Integration of transcriptional and metabolic control in macrophage activation

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Abstract

Macrophages react to microbial and endogenous danger signals by activating a broad panel of effector and homeostatic responses. Such responses entail rapid and stimulus-specific changes in gene expression programs accompanied by extensive rewiring of metabolism, with alterations in chromatin modifications providing one layer of integration of transcriptional and metabolic regulation. A systematic and mechanistic understanding of the mutual influences between signal-induced metabolic changes and gene expression is still lacking. Here, we discuss current evidence, controversies, knowledge gaps, and future areas of investigation on how metabolic and transcriptional changes are dynamically integrated during macrophage activation. The cross-talk between metabolism and inflammatory gene expression is in part accounted for by alterations in the production, usage, and availability of metabolic intermediates that impact the macrophage epigenome. In addition, stimulus-inducible gene expression changes alter the production of inflammatory mediators, such as nitric oxide, that in turn modulate the activity of metabolic enzymes thus determining complex regulatory loops. Critical issues remain to be understood, notably whether and how metabolic rewiring can bring about gene-specific (as opposed to global) expression changes.

See the Glossary for abbreviations used in this article.

Introduction

The different functions exerted by macrophages are regulated and tuned by the signals they are exposed to in different tissues, during homeostasis or in response to acute or chronic damage. In the past few years, the availability of a large panel of unbiased, high-throughput data, including transcriptomic and epigenomic datasets, has greatly increased our understanding of the mechanisms by which tissue-microenvironmental signals determine distinct transcriptional programs in macrophages already early in organ development (Gautier *et al*, 2012; Yona *et al*, 2013; Gosselin *et al*, 2014; Lavin *et al*, 2014; Mass *et al*, 2016; Bonnardel *et al*, 2019; Sakai *et al*, 2019). Different macrophage gene expression programs are enabled by signal-regulated transcription factors (TFs) binding to thousands of *cis*-regulatory regions maintained accessible and functional by macrophage lineage-determining TFs such as PU.1 and IRF8 (Barish *et al*, 2010; Ghisletti *et al*, 2010; Heinz *et al*, 2010; Mancino *et al*, 2015). The complex interplay among TFs, coregulators, and chromatin modifications implicated in the control of specific macrophage functions and the overall principles regulating inducible gene expression have been extensively reviewed elsewhere (Heinz *et al*, 2015; Amit *et al*, 2016; Glass & Natoli, 2016; Monticelli & Natoli, 2017; Barrat *et al*, 2019; Stadhouders *et al*, 2019).

Concomitantly to the induction of gene expression changes, the detection of danger signals or other regulatory stimuli rapidly activates an extensive reprogramming of macrophage metabolism (Artyomov *et al*, 2016; Jung *et al*, 2019; O'Neill *et al*, 2016; O'Neill & Pearce, 2016; Russell *et al*, 2019; van Teijlingen & Pearce, 2020). How such metabolic reprogramming impacts macrophage phenotypes has only recently begun to emerge. As discussed below, it is now clear that metabolic rewiring not only aims at fulfilling energy requirements, but also supports the distinctive changes in macrophage functions determined by different stimuli.

Macrophage activation and polarization

Classical *in vitro* models for the elucidation of signal-dependent gene expression and metabolic reprogramming include treatment of mouse bone marrow- or human monocyte-derived macrophages with lipopolysaccharide (LPS) and/or interferon γ (IFNγ) to induce a "classically" activated (M1) phenotype or treatment with interleukin-4 (IL-4) to induce an "alternatively" activated (M2) phenotype (Murray *et al*, 2014). M1 macrophages express proinflammatory cytokines and chemokines and produce reactive oxygen species (ROS) and molecules with microbicidal activity such as

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Glossary

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ACLY	ATP citrate lyase
ACO2	Aconitase
αKT	alpha-ketoglutarate
AKT	Protein Kinase B
ALDOA	Fructose-bisphosphate aldolase A
AP-1	Activator protein 1
ARG1	Arginase 1
ATM	Adipose tissue macrophage
CBP	CREB-binding protein
CD36	Cluster of differentiation 36
CD206	Cluster of differentiation 206
COBRA	Constraint-based reconstruction and analysis
CXCL1	Chemokine (C-X-C motif) ligand 1
DHAP	Dihydroxyacetone phosphate
DIC	Mitochondrial dicarboxylate carrier
eIF5A	Eukaryotic translation initiation factor 5A
ETC	Electron transport chain
FAO	Fatty acid oxidation
G3P	Glycerol 3-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN5	General control non-repressed 5
GPD1	Glycerol-3-phosphate dehydrogenase 1
GPD2	Glycerol-3-phosphate dehydrogenase 2
GPS	Glycerol phosphate shuttle
H3K27ac	Histone H3 acetylation at lysine 27
H3K18la	Histone H3 lactylation at lysine 18
H3K27me3	Histone 3 tri-methylation at Lysine 27
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HIF1α	Hypoxia-inducible factor 1 alpha
IDH	Isocitrate dehydrogenase
IFN	Interferon
IL-4	Interleukin 4
IL12β	Interleukin 12 beta
IL1β	Interleukin 1 beta
IL6	Interleukin 6
iNOS	inducible nitric oxide synthase
IRF	Interferon regulatory factor (1-9)
IRG1	Immune responsive gene 1
JMJD3	Jumonji domain-containing protein D3
Km	Michaelis–Menten constant
LAL	Lysosomal acid lipase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MGL	Macrophage galactose C-type Lectin
MPC	Mitochondrial pyruvate carrier
mTORC	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
OGDC	Oxoglutarate/α-ketoglutarate dehydrogenase complex
oxLDL	Oxidized low-density lipoproteins
oxPAPC	Oxidized phospholipids
OXPHOS	Oxidative phosphorylation
P/CAF	P300/CBP-associated factor
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PFK1	Phosphofructokinase 1
PFK2	6-phosphofructo-2-kinase
PGC1β	Peroxisome proliferator-activated receptor gamma (PPAR-gamma) coactivator-1 beta
PGE2	Prostaglandin E2
PPARγ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SAM	S-adenosyl-methionine
SLC	Solute carrier family protein
SSP	Serine-synthesis pathway
STAT	Signal transducer and activator of transcription
SUCNR1	Succinate receptor 1
TCA	Tricarboxylic acid cycle
TET	Ten-eleven translocation
TNFα	Tumor necrosis factor alpha

nitric oxide (NO), while M2 macrophages activate the transcription of genes involved in wound healing and defense from parasitic and fungal infections. M1- and M2-polarized states are an oversimplification of more complex biological responses (Mantovani, 2016) whereby combinations of stimuli transiently and usually reversibly instruct macrophages to acquire a variety of functional states (Murray *et al*, 2014). However, for the sake of simplicity, the M1/M2 nomenclature is still used to describe functionally opposite macrophage states.

Mechanistically, similar but also clearly distinct M1 phenotypes can be triggered by IFNγ or LPS. In the first case, the response is mainly driven by STAT1 activation and a few partner TFs such as IRF1/2 (Interferon Regulatory Factors1/2), while in the case of LPS it is driven by multiple signal-induced TFs, such as NF-kB, AP-1, and IRFs that activate the IFNβ1 gene, followed by IFNβ release and a secondary response driven by IFNβ itself and by other inflammatory cytokines released upon activation, acting in a paracrine and/or autocrine manner. Although most changes in epigenomic features and TF binding induced by LPS or IFNγ occur at pre-accessible regulatory regions, initially inactive genomic loci can acquire competence to activate transcription upon stimulation, so-called "latent" or "de novo" enhancers (Kaikkonen *et al*, 2013; Ostuni *et al*, 2013). Therefore, the usage of the genomic regulatory elements responsible for macrophage activation is flexibly adjusted in response to stimulation.

