanga, M.M., Mulder, W.J.M., et al. (2020). Defining trained immunity and its role in health and disease. Nat. Rev. Immunol. *20*, 375-388.

Netea, M. G., Giamarellos-Bourboulis, E. J., Domínguez-Andrés, J., Curtis, N., van Crevel, R., van de Veerdonk, F. L., & Bonten, M. (2020). Trained Immunity: a Tool for Reducing Susceptibility to and the Severity of SARS-CoV-2 Infection. *Cell*, *181*(5), 969–977.

Novakovic, B., Habibi, E., Wang, S.-Y., Arts, R.J.W., Davar, R., Megchelenbrink, W., Kim, B., Kuznetsova, T., Kox, M., Zwaag, J., et al. (2016). β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. Cell *167*, 1354-1368.e14.

O'Leary, J.G., Goodarzi, M., Drayton, D.L., and von Andrian, U.H. (2006). T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat. Immunol. *7*, 507–516.

O'Neill, L., & Netea, M. G. (2020). BCG-induced trained immunity: can it offer protection against COVID-19?. *Nature reviews. Immunology*, *20*(6), 335–337.

Ordovas-Montanes, J., Dwyer, D.F., Nyquist, S.K., Buchheit, K.M., Vukovic, M., Deb, C., Wadsworth, M.H., Hughes, T.K., Kazer, S.W., Yoshimoto, E., et al. (2018). Allergic inflammatory memory in human respiratory epithelial progenitor cells. Nature *560*, 649–654.

Quinodoz, S., and Guttman, M. (2014). Long noncoding RNAs: an emerging link between gene regulation and nuclear organization. Trends Cell Biol. *24*, 651–663.

Quintin, J., Saeed, S., Martens, J.H.A., Giamarellos-Bourboulis, E.J., Ifrim, D.C., Logie, C., Jacobs, L., Jansen, T., Kullberg, B.-J., Wijmenga, C., et al. (2012). Candida albicans Infection Affords Protection against Reinfection via Functional Reprogramming of Monocytes. Cell Host Microbe *12*, 223–232.

Ratter, J.M., Rooijackers, H.M.M., Hooiveld, G.J., Hijmans, A.G.M., de Galan, B.E., Tack, C.J., and Stienstra, R. (2018). In vitro and in vivo Effects of Lactate on Metabolism and Cytokine Production of Human Primary PBMCs and Monocytes. Front. Immunol. *9*, 2564.

Reeves, R.K., Li, H., Jost, S., Blass, E., Li, H., Schafer, J.L., Varner, V., Manickam, C., Eslamizar, L., Altfeld, M., et al. (2015). Antigen-specific NK cell memory in rhesus macaques. Nat. Immunol. *16*, 927–932.

Saeed, S., Quintin, J., Kerstens, H.H.D., Rao, N.A., Aghajanirefah, A., Matarese, F., Cheng, S.-C., Ratter, J., Berentsen, K., van der Ent, M.A., et al. (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science (80-.). *345*, 1251086–1251086.

Lo Sasso, G., Menzies, K.J., Mottis, A., Piersigilli, A., Perino, A., Yamamoto, H., Schoonjans, K., and Auwerx, J. (2014). SIRT2 deficiency modulates macrophage polarization and susceptibility to experimental colitis. PLoS One *9*, e103573.

Schulthess, J., Pandey, S., Capitani, M., Rue-Albrecht, K.C., Arnold, I., Franchini, F., Chomka, A., Ilott, N.E., Johnston, D.G.W., Pires, E., et al. (2019). The Short Chain Fatty Acid Butyrate Imprints an Antimicrobial Program in Macrophages. Immunity *50*, 432-445.e7.

Shao, W., and Zeitlinger, J. (2017). Paused RNA polymerase II inhibits new transcriptional initiation. Nat. Genet. *49*, 1045–1051.

Shen, H., Campanello, G.C., Flicker, D., Grabarek, Z., Hu, J., Luo, C., Banerjee, R., and Mootha, V.K. (2017). The Human Knockout Gene CLYBL Connects Itaconate to Vitamin B12. Cell *171*, 771-782.e11.

Sigova AA, Abraham BJ, Ji X, Molinie B, Hannett NM, Guo YE, Jangi M, Giallourakis CC, Sharp PA, Young RA. Transcription factor trapping by RNA in gene regulatory elements. Science. 2015 Nov 20;350(6263):978-81.

Sowter, H.M., Raval, R.R., Moore, J.W., Ratcliffe, P.J., and Harris, A.L. (2003). Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. Cancer Res. *63*, 6130–6134.

Spurlock, C.F. 3rd, Tossberg, J.T., Guo, Y., Collier, S.P., Crooke, P.S. 3rd, Aune, T.M. (2015). Expression and functions of long noncoding RNAs during human T helper cell differentiation. Nat Commun. 23;6:6932.

Sun, J.C., Beilke, J.N., and Lanier, L.L. (2009). Adaptive immune features of natural killer cells. Nature *457*, 557–561.

Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., et al. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science (80-.). *324*, 930–935.

Takeuchi, O., Akira, S., Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K.A., Hornung, V., Akira, S., Uematsu, S., et al. (2010). Pattern recognition receptors and inflammation. Cell *140*, 805–820.

Tantale, K., Mueller, F., Kozulic-Pirher, A., Lesne, A., Victor, J.M., Robert, M.C., Capozi, S., Chouaib, R., Bäcker, V., Mateos-Langerak, J., et al. (2016). A

single-molecule view of transcription reveals convoys of RNA polymerases and multi-scale bursting. Nat. Commun. *7*, 12248.

Wang, K. C., Yang, Y. W., Liu, B., Sanyal, A., Corces-Zimmerman, R., Chen, Y., et al. (2011). A long non-coding RNA maintains active chromatin to coordinate homeotic gene expression. Nature 472, 120–124.

Weizman, O.-E., Song, E., Adams, N.M., Hildreth, A.D., Riggan, L., Krishna, C., Aguilar, O.A., Leslie, C.S., Carlyle, J.R., Sun, J.C., et al. (2019). Mouse cytomegalovirus-experienced ILC1s acquire a memory response dependent on the viral glycoprotein m12. Nat. Immunol. *20*, 1004–1011.

Wellen, K.E., Hatzivassiliou, G., Sachdeva, U.M., Bui, T. V., Cross, J.R., and Thompson, C.B. (2009). ATP-Citrate Lyase Links Cellular Metabolism to Histone Acetylation. Science (80-.). *324*, 1076–1080.

Xiao, M., Yang, H., Xu, W., Ma, S., Lin, H., Zhu, H., Liu, L., Liu, Y., Yang, C., Xu, Y., et al. (2012). Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes Dev. *26*, 1326–1338.

Yeung, F., Hoberg, J.E., Ramsey, C.S., Keller, M.D., Jones, D.R., Frye, R.A., and Mayo, M.W. (2004). Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. *23*, 2369–2380.

Zhang, D., Tang, Z., Huang, H., Zhou, G., Cui, C., Weng, Y., Liu, W., Kim, S., Lee, S., Perez-Neut, M., et al. (2019). Metabolic regulation of gene expression by histone lactylation. Nature *574*, 575–580.

Zhong, L., D'Urso, A., Toiber, D., Sebastian, C., Henry, R.E., Vadysirisack, D.D., Guimaraes, A., Marinelli, B., Wikstrom, J.D., Nir, T., et al. (2010). The histone

deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. Cell *140*, 280–293.