Regulation of Inflammatory Macrophage Activation and Tolerance by Modulation of Glucose Metabolism

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REGULATION OF INFLAMMATORY MACROPHAGE ACTIVATION AND TOLERANCE BY MODULATION OF GLUCOSE METABOLISM

A DISSERTATION PRESENTED
BY
P. KENT LANGSTON
TO
THE COMMITTEE ON HIGHER DEGREES IN BIOLOGICAL SCIENCES IN PUBLIC HEALTH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE SUBJECT OF BIOLOGICAL SCIENCES IN PUBLIC HEALTH

HARVARD UNIVERSITY
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REGULATION OF INFLAMMATORY MACROPHAGE ACTIVATION AND TOLERANCE BY MODULATION OF GLUCOSE METABOLISM

ABSTRACT

The reactions in an organism’s metabolic network take on dynamic changes in fluxes in response to imposed stress to support calibrated functional responses. Macrophages, phagocytic cells of the myeloid lineage, are activated during microbial infection to coordinate inflammatory responses and host defense. Here we show that in macrophages activated by bacterial lipopolysaccharide (LPS), mitochondrial glycerol 3-phosphate dehydrogenase (GPD2) regulates glucose oxidation to drive inflammatory responses. GPD2, a component of the glycerol phosphate shuttle (GPS), boosts glucose oxidation to fuel acetyl-CoA (Ac-CoA) production, histone acetylation and inflammatory gene induction. While acute LPS exposure drives macrophage activation, prolonged exposure triggers entry into LPS tolerance, where macrophages orchestrate immunosuppression to limit the detrimental effects of sustained inflammation. We find that the shift from activation to tolerance is modulated by GPD2, which coordinates a shutdown of oxidative metabolism that limits Ac-CoA availability for histone acetylation at inflammatory genes, thus contributing to suppression of inflammatory responses. Therefore, GPD2 and the GPS integrate the extent of microbial stimulation with glucose oxidation to balance the beneficial and detrimental effects of the inflammatory response. In addition, we show that LPS-inducible glucose oxidation is downstream of the serine/threonine kinase Akt. We find that Akt signaling controls glucose oxidation to couple the strength and duration of LPS exposure to the GPD2-dependent increase and decrease in
mitochondrial respiration, promoter region histone acetylation, and inflammatory gene induction in macrophages over the transition from activation to tolerance. Furthermore, transcriptional profiling of tolerant macrophages re-exposed to LPS revealed a subset of highly-responsive, primed (P) genes, important for antimicrobial activity and mitochondrial respiration. Induction of P genes is Akt-dependent and fueled by acetate-derived Ac-CoA production catalyzed by nucleocytosolic Ac-CoA synthetase (ACSS2) rather than by citrate-derived Ac-CoA from glucose oxidation. Therefore, Akt and ACSS2 act as additional regulatory modules integrating TLR signaling with shifts in macrophage metabolism to induce gene-specific responses to microbial encounter. Taken together, our findings support a paradigm shift in immunometabolism, demonstrating that glucose oxidation regulates induction and suppression of macrophage inflammatory responses while also identifying several novel metabolic pathways controlling gene-specific transcriptional responses in tolerant macrophages.
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Chapter 1

Introduction
Sepsis: a tale of two syndromes

Systemic microbial infection in vertebrate animals elicits an acute systemic inflammatory response syndrome\(^1\), characterized by activation of the innate and adaptive arms of the immune cells and elevated local and circulating pro-inflammatory cytokines (e.g. IL-6, IL-1\(\beta\), and TNF\(\alpha\)) and enhanced antimicrobial activity, including phagocytosis and production of reactive oxygen species (ROS), nitric oxide (NO), and hypochlorous acid\(^2\). The clinical presentation of SIRS in patients with a documented pathogen is referred to as sepsis, or septic shock, and is diagnosed by elevated temperature, heart rate, respiratory rate, and/or blood leukocyte count\(^2\). Hospital mortality statistics for sepsis give pause, as sepsis-related illness contributes to nearly half of all hospital deaths despite presentation in only 10% of patients\(^3\). Although SIRS may progress to multiorgan dysfunction syndrome resulting from tissue damage in the wake of the primary pro-inflammatory response, standard care practices and advances in medical technology in intensive care units have minimized acute risk of mortality from sepsis\(^2,4\). However, patients who survive SIRS are at an elevated risk of succumbing to secondary nosocomial infections or reactivated latent infections\(^5,6\), as the initial robust pro-inflammatory response is followed by prolonged immunoparalysis, characterized by general immunosuppression due to decreased pro-inflammatory cytokine production, impaired antigen presentation and costimulation, and marked T cell anergy\(^7-10\). Immunosuppression in this context, referred to as compensatory anti-inflammatory response syndrome (CARS)\(^11\), is believed to be a compensatory mechanism to limit damage to the host; however, protracted immunosuppression contributes a high percentage of sepsis-related hospital deaths\(^12\).
There have been many attempts at suppressing inflammation during sepsis, resulting in numerous phase-two clinical trials, including anti-TNFα antibodies (Abs)\textsuperscript{13–15}, IL-1 receptor antagonists\textsuperscript{16,17}, and endotoxin-neutralizing Abs\textsuperscript{18}. Such attempts have so far been unsuccessful, turning attention toward preventing immunoparalysis and restoring immunocompetence during CARS\textsuperscript{12,19,20}. Providing clarity to known mechanisms of immunosuppression, as well as uncovering novel modules of regulation, may inform the design of targeted therapies to improve patient outcome.