Macrophage activation and metabolic reprogramming

Upregulation of glucose utilization and decrease in oxygen consumption are hallmarks of classically activated macrophages (O'Neill & Pearce, 2016). The metabolic state of an activated macrophage after *ca*. 24 h LPS treatment is characterized by impaired mitochondrial oxidative phosphorylation (OXPHOS) coupled with increased aerobic glycolysis (Fig 1) (Rodríguez-Prados *et al*, 2010; Freemerman *et al*, 2014). In this setting, pyruvate produced as an end product of glycolysis is reduced to lactate instead of being oxidized to acetyl-CoA by the pyruvate dehydrogenase complex (PDC) to be eventually fed into the tricarboxylic acid (TCA) cycle in mitochondria (Fig 1). An important player in this metabolic rewiring is nitric oxide (NO), whose increased production is ascribed to the transcriptional activation of the inducible nitric oxide synthase (encoded by the *Nos2* gene). NO and NO-derived reactive nitrogen species can inactivate the iron-sulfur-containing complexes of the mitochondrial electron transport chain, thus eventually promoting mitochondrial dysfunction (Fig 1) (Chouchani *et al*, 2013; Clementi *et al*, 1998; Van den Bossche *et al*, 2016). The increase in glycolytic metabolism is thus first of all an adaptive response aimed at maintaining a sufficient level of ATP
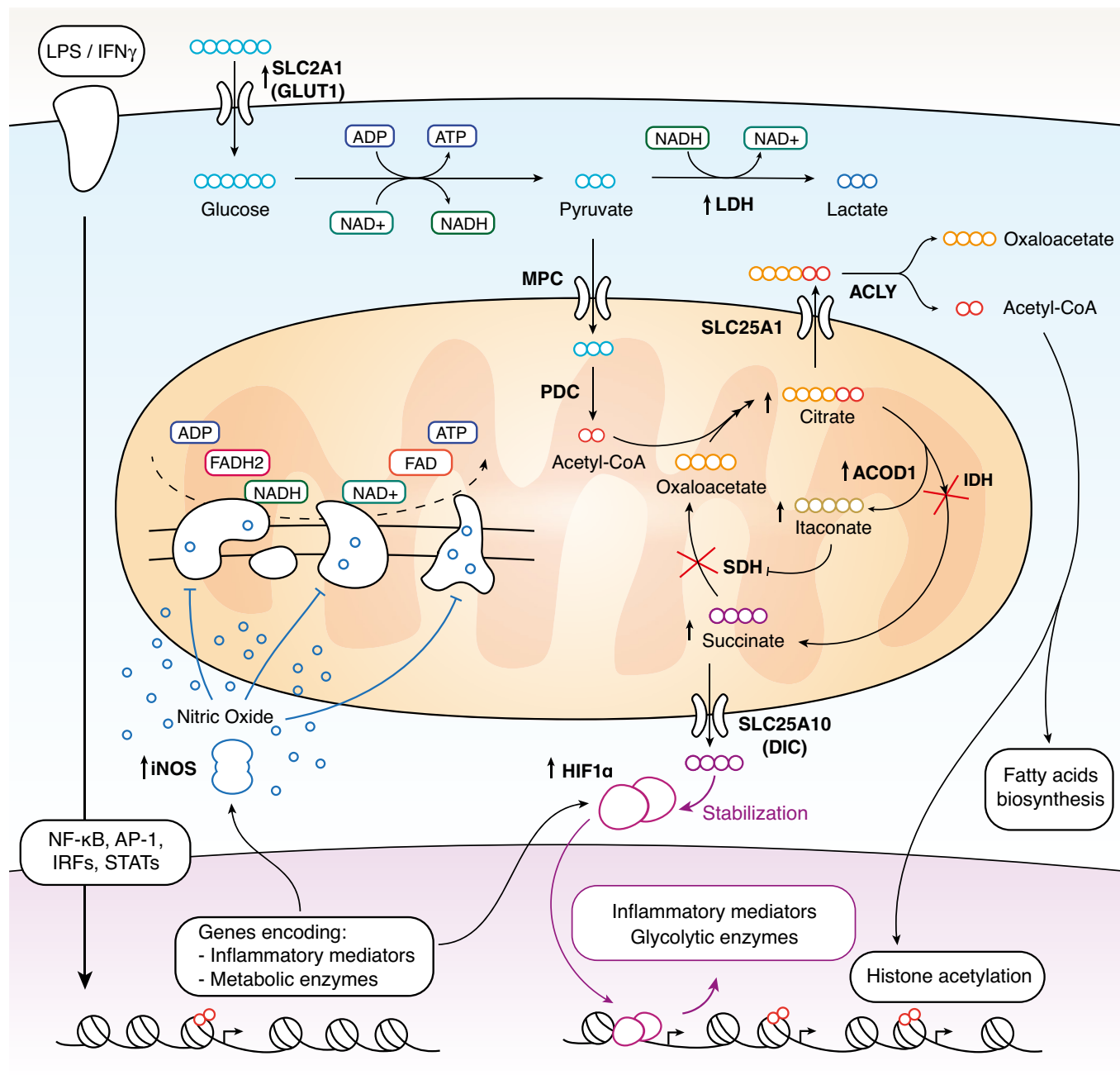
Figure 1. An overview of metabolic and transcriptional reprogramming in LPS-activated macrophages.

Macrophages respond to proinflammatory stimuli by extensively reprogramming their transcriptional and metabolic activity. The glycolytic flux is enhanced, and oxygen consumption is reduced, while several pathways, such as the TCA cycle, are altered and specific metabolites accumulate over time. Both glucose uptake and metabolism are rapidly upregulated, and the pyruvate produced is converted into lactate. The induction of the *Nos2* gene leads to a massive burst of NO production. NO acts as an antimicrobial molecule and impairs the activity of the electron transport chain (ETC), eventually reducing cellular oxygen consumption. The TCA cycle is remodeled because of the impairment of IDH activity, leading to the accumulation of citrate which is exported to the cytoplasm via the mitochondrial citrate carrier (SLC25A1). Cleavage of cytoplasmic citrate by ACLY equilibrates the mitochondrial and the cytosolic acetyl-CoA pool. ACOD1 induction leads to aconitate being diverted to itaconate, which functions as an immunomodulatory molecule and also affects succinate dehydrogenase (SDH), blocking its activity and thus promoting succinate accumulation. Among key transcription factors produced, HIF1α is stabilized by the accumulation of succinate and it is able to induce several key metabolic genes. Together with key transcription factors, the second wave of inflammatory mediators results from the activity of histone acetyltransferases, which rely on the acetyl-CoA produced by the cell to support activation of gene expression.

production in cells with impaired mitochondrial respiration (Everts *et al*, 2012). In parallel, the response to LPS or IFNγ results in three main alterations in the TCA cycle that can be summarized as follows.

First, downregulation of the activity of isocitrate dehydrogenases, which convert isocitrate to α-ketoglutarate, leads to the accumulation of citrate (Fig 1) (Tannahill *et al*, 2013; Jha *et al*, 2015).

Accumulation of citrate inside mitochondria favors its export to the cytoplasm by the mitochondrial citrate transporter (encoded by *SLC25A1*) (Infantino *et al*, 2013). In the cytoplasm, citrate undergoes cleavage by the ATP citrate lyase (ACLY) leading to the production of oxaloacetate and acetyl-coenzyme A (acetyl-CoA), a central metabolic intermediate for the synthesis of fatty acids and prostaglandins (Fig 1). In addition, a most prominent signaling function of acetyl-CoA is to provide the acetyl groups required for acetylation of histones (Lauterbach *et al*, 2019), which undergo hyperacetylation at thousands of *cis*-regulatory elements activated in response to the recruitment of TFs induced by macrophage stimulation (Ghisletti *et al*, 2010; Heinz *et al*, 2010; Kaikkonen *et al*, 2013; Ostuni *et al*, 2013).

Second, the strong LPS-mediated induction of the *ACOD1* gene (previously known as *IRG1*, Immune Responsive Gene 1) encoding aconitate decarboxylase 1 (Michelucci *et al*, 2013) allows the synthesis of the metabolite itaconate (Strelko *et al*, 2011), from the TCA cycle intermediate *cis*-aconitate (Fig 1). Itaconate, a cysteine-reactive electrophile (Bambouskova *et al*, 2018; Mills *et al*, 2018), starts accumulating within 4 h after in vitro LPS stimulation of macrophages, reaches concentrations in the low millimolar range at approx. 24 h (Lauterbach *et al*, 2019; Seim *et al*, 2019), and exerts complex anti-bacterial (McFadden & Purohit, 1977; Michelucci *et al*, 2013) as well as various immunoregulatory effects (Hooftman *et al*, 2020; Swain *et al*, 2020) that are outside of our main focus and have been extensively covered in recent review articles (Hooftman & O'Neill, 2019; O'Neill & Artyomov, 2019; Zaslona & O'Neill, 2020; Cordes & Metallo, 2021).

Accumulation of itaconate also causes extensive metabolic reprogramming at least at two distinct levels: It impairs glycolysis mainly by modifying and inhibiting ALDOA (fructose-bisphosphate aldolase A) (Qin *et al*, 2019) and attenuates fluxes in the TCA cycle by inhibiting the succinate dehydrogenase (SDH), eventually resulting in succinate accumulation (Fig 1) (Jha *et al*, 2015; Cordes *et al*, 2016; Lampropoulou *et al*, 2016). Accumulation of succinate has two consequences. Extracellularly, released succinate triggers succinate receptor 1 (SUCNR1), eventually causing context-dependent anti-inflammatory effects (Peruzzotti-Jametti *et al*, 2018; Keiran *et al*, 2019; Harber *et al*, 2020; Wu *et al*, 2020). Intracellularly, the increased concentrations of succinate inhibit prolyl hydroxylases that generate succinate as reaction product and are thus subjected to product inhibition (Tannahill *et al*, 2013). These oxygen-regulated dioxygenases induce prolyl hydroxylation of hypoxia-inducible factor 1 alpha (HIF1α), targeting it to degradation in normoxic conditions; therefore, their inhibition by succinate increases HIF1α protein levels. In turn, the accumulation of HIF1α leads to transcriptional activation of the interleukin 1β (IL-1β) gene (Tannahill *et al*, 2013) and other HIF1α-dependent genes, including those encoding several glycolytic enzymes (Cheng *et al*, 2014). HIF1α accumulation and downstream changes in gene expression thus represent a paradigm of how a metabolic alteration can bring about gene-specific transcriptional effects. Notably, itaconate has been recently shown to covalently modify ("itaconation") cysteine residues of proteins (Qin *et al*, 2019; Qin *et al*, 2020). Given the abundance of this metabolite in M1-polarized macrophages, it will be crucial to understand whether itaconate can also directly modify histones and regulate the transcription of inflammatory genes.

Compared with inflammatory macrophages, alternatively activated (M2) macrophages show a milder metabolic reprogramming, with an intact TCA cycle and a prevalent use of fatty acid oxidation (FAO, also known as β-oxidation) to fuel mitochondrial oxidative phosphorylation (OXPHOS) (Fig 2A) (Jha *et al*, 2015). Mechanistically, IL-4 potently enhances the uptake of fatty acids and induces the transcription of oxidative metabolism genes by the direct activation of STAT6 and its partner PPARγ with its coactivator PGC1β (Vats *et al*, 2006; Odegaard *et al*, 2007; Nomura *et al*, 2016; Piccolo *et al*, 2017; Czimmerer *et al*, 2018; Nelson *et al*, 2018). The substrates for FAO are exogenous lipids taken up by CD36, a scavenger receptor transcriptionally regulated by PPARγ and with high affinity for long-chain fatty acids and triacylglycerols (Fig 2A) (Feng *et al*, 2000; Chawla *et al*, 2001). In turn, triacylglycerols must be metabolized by the lysosomal acid lipase (LAL), and the impairment of this lipolysis step decreases macrophage oxidative respiration and impairs M2 polarization (Huang *et al*, 2014). Essential for OXPHOS and FAO metabolism in M2 macrophages is the generation of α-ketoglutarate from glutamine, the most abundant extracellular amino acid (Liu *et al*, 2017). α-ketoglutarate is a co-substrate required for the activity of the histone demethylase Jmjd3, which removes the repressive histone mark H3K27me3 (De Santa *et al*, 2007), thus promoting the activation of M2 genes, as discussed below (Fig 2B) (Liu *et al*, 2017). Glutamine has also been reported to support M2 macrophage polarization through the glutamine-UDP-GlcNac pathway, which is fundamental for glycosylation of proteins relevant for M2 macrophage function, such as the macrophage mannose receptor and the macrophage galactose-binding lectin (Fig 2C; Jha *et al*, 2015).

The role of glycolysis in M2 macrophage functions is not completely clear yet. Macrophage stimulation with IL-4 leads to the activation of the mTORC2 pathway and the upregulation of glycolysis by IRF4, and the decrease in this pathway may inhibit M2 polarization and function (Fig 2A) (Huang *et al*, 2016). On the contrary, more recent data suggest that glycolysis is not required for M2 functions, as long as OXPHOS remains intact (Wang *et al*, 2018).

A hallmark of mouse M2 macrophages is the expression of the arginase 1 (*Arg1*) gene (Munder *et al*, 1998), which converts arginine to ornithine, resulting in several effects: (i) It provides the substrate for the synthesis of polyamines, which negatively impact pathogen fitness (Esser-von Bieren *et al*, 2013); (ii) it depletes arginine from the extracellular milieu, preventing deleterious consequences of sustained activation of Th2 lymphocytes (Pesce *et al*, 2009); and (iii) it promotes efferocytosis (engulfment of apoptotic cells) in a feed-forward loop in which arginine and ornithine taken up from engulfed apoptotic cells are converted into putrescine, which in turn enhances subsequent rounds of efferocytosis (Yurdagul *et al*, 2020). A complete understanding of how arginine metabolism impacts M2 macrophage activation is still lacking. However, arginine-derived spermidine was recently shown to be involved in the hypusination of the translation factor eIF5A, which controls the production of a subset of mitochondrial proteins involved in the TCA cycle and OXPHOS: Inactivation of this pathway selectively impairs OXPHOS-dependent alternative macrophage activation, hinting at a close link between IL-4-mediated *Arg1* gene induction and metabolic reprogramming in of M2 macrophages (Fig 2D) (Puleston *et al*, 2019).
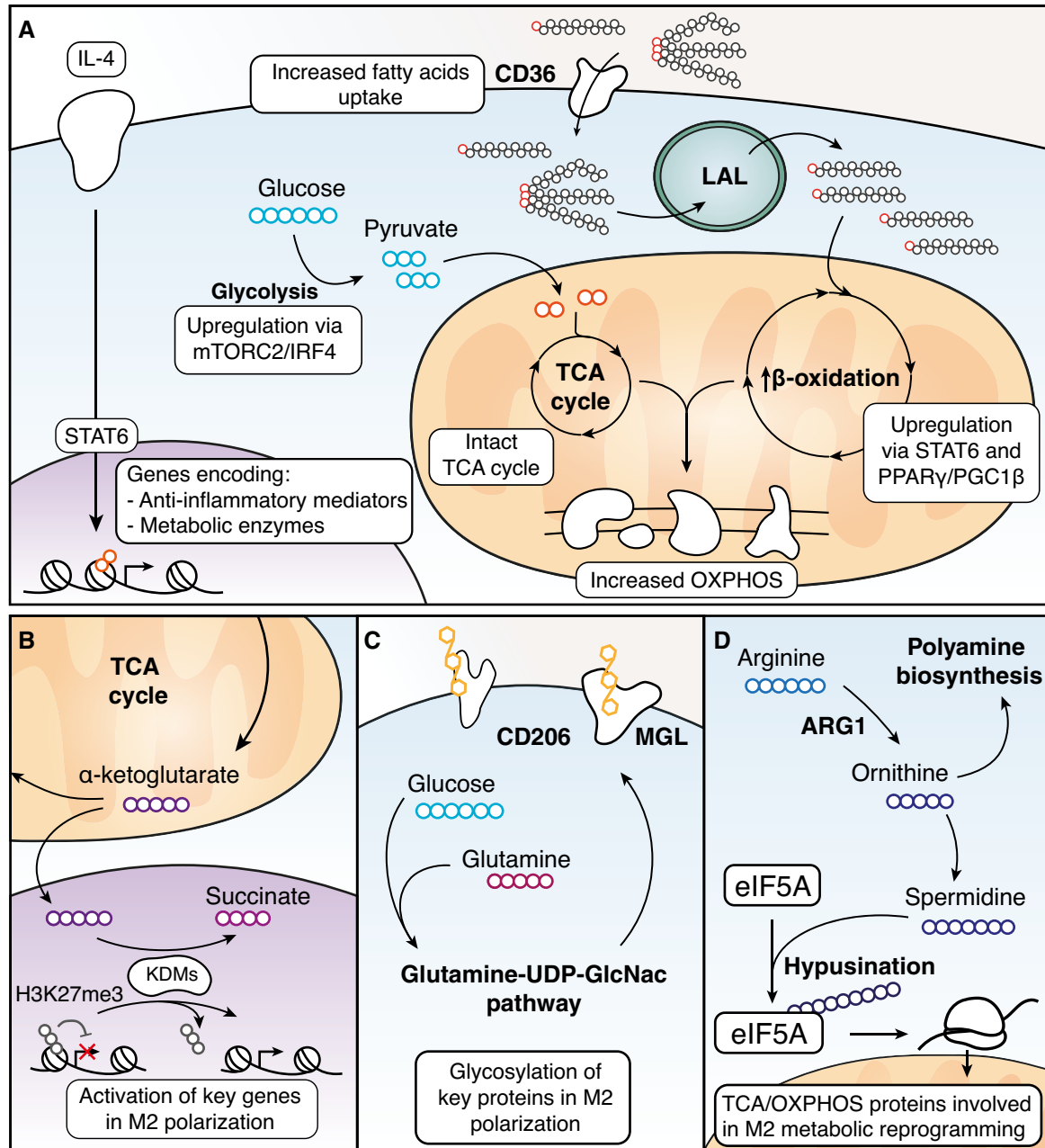
Figure 2. IL-4-induced changes in metabolism and their effects on gene expression.