**Innate response to microbial encounter: activation**

Macrophages, phagocytic cells of the innate immune system, are a critical component of the biphasic host response during sepsis, mounting an initial pro-inflammatory response to microbial encounter followed by a persistent state of tolerance to subsequent challenges. First described in the late 19\textsuperscript{th} century by Metchnikoff, macrophages discriminate between invading microbes and the host’s own tissues through detection of various structures common to pathogens, including lipopolysaccharide (LPS), peptidoglycans, and mannose-rich oligosaccharides\textsuperscript{21}. Collectively, these structures are referred to as pathogen-associated molecular patterns (PAMPs), and the receptors expressed on macrophages that identify PAMPs are called pattern recognition receptors (PRRs). There are many types of PRRs in different cell compartments developed to generate specific responses to a wide array of PAMPs from viruses, bacteria, fungi, and parasites\textsuperscript{21–23}.

Toll-like receptors (TLRs), originally identified in *Drosophila*\textsuperscript{24}, are the best-characterized of the many PRRs. The TLR family consists of 10 members in humans and 12 members in mice\textsuperscript{25}. This repertoire allows cells of the innate immune system to sense a wide array of PAMPs to drive inflammatory responses and enhance adaptive immunity. Signaling through TLRs is initiated by
homo- or heterodimer formation, followed by transduction through a cytoplasmic Toll/IL-1 receptor (TIR) domain to adaptor proteins capable of sorting signals from multiple TLRs into a broad range of transcriptional responses. All TLRs except for TLR3, which senses specific viral components, signal through the adaptor protein MyD88 (myeloid differentiation primary response 88), which interacts through its TIR domain with the TIR domain of TLRs. This MyD88-dependent pathway signals through activation of downstream IL-1 receptor-associated kinases (IRAK), of which there are 4 members (1, 2, M, and 4). IRAK4 is first recruited to MyD88, followed by activation and phosphorylation of IRAK1. Both IRAKs interact with MyD88 through their death domains and then associate with TRAF6 (tumor-necrosis factor-receptor-associated factor 6), leading to activation of the transcription factors NF-κB and AP-1 through activation of IKKs α/β/γ (inhibitor of NF-κB (IκB) kinases) and JNK, respectively. TLRs also signal through a MyD88-independent pathway, which ultimately activates IRF3 (IFN-regulatory factor 3) to induce IFN-β and, consequently, IFN-inducible genes. IRF3 activation occurs as a result of phosphorylation by the non-canonical IKKs TANK-binding kinase 1 (TBK1) and IKKε/IκK1.

As mentioned above, transcriptional responses induced downstream of TLR signaling are further calibrated to specific PAMPs through the incorporation of additional adaptor molecules. In the MyD88-dependent pathway, MyD88 functions with TIRAP (TIR domain-containing adaptor protein) to constitute an adapter set, while the proteins TRIF (TIR domain-containing adaptor inducing IFN-β) and TRAM (TRIF-related adapter molecule) form a second adapter set. Importantly, TLR4, which senses lipopolysaccharide (LPS) from gram-negative bacteria, has the unique ability to signal through both of these adapter sets, first through TIRAP/MyD88 at the plasma membrane and then through TRIF/TRAM from endosomes following CD14-dependent
endocytosis of TLR4\textsuperscript{42,43}. Notably, CD14 is also involved in signaling from the cell surface, transferring LPS from LPS-binding protein (LBP) to TLR4-bound MD-2, which promotes dimerization and activation of TLR4\textsuperscript{44–47}. This spatiotemporal regulation of signaling from TLR4 results in early and late phases of inflammatory gene induction through NF-κB (late phase being TRIF/TRAM-dependent) and induction of antiviral immunity through IRF3.