(A) In response to IL-4 stimulation, glycolysis is increased; the TCA cycle remains intact and both fatty acid uptake and metabolism are enhanced via regulation of metabolic genes by STAT6 and PPARγ. (B) Alpha-ketoglutarate, an intermediate of the TCA cycle, acts as a co-substrate of lysine demethylases such as JMJD3 to remove the repressive H3K27me3 mark, eventually activating gene transcription. (C) Glutamine and glucose are employed *via* the Glutamine-UDP-GlcNac pathway to glycosylate proteins involved in M2 macrophage function such as CD206 and MGL. (D) Arginase 1, upregulated by IL-4, converts arginine to ornithine which is used in polyamine biosynthesis. Ornithine can be also further metabolized to spermidine which was found to be involved in the hypusination of translation factor eIF5A, which controls the production of key mitochondrial proteins involved in the TCA cycle and OXPHOS.

Of note, most of the findings on M1 and M2 macrophage activation and metabolic reprogramming reported so far come from studies using mouse macrophages. However, it is important to consider that substantial differences have been reported in the response of human and mouse macrophages to inflammatory stimuli *in vitro*. Particularly, M1 stimulation of mouse macrophages leads to *Nos2* gene expression and NO release, while human macrophages do not

seem to release NO *in vitro* (Schneemann *et al*, 1993). Moreover, human peripheral blood monocyte-derived macrophages (hMDMs) do not exhibit the switch to glycolysis and mitochondrial dysfunction that has been reported for mouse macrophages upon LPS treatment (Vijayan *et al*, 2019). Thus, activated hMDMs rely on OXPHOS rather than glycolysis for ATP production unlike mouse macrophages (Vijayan *et al*, 2019). Finally, human monocyte-derived

macrophages do not upregulate *ARG1* expression upon IL-4 treatment (Raes *et al*, 2005). However, there may exist inflammatory settings *in vivo* characterized by elevated *NOS2* and *ARG1* expression in human macrophages (Anstey *et al*, 1996; Nicholson *et al*, 1996; St Clair *et al*, 1996; Thomas & Mattila, 2014). In conclusion, it is crucial to be aware of substantial differences between mouse and human macrophages and to verify to what extent *in vitro* experimental models recapitulate *in vivo* phenomena.

Coordination of transcriptional and metabolic changes

As discussed above, on the one hand stimulus-dependent changes in transcriptional programs can result in differential expression of genes encoding components of metabolic pathways; on the other hand, metabolic reprogramming can bring about gene-specific transcriptional changes (such as those due to HIF1α accumulation) as well as changes in the abundance of intermediates such as acetyl-CoA that are required for chromatin modifications and may thus exert a pervasive impact on transcription. Availability of metabolites required for chromatin modifications appears to play a central role in the integration of metabolic information and transcriptional control. Specifically, the enzymatic activity of many chromatin modifiers is regulated, at least in part, by the concentration of substrates and cofactors, whose availability may change depending on the stimulus macrophages are exposed to. For example, metabolic intermediates serve as cofactors (e.g., NAD+) or substrates (e.g., acetyl-CoA) for numerous histone deacetylases (HDACs) and histone acetyl transferases (HATs), respectively (Houtkooper *et al*, 2012; Kinnaird *et al*, 2016). Moreover, the activity of lysine demethylases and cytosine-hydroxylases is influenced by levels of α-ketoglutarate (αKG) and succinate. αKG is a required cofactor for Jumonji-C (JmjC) domain-containing histone lysine demethylases (JMJDs) and ten-eleven translocation (TET) families of 5mC hydroxylases (Tsukada *et al*, 2006; Tahiliani *et al*, 2009) and is converted into succinate during catalysis. Generally, these enzymes are subjected to product-mediated inhibition by succinate, and therefore, their activity is dependent on the cofactor-to-product (αKG/succinate) ratio (Carey *et al*, 2015; Sciacovelli *et al*, 2016). For example, IL-4 stimulation promotes a high αKG/succinate ratio, substrate, and inhibitor, respectively, of histone H3K27me3 demethylase JMJD3 (De Santa *et al*, 2007) thus potentiating gene induction in response to this cytokine (Liu *et al*, 2017). More recently, type I IFN has been linked to epigenetic regulation in macrophages *via* control of amino acid metabolism. Type I IFN was shown to suppress the serine-synthesis pathway (SSP), thus negatively impacting on the production of S-adenosyl-methionine (SAM), the only substrate for histone methylation, and eventually deposition of H3K27me3 on gene promoters (Shen *et al*, 2021).

The link between metabolites and chromatin modifiers, and more in general the effects of metabolic reprogramming on macrophage function, have been extensively reviewed (O'Neill *et al*, 2016; Phan *et al*, 2017; Williams & O'Neill, 2018; Jung *et al*, 2019). In this review, we will instead focus on recent studies providing novel aspects of the interplay between metabolism and transcription, and we will highlight the most relevant knowledge gaps in the field. We will first discuss novel data on glucose metabolism changes over the course of the inflammatory response and their impact on inflammatory gene transcription. We will then explore new roles of the inflammatory mediator nitric oxide (NO) in the regulation of the

inflammatory response through the modulation of metabolite production, and we will speculate on how this may influence epigenomic modifications and transcriptional programs. In the last part, we will examine selected examples of the influence of tissue environment on macrophage metabolic and transcriptional reprogramming, showing how its dysregulation can lead to disease states characterized by chronic inflammation. Finally, we will provide a long-term perspective on this field of investigation.

Glucose metabolism as driver of inflammatory gene expression

Glucose utilization is crucial for classical macrophage activation, and its inhibition affects many functions typical of their inflammatory phenotype, including phagocytosis, ROS production, and secretion of proinflammatory cytokines. Upgrade of glycolysis in LPS-activated macrophages occurs in sequential phases involving multiple mechanisms. First, a rapid increase in glucose uptake that reaches a plateau already 20 min after LPS stimulation (Fukuzumi *et al*, 1996) was reported to be mediated by the early induction of SLC2A1 (GLUT1), the main glucose transporter in myeloid cells (Fig 1) (Freemerman *et al*, 2014). Although the transcriptional regulation of *SLC2A1* has historically been linked to HIF1α (Ebert *et al*, 1995), the rapid kinetics of *SLC2A1* induction by LPS is not compatible with a role for HIF1α and imposes that alternative mechanisms are considered.

Second, transcriptional induction of *PFK2*, encoding the 6-phosphofructo-2-kinase, determines the accumulation of fructose 2,6-bisphosphate, an allosteric activator of the rate-limiting glycolytic enzyme PFK1 (phosphofructokinase 1), eventually leading to increased glycolytic fluxes (Rodríguez-Prados *et al*, 2010). Third, HIF1α was shown to play a critical role in inflammatory macrophage activation *in vivo* (Cramer *et al*, 2003), an effect that also involves regulation of glycolytic metabolism.

HIF1α accumulation in LPS-activated macrophages is a two-step process involving first its transcriptional induction by NF-kB (Rius *et al*, 2008), and then the stabilization of the HIF1α protein *via* succinate-mediated inactivation of prolyl hydroxylases (Fig 1) (Tannahill *et al*, 2013). The essential role of HIF1α in inflammatory macrophage activation may in part be linked to the control of the expression of multiple glycolytic enzymes (Cheng *et al*, 2014) and in part to the control of IL-1β expression (Tannahill *et al*, 2013) (Fig 1). However, the slow kinetics of HIF1α accumulation implies that early reprogramming of energy metabolism in LPS-activated macrophages must be HIF1α-independent, as already reported in the specific case of PFK2 induction (Rodríguez-Prados *et al*, 2010). HIF1α also induces the expression of the lactate dehydrogenase (LDH), which produces lactate from pyruvate, and of the pyruvate dehydrogenase kinase (PDK), which inactivates the pyruvate dehydrogenase thus limiting pyruvate conversion to acetyl-CoA (Fig 1) (Semenza *et al*, 1996; Kim *et al*, 2006; Palsson-McDermott *et al*, 2015). Enhanced LDH activity appears to have a critical role in this context: Since in classically activated macrophages OXPHOS is progressively impaired, maintenance of the flux through the glycolytic pathway is critical and this involves the regeneration of NAD+ from NADH in the LDH-mediated conversion of pyruvate into lactate (Fig 1).

Glucose oxidation controls transcriptional regulation of inflammatory mediators

It has been recently reported that oxidative metabolism of macrophages is increased within the first hour and until eight hours post-LPS stimulation, in contrast to the reduced oxygen consumption occurring at later time points (Langston *et al*, 2019; Lauterbach *et al*, 2019). This boost in oxidative metabolism in the early phases of LPS stimulation depends on increased glycolysis fueling the TCA cycle and is regulated by the mitochondrial glycerol 3-phosphate dehydrogenase (GPD2) (Fig 3A). GPD2 is a rate-limiting enzyme in the glycerol phosphate shuttle (GPS), which includes the glycolytic enzyme GAPDH and GPD1, located on the cytoplasmic side of the mitochondrial membrane. In the GPS, the NADH produced by GAPDH during glycolysis is used by GPD1 to reduce dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G3P), thus regenerating NAD+ required for glycolysis. Inside mitochondria, electrons from G3P are directly transferred to the electron transport chain (ETC) through the FAD cofactor of GPD2 (Fig 3A). Upon LPS-induced activation, the expression of GDP2 and the activity of the GPS are increased, resulting in both an increased NAD+ regeneration, which maximizes glycolysis, and an enhanced flux of electrons in the ETC (Langston *et al*, 2019). The relevance of this enzyme for macrophage activation is underscored by the observation that loss of GPD2 impairs the secretion of critical inflammatory cytokines upon LPS activation (Langston *et al*, 2019).

Interestingly, a GPD2-dependent metabolic shift from increased (within the first 3 h of LPS-induced macrophage activation) to decreased oxidative metabolism occurs over the course of 24 h of LPS exposure (Langston *et al*, 2019). This process appears to be relevant for LPS tolerance, the process whereby macrophages exposed to LPS for an extended time are subsequently unable to mount a full transcriptional response upon restimulation (Foster *et al*, 2007). A variety of mechanisms has been shown to contribute to the LPS-tolerant state of macrophages, including receptor desensitization, secondary activation of negative signaling molecules, and induction of anti-inflammatory microRNAs (Medvedev *et al*, 2000; Mages *et al*, 2008; Nahid *et al*, 2009; Doxaki *et al*, 2015; Seeley *et al*, 2018). In this context, the lack of re-induction of tolerized genes correlates with defective deposition of histone marks associated with active transcription, chromatin accessibility, and nucleosome remodeling at promoters and enhancers of tolerized genes (Foster *et al*, 2007; Chen & Ivashkiv, 2010; Saeed *et al*, 2014). Emerging evidence indicates that an additional mechanism involves the metabolic regulation of acetyl-CoA production by GPD2 (Langston *et al*, 2019). Upon prolonged exposure to LPS, GPD2-dependent shutdown of oxidative metabolism limits the ability of glucose oxidation to support acetyl-CoA production, thus contributing to the inability to induce specific proinflammatory mediators. However, whether and how GPD2-mediated glucose oxidation and acetyl-CoA production can modulate histone acetylation and the expression of a specific subset of LPS-inducible genes as opposed to a global reduction in transcriptional activity remains unclear.

Links between glucose metabolism and histone acetylation

Histone acetyl transferases (HATs) catalyze the addition of acetyl groups to the histone N-terminal lysines using acetyl-CoA as donor (Choudhary *et al*, 2009). There are two separate pools of cellular acetyl-CoA: the mitochondrial pool, generated by pyruvate oxidization to acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex (PDC), and the cytosolic pool. Since the inner mitochondrial membrane does not contain an acetyl-CoA transporter, acetyl units are transported into the cytosol as part of a larger molecule, citrate. Briefly, immediately after LPS stimulation, increased glycolysis leads to augmented production of pyruvate that is fed into the Krebs' cycle by the PDC, leading to increased citrate synthesis (Lauterbach *et al*, 2019). Citrate is then exported from the mitochondria to the cytosol by the citrate-malate transporter, SLC25A1. Subsequently, the ATP citrate lyase (ACLY) cleaves citrate into oxaloacetate and acetyl-CoA which then reaches an equilibrium in the cytoplasm and the nucleus. Cellular acetyl-CoA fluctuates in response to a number of factors and changes in cytoplasmic acetyl-CoA levels produced by ACLY-mediated cleavage of citrate are sufficient to regulate global HAT activity (Wellen *et al*, 2009).

In various systems, high levels of glucose have been shown to increase the cellular amount of acetyl-CoA, eventually affecting global histone acetylation (Lee *et al*, 2014; Moussaieff *et al*, 2015; Sivanand *et al*, 2017). ACLY appears to be an important bridge linking glucose metabolism to macrophage inflammatory gene expression programs (Langston *et al*, 2019; Lauterbach *et al*, 2019). These recent studies show that treatment of primary macrophages with LPS for 0.5-2h results in AKT-dependent activation of ACLY (Fig 3B) (Langston *et al*, 2019; Lauterbach *et al*, 2019). Blocking ACLY activity with structurally distinct inhibitors alters the transcription of specific subsets of inflammatory genes *in vitro* as well as in an *in vivo* model of peritonitis (Lauterbach *et al*, 2019). Along the same line, upregulation of ACLY by LPS and the consequent increase in cytosolic acetyl-CoA levels were previously shown to be required for the transcriptional upregulation of proinflammatory mediators, such as nitric oxide (NO), ROS, and prostaglandin E2 (PGE2) (Infantino *et al*, 2013). Mitochondrial export of citrate and ACLY activity are also induced in an AKT-dependent manner in response to IL-4 treatment of macrophages, and they contribute to the activation of signature M2 genes (Covarrubias *et al*, 2016).

A key question is how fluctuations in acetyl-CoA levels mediated by ACLY trigger a gene-specific transcriptional response rather than global effects on chromatin acetylation and accessibility. ACLY inhibition seems to preferentially impact "late" genes, whose promoter undergoes extensive *de novo* acetylation in response to stimulation (Hargreaves *et al*, 2009; Ramirez-Carrozzi *et al*, 2009). This observation has been interpreted as evidence that constitutively acetylated early genes may be less dependent on this pathway. Indeed, the genes regulated by ACLY, such as *IL6* and *IL12b*, are secondary response genes, namely genes induced in a protein synthesis-dependent manner, while primary response genes, such as *CXCL1*, remain unaffected (Lauterbach *et al*, 2019). However, *IL1b*, an early response gene, in one case was found to be ACLY dependent (Langston *et al*, 2019), while in another report it did not change in response to ACLY inhibition (Lauterbach *et al*, 2019). Moreover, *TNF*, an early gene, was also found to be regulated by ACLY inhibition (Lauterbach *et al*, 2019). Clearly, whereas activation of early LPS-inducible genes is comparatively less demanding than that of late genes, as they are constitutively acetylated and bound by RNA Polymerase II, the mere induction kinetics and the basal chromatin status at a gene locus do not suffice to explain the gene-selective
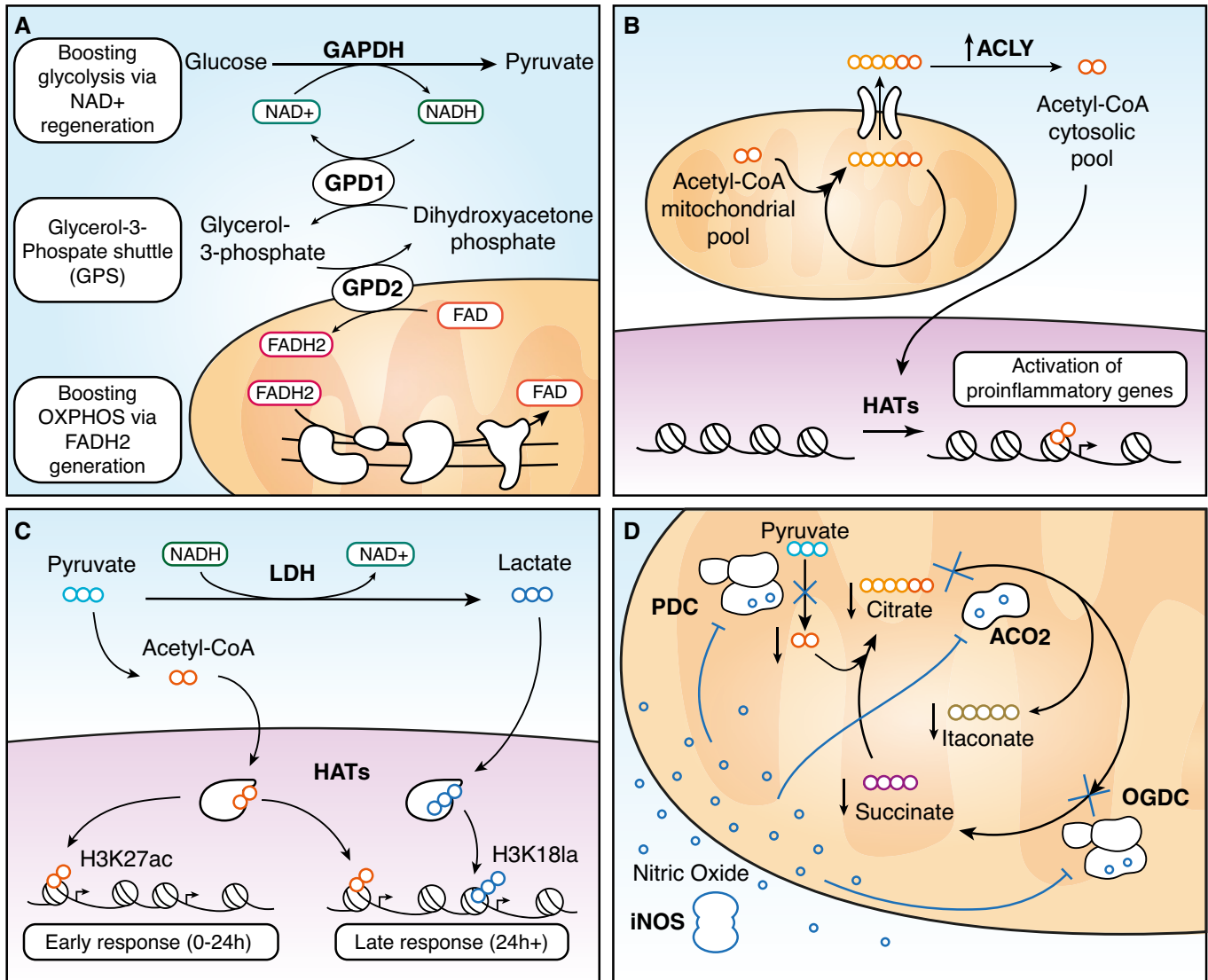
Figure 3. Novel aspects of metabolic remodeling and its transcriptional consequences in macrophages.

(A) The glycerol phosphate shuttle plays a key role in linking increased glycolytic flux to increased oxygen consumption in the early phases of the LPS response. (B) ACLY, a key gene able to regulate global acetyl-CoA levels in the cell, is activated via AKT in the early phase (< 3 h) after LPS exposure, and its activity is crucial for the upregulation of several proinflammatory mediators. (C) Lactate produced from pyruvate is used to generate a novel post transcriptional modification on histones, H3K18la (lactylation of K18 on histone H3), which is deposited on genes activated at late time points in the LPS response. (D) The burst of NO production inactivates PDC, ACO2, and OGDC at late time points in the LPS response by nitrosylating one of their subunits (blue circles), leading to the normalization of citrate, itaconate, and succinate levels over time.

effects of ACLY inhibition. Indeed, histone acetylation is an extremely dynamic modification and the high levels observed at the promoters of constitutively acetylated, early inducible genes reflect a dynamic equilibrium of acetylation and deacetylation rather than a static acetylated state. Overall, it seems unlikely that higher amounts of acetyl-CoA are selectively required to acetylate the promoters of late but not early inducible genes (Escoubet-Lozach et al, 2011). Another mechanism proposed to explain the different ACLY dependence of specific inflammatory genes is the selective recruitment of distinct HATs with different Km to distinct regulatory regions (Fig 4, upper panel) (Lauterbach et al, 2019). In this framework, selectivity may result from the gene-specific recruitment of

HATs that have a differential response to fluctuating acetyl-CoA concentrations. In mammalian cell lines, the concentration of acetyl-CoA has been reported to range between 2 and 20 μM (Lee et al, 2014) which is well above the Km of several HATs including Gcn5 and P/CAF (Tanner et al, 2000; Langer et al, 2002), whose activity would thus be unaffected by acetyl-CoA fluctuations within such range. On the contrary, the concentration of acetyl-CoA is near the Km of p300/CBP, which is 6.7 μM (Lau et al, 2000; Liu et al, 2008), which makes this specific HAT a candidate transducer of acetyl-CoA fluctuations into gene expression changes (Fan et al, 2015). Indeed, the autoacetylation of p300 (which reflects its acetyltransferase activity), as well as its capacity to acetylate other

substrates, is influenced by cytoplasmic acetyl-CoA concentrations (Marino *et al*, 2014). p300 was previously found to associate in an LPS-regulated manner to early and late inducible gene promoters (Ghisletti *et al*, 2010; Escoubet-Lozach *et al*, 2011). Moreover, p300 may similarly be involved in alternative macrophage activation as it has been shown to preferentially regulate a subset of Akt-dependent M2 genes (Covarrubias *et al*, 2016). Overall, it appears that specific gene subsets may be more sensitive than others to acetyl-CoA fluctuation and this differential sensitivity may in turn be a major determinant of differential ACLY dependence.

Importantly, while acetyl-CoA synthesis in the nucleus is not required as it can diffuse freely through the nuclear pores, multiple acetyl-CoA producing enzymes, such as ACLY and PDC, have also been found in the nucleus (Fig 4, lower panel) (Takahashi *et al*, 2006; Wellen *et al*, 2009; Ariyannur *et al*, 2010; Comerford *et al*, 2014). These observations suggest that histone acetylation may also depend on acetyl-CoA produced locally and possibly in proximity of genomic *cis*-regulatory elements.

A key question that needs to be addressed is whether and how these enzymes are recruited onto specific genomic regulatory elements by sequence-specific TFs, which could provide a simple mechanism for selective dependencies. In this model, genes not requiring a cytoplasmic source of acetyl-CoA would be those able to recruit enzymes to their *cis*-regulatory elements to locally generate acetyl-CoA (Fig 4, lower panel). In conclusion, ACLY preferentially regulates the expression of specific inflammatory gene subsets but the mechanisms accounting for this selectivity remain unclear.

Lactate regulates macrophage phenotypic states

As discussed above, at late stages (24 h) of LPS activation, macrophages exhibit high rates of aerobic glycolysis resulting in high production of lactate (Zaslona & O'Neill, 2020). While the shutdown
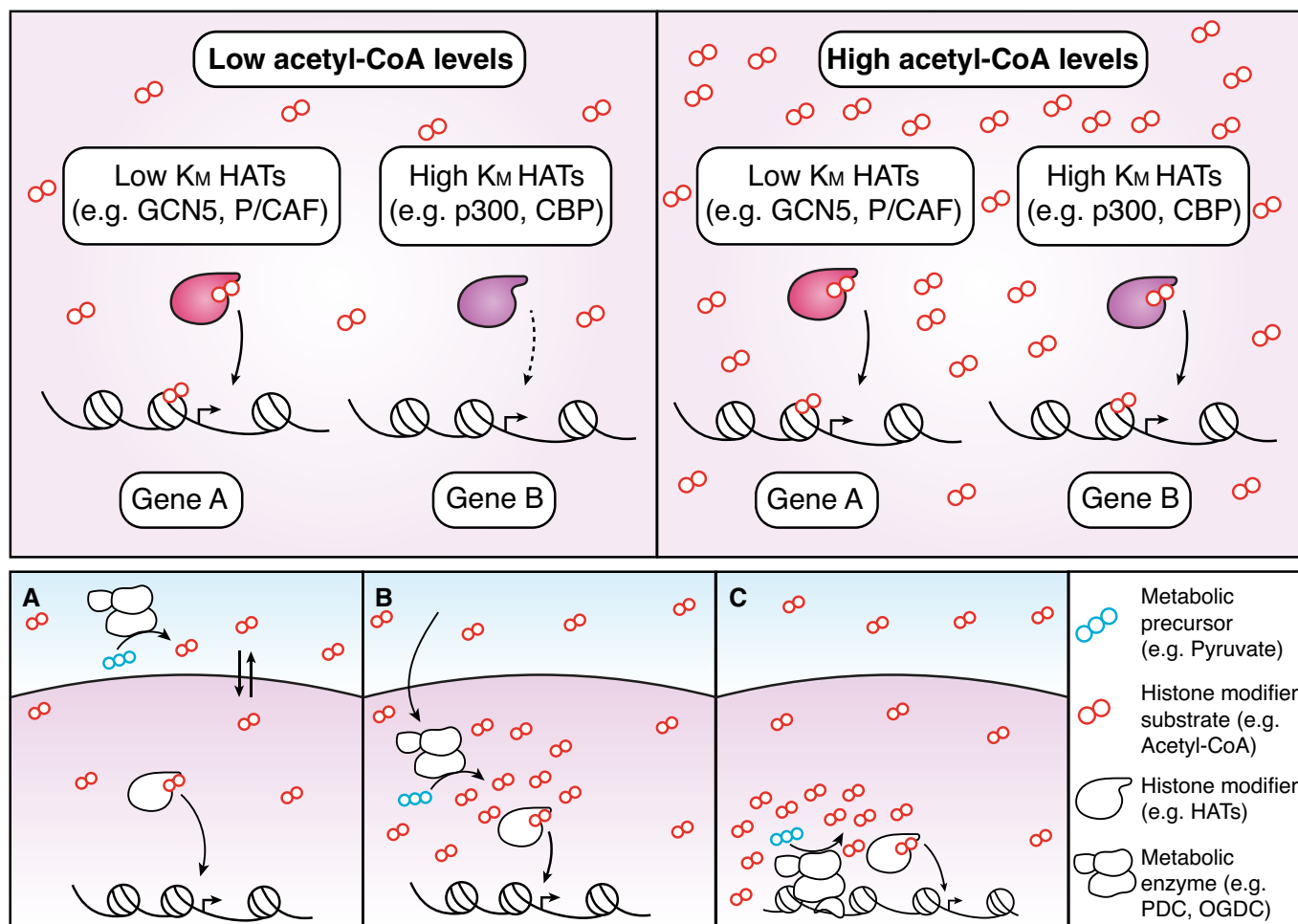
Figure 4. Putative mechanisms linking global or local changes in metabolites concentration to changes in gene expression.

Upper panel. Changes in global availability of the substrate (e.g., acetyl-CoA) of a histone modifier (e.g., a histone acetyl transferase, HAT) differentially regulate enzymes with distinct properties. For instance, HATs such as GCN5 or P/CAF have a low K_m and are thus largely insensitive to broad changes in cellular substrate concentration. Conversely, HATs such as p300/CBP have a K_m which is within the range of cellular concentration of the enzyme, making them able to transduce an increase in acetyl-CoA concentration into increased histone acetylation levels. Lower panel. Three possible mechanisms explaining the impact of metabolite concentration on gene activity. (A) A metabolite produced outside of the nucleus diffuses through the nuclear membrane and equilibrates between different compartments. Changes in its cellular concentration thus affect histone modification activity inside the nucleus. (B) Translocation of a metabolic enzyme into the nucleus increases local production of a metabolite (e.g., acetyl-CoA), modulating the activity of histone-modifying enzymes. (C) Direct recruitment of a metabolic enzyme onto chromatin increases the local production of substrate, enhancing the deposition of histone modifications at specific loci.

of oxidative metabolism is a central component of the metabolic reprogramming during prolonged exposure to LPS, the induction of aerobic glycolysis was previously considered just a compensatory mechanism to support ATP production. However, lactate production has important biological consequences linked to its activity as a signaling mediator (Brooks, 2009), an energy source (Bergman *et al*, 1999; Hirschhaeuser *et al*, 2011), and an immunosuppressive molecule capable of inhibiting proliferation, activation, and cytokine production in T cells, dendritic cells, and macrophages (Gottfried *et al*, 2006; Fischer *et al*, 2007; Dietl *et al*, 2010; Goetze *et al*, 2011; Colegio *et al*, 2014; Peter *et al*, 2015; Pucino *et al*, 2017; Nolt *et al*, 2018).

High lactate amounts produced by enhanced glycolysis in M1-polarized cells also enable a recently described modification of histones, lactylation (Zhang *et al*, 2019) (Fig 3C). Histone H3 lactylation at lysine 18 (H3K18la) is deposited at the promoters of genes involved in tissue repair and wound healing at late time points (16-24h) after stimulation, promoting their activation (Fig 3C). By contrast, histone lactylation does not occur at promoters of early inducible genes whose induction peaks when lactate levels are still low (Fig 3C) (Zhang *et al*, 2019). Thus, metabolic rewiring leads to increased production and accumulation of lactate, histone lactylation, and eventually a switch to a homeostatic gene expression program favoring the resolution of inflammation. Along the same line, increased intracellular lactate in macrophages was shown to induce an M2-like tumor growth-promoting phenotype (Bohn *et al*, 2018; Morioka *et al*, 2018; Liu *et al*, 2019). Mechanistically, the p300 HAT seems to be responsible for histone lactylation (Fig 3C) (Zhang *et al*, 2019) although the contribution of additional enzymes cannot be ruled out at this stage. Moreover, a most interesting question relates to the accumulation of lactate in the nucleus. Lactate can freely diffuse from the cytoplasm, where it is produced, into the nucleus (Zheng *et al*, 2003) but there is also evidence of LDH in the nucleus (Ronai, 1993), suggesting the possibility of a local production. It is still unclear what are the nuclear lactate concentrations required for this epigenetic modification and what mechanisms account for its selective deposition at a subset of genomic regions. Finally, it is worth mentioning that also non-histone proteins are lactylated on lysine residues; specifically, glycolytic enzymes have been found to be the major targets of this modification, suggesting that the effects of increased lactate production in classically activated macrophages may be pervasive (Gaffney *et al*, 2020).

Nitric oxide-dependent metabolic remodeling and chromatin regulation

Central to the metabolic switch of mouse M1 macrophages is the expression of the inducible nitric oxide synthase (iNOS) encoded by *Nos2*, a secondary response gene. iNOS produces large amounts of nitric oxide (NO), whose antimicrobial activity is linked to the nitrosation or nitrosylation and the consequent inactivation of essential enzymatic systems of intracellular bacterial pathogens (Fig 1) (Richardson *et al*, 2011). A metabolic side effect of increased NO production is the inhibition of mitochondrial respiration by nitrosation of the NADH dehydrogenase (Complex I) of the ETC and the competitive inhibition of O_2-consuming cytochrome c oxidase (Complex IV) (Clementi *et al*, 1998; Chouchani *et al*, 2013). In addition, NO nitrosylates and inactivates all iron-sulfur proteins of the ETC, thereby inhibiting electron transport and mitochondrial ATP production (Fig 1) (Van den Bossche *et al*, 2016).

Recently, NO has also been shown to inhibit the pyruvate dehydrogenases complex (PDC), the oxoglutarate/α-ketoglutarate dehydrogenase complex (OGDC), which converts α-ketoglutarate to succinyl-CoA in the Krebs' cycle, and aconitase (ACO2) (Seim *et al*, 2019; Palmieri *et al*, 2020) (Fig 3D). Moreover, isocitrate dehydrogenase (IDH) activity has also been shown to be inhibited by NO (Yang *et al*, 2002; Lee *et al*, 2003; Bailey *et al*, 2019). At late stages (48–72 h) of LPS and IFNγ stimulation, an impaired flux through PDC and OGDC was observed and this reduced flux through the TCA cycle leads to the normalization of citrate, itaconate, and succinate levels and eventually decreased levels of acetyl-CoA and succinyl-CoA (Seim *et al*, 2019). Mechanistically, NO-dependent suppression of PDC occurs via direct S-nitrosation of one of its subunits during macrophage polarization (Palmieri *et al*, 2020). These data suggest that NO-mediated inhibition of PDC may contribute to the late decline in acetyl-CoA production (Fig 3D; Palmieri *et al*, 2020). Thus, NO takes center stage not only as an orchestrator of changes in macrophage mitochondrial metabolism during prolonged stimulation, but also as a potential regulator of downstream epigenomic changes.

As discussed above, metabolic enzymes, including glycolytic enzymes, have been observed in the nucleus (Boukouris *et al*, 2016). Among them, PDC and OGDC have been shown to generate acetyl-CoA and succinyl-CoA in the nucleus and local synthesis appears to be important for histone acetylation and succinylation (Sutendra *et al*, 2014). A functional PDC, in spite of its considerable size, has been found to be able to translocate from the mitochondria to the nucleus in response to diverse stimuli, and the nuclear localization seems to be required for basal histone acetylation (Fig 3D) (Sutendra *et al*, 2014). Pyruvate can diffuse into the nucleus from the cytosol and then serves as a substrate for nuclear PDC. The detailed mechanisms involved in the regulation of nuclear processes by these enzymes and their possible involvement in dynamic gene expression changes associated with macrophage activation remain to be defined.

Endogenous inflammatory signals reprogram macrophage metabolism

Each tissue-resident macrophage population contributes to tissue-specific homeostatic functions (Ginhoux & Guilliams, 2016). Consistent with this functional specialization, different tissue-resident macrophages express distinct and to some extent tissue-specific gene expression programs (Gautier *et al*, 2012). These differences in gene expression are associated with the selection and usage of partially non-overlapping sets of tissue-specific enhancers (Gosselin *et al*, 2014; Lavin *et al*, 2014; Sakai *et al*, 2019).

Notably, tissue-resident macrophages can also be distinguished by differential expression of genes regulating metabolism, as different metabolic genes are associated with distinct populations. For example, genes regulating oxidative metabolism are enriched in microglia, where they play fundamental roles for microglia homeostatic functions such as trophic factor release and debris clearance by phagocytosis. Lipid metabolism signatures are enriched in alveolar

macrophages, in which the high lipid content of surfactant imposes dedicated control mechanisms. Finally, eicosanoid and lipid signaling are enriched in peritoneal macrophages (Gautier *et al*, 2012; Gautier *et al*, 2014; Amit *et al*, 2016). Recently, the analysis of transcriptomes of six tissue-resident macrophage populations also showed distinct responses to short- and long-term metabolic challenge (Brykczynska *et al*, 2020). In this study, distinct or similar responses of macrophage subtypes to acute vs. chronic nutritional stress were identified, such as in the case of adipose tissue macrophages (ATMs), where feeding upregulated a local, low-grade inflammatory response selectively in ATMs (Brykczynska *et al*, 2020).

Overall, the tissue environment is a critical determinant of tissue-specific macrophage gene expression (Gautier *et al*, 2012; Gosselin *et al*, 2014; Lavin *et al*, 2014; Sakai *et al*, 2019) and its dysregulation contributes to a diverse range of inflammatory associated diseases. A paradigmatic example is represented by the atherosclerotic plaque microenvironment, where macrophages are exposed to oxidized lipids, cytokines, and cholesterol crystals that can greatly affect their activation state. In this peculiar environment, macrophages ingest and accumulate oxidized lipid particles and become overloaded foam cells (Ross, 1993). Foam cells display an activated state as (oxidized) lipids act as TLR agonists, leading to the activation of a proinflammatory response. In a recent report, the oxidized phospholipids (collectively known as oxPAPC) have been shown to induce a unique hypermetabolic state in macrophages exposed to LPS (Di Gioia *et al*, 2020). oxPAPC are produced at injury sites and are also part of the oxidized low-density lipoproteins (oxLDL) that promote atherosclerosis (Bochkov *et al*, 2002; Berliner *et al*, 2009; Que *et al*, 2018). When primary macrophages are primed with LPS and exposed to oxPAPC, glycolysis is increased but without the classical decrease in OXPHOS that occurs in response to LPS alone (Di Gioia *et al*, 2020). From a mechanistic point of view, macrophages exposed to oxPAPC and LPS are able to feed the TCA cycle with glutamine and favor the accumulation of oxaloacetate (OAA) in the cytoplasm, as a result of increased cytoplasmic metabolism of citrate. In turn, OAA accumulation stabilizes HIF1α and increases IL-1β production, by inactivating HIF prolyl hydroxylases which target HIF1α for rapid proteasomal degradation (Koivunen *et al*, 2007; Di Gioia *et al*, 2020). In addition, oxPAPC exposure promotes the transcriptional activation of *Acly*, driving a hyperinflammatory phenotype (Di Gioia *et al*, 2020). This mechanism is relevant *in vivo*, as similar metabolic adaptations occur in atherosclerotic mice and in hypercholesterolemic human subjects (Baardman *et al*, 2020; Di Gioia *et al*, 2020).

Overall, the main concept discussed above is that the exposure to oxidized phospholipids, altering metabolism and consequently inflammatory gene transcription, can drive a hypermetabolic, hyperinflammatory macrophage phenotype, which is critical in inflammation-associated diseases (Di Gioia *et al*, 2020).

A long-term perspective: modeling macrophage activation

While major technical advances led to the identification of mechanisms controlling signal-induced transcriptional and metabolic changes in activated macrophages, a rigorous, quantitative, and system-wide integration of these two regulatory layers is yet to be established.

While extremely valuable for the systematic description of cellular behavior, genome-wide, and high-throughput data are usually combined and interpreted using associative statistical approaches (such as correlations among variables, clustering, and grouping), that are useful to provide an unbiased description of biological systems but is also intrinsically not suitable to predict changes in cell states. Notably, data-driven associations do not necessarily imply causality among the variables under investigation and therefore they are not necessarily informative of the molecular mechanism underlying a given phenotype. The application of these methodologies is in fact most appropriate to describe and interpret genome-wide transcriptional changes. Thus, TF-mediated gene expression, within a specific timeframe, can be considered as not constrained by most other changes observed in the cell. To clarify this concept, the activation of one gene (e.g., *TNF*) does not generate any intrinsic limitation to the activation of a second gene (e.g., *IL1B*). However, such approximation is not suitable to interpret changes in metabolites detected in high-throughput metabolomics as those result from the adjustment of thousands of interlinked reactions all participating in the consumption and production of common metabolite pools. Indeed, while high-throughput transcriptomics assesses the absolute and relative levels of expression gene by gene, scalable high-throughput techniques reporting flux rates for all individual biochemical reactions within a cell at once are yet to be established.

Nevertheless, recent advances have highlighted the possibility to integrate a variety of omics datasets in the context of interpretable and predictive human models (Bordbar *et al*, 2014; Brunk *et al*, 2018; Robinson *et al*, 2020). A central tenet in these approaches is that all simultaneous molecular circuits occurring in a cell function coherently as they are all operating under countless *constraints*. Constraints can be divided into specific categories, namely physical–chemical (e.g., conservation of mass, elements, energy), spatial (e.g., cellular compartmentalization of molecules), environmental (e.g., nutrients, temperature, pH), and regulatory constraints, and they are all inviolable. Constraint-based modeling and reconstruction analysis (COBRA) relates to approaches aiming to collect, integrate, and analyze in a bottom-up (i.e., operator-supervised) fashion all known biochemical transformations belonging to the network under investigation (Palsson, 2015). COBRA approaches have been used mostly for metabolic networks, including those of macrophages, as the thousands of known biochemical reactions occurring in cells can be explicitly formulated and combined in mathematical terms. These strategies have already been adopted in key works to reconstruct macrophage metabolic networks and coherently integrate condition-specific changes (Bordbar *et al*, 2010; Bordbar *et al*, 2012; Jha *et al*, 2015).

Far from being a mere technological and modeling *tour de force*, this approach would instead provide researchers with accurate *predictions* of system-level changes in macrophage biology that may also have translational impact, in principle enabling controlled and highly predictable reprogramming of macrophage properties. However, stoichiometric COBRA models face challenges when modeling dynamic cellular states. In this context, it is critical to complement COBRA models with kinetic models assembled to simulate changes in metabolite concentrations over time combining stoichiometric, reaction rate laws, kinetic parameters, and enzyme concentrations (Volkova *et al*, 2020). Nevertheless, given the

Box 1. In need of answers

i   What are the mechanisms by which dynamic changes in concentration of metabolites during macrophage activation trigger gene-specific transcriptional responses rather than global effects?

ii What is the subcellular localization of key metabolites impacting epigenomic and transcriptional responses of macrophages exposed to different stimuli? Since the abundance and/or the distribution of metabolites might considerably change, it is crucial to measure metabolite fluctuations within distinct subcellular compartments.

iii Given the heterogeneity of macrophage functional states, to what extent do metabolic and transcriptional programs show heterogeneity in dynamic *in vivo* settings?

iv What is the full range and functional impact of chromatin modifications in the nuclear compartment that are impacted by dynamic changes in metabolite availability? A wide variety of novel modifications is increasingly being discovered, but their functional impact on transcriptional regulation requires extensive additional work.

v   How will integrated system-level analyses change our understanding of macrophage activation? While a plethora of transcriptomics studies has been performed in many different macrophage types, also at single-cell levels, metabolomic approaches reporting flux rates for all individual biochemical reactions at once are yet to be generated. In this way, system-level changes can be accurately predicted, explaining the different behavior of polarized macrophages in the early activation phase compared with their long-term phenotype.

amount of information required to construct a kinetic model, this strategy has so far been successfully adopted only to model cells with low complexity such as prokaryotes. Recent computational efforts provide promising solutions to model dynamic cellular states for complex genome-wide networks exploiting the integration of time-resolved multi-omics datasets with constrained models, in order to predict reaction flux rates (for an overview see Basler *et al* (2018)). Time-series metabolomics have been integrated to model flux distribution in red blood cells (Bordbar *et al*, 2017) and similar approaches have been applied to model changes in the transition from naïve to primed murine pluripotent stem cells (Chandrasekaran *et al*, 2017).

The development and application of similar strategies to time-resolved multi-omics datasets describing the process of macrophage activation are yet to be tested. These approaches will shed light on cause–effect relationships between changes in signaling, transcriptional, and metabolic changes from the early activation phase to the long-term phenotype of polarized macrophages, providing a critical contribution to unraveling outstanding questions in this field (Box 1).

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

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