The enhanced production of pro-inflammatory cytokines, antimicrobial species, and expression of costimulatory molecules by innate immune cells as a result of TLR-dependent signaling is critical for mounting an appropriate and effective defense against microbial invasion. Indeed, disruption of pathogen sensing and signaling by mutations in TLRs or deficiencies in adaptor molecules and associated kinases results in incomplete protection\textsuperscript{48}. However, TLR signaling is also involved in the pathogenesis of autoimmune, chronic inflammatory, and infectious diseases\textsuperscript{49}. As described at the beginning of this chapter, systemic bacterial infection induces a robust pro-inflammatory response, often referred to as a “cytokine storm,” which is necessary to control and eliminate the microbe by enhancing innate and adaptive immunity at sites of infection. However, if the magnitude and duration of the inflammatory response are left unchecked, then serious damage to the host’s own tissues will ensue, resulting in fatal multi-organ dysfunction\textsuperscript{4,12}. Therefore, many mechanisms of attenuating the pro-inflammatory response have evolved to balance the double-edged sword of TLR activation.
Innate response to microbial encounter: suppression and tolerance

The myriad mechanisms of negative regulation of early TLR-driven inflammatory responses can be conveniently grouped into three classifications based on spatial distribution: extracellular, transmembrane, and intracellular⁵⁰. Extracellular mechanisms of negative regulation of TLR signaling involve competition for binding PAMPs and co-receptor complexes by soluble decoy TLRs (sTLRs). sTLR2 and sTLR4 have been identified as a first layer of negative regulation of inflammatory responses induced by TLR2 and TLR4 agonists⁵¹,⁵². sTLR2 exists in six isoforms that each result from post-translational modification of transmembrane TLR2. In contrast, sTLR4 is encoded by several mRNAs. Expression of the orphan receptor SIGIRR (single immunoglobulin IL-1-related receptor) constitutes a layer of negative regulation at the transmembrane level, as the level of surface residency of SIGIRR is negatively correlated with NF-kB activation⁵³,⁵⁴. Supportive of a suppressive role in inflammatory responses, SIGIRR-deficient mice are more susceptible to septic shock, and DCs from the bone marrow of these mice are more sensitive to TLR4 and TLR9 stimulation. Finally, intracellular mechanisms represent the largest class of negative regulators, including truncated MyD88, catalytically-inactive IRAKs, SOCS1, TOLLIP, and A20. The first in this list, truncated MyD88, or short form MyD88 (MyD88s), is a splice variant of MyD88 that lacks the normal interdomain. When MyD88s is expressed at high levels (as is true in the spleen), it forms a heterodimer with MyD88 and prevents the association with IRAK4 that is required for IRAK1 phosphorylation and ultimately for activation of NF-kB⁵⁵,⁵⁶. Second on the list, are IRAK2 and IRAKM. Expression of splice variants of IRAK2 (Irak2a, b, c, and d) and IRAKM attenuates LPS-induced inflammatory gene induction⁵¹,⁵⁷. Perhaps most striking of the intracellular negative regulators is SOCS1, as deficiency is lethal within 3 weeks of life, and SOCS1-deficient mice are also profoundly susceptible to sepsis-induced multi-organ
dysfunction and mortality\textsuperscript{58,59}. The remaining two intracellular negative regulators, TOLLIP (Toll-interacting protein) and A20 act at IRAK1 and TRAF6, respectively. TOLLIP is phosphorylated by IRAK1 upon LPS stimulation but impinges on IRAK1 phosphorylation in a negative feedback loop, while TLR-induced expression of A20 leads to enhanced deubiquitination of TRAF6, attenuating MyD88-dependent and -independent signaling\textsuperscript{60–63}.

While modulation of the negative regulators of TLR signaling discussed above leads to important changes in inflammation, both in normal animals (i.e. SOCS1) and in animals subjected to septic shock induced by TLR agonists (i.e. A20), these mechanisms do not provide protection against subsequent exposures to PAMPs\textsuperscript{50}. However, in addition to fine tuning the magnitude and duration of inflammation, macrophages have also evolved mechanisms that drive entry into a state of profound refractoriness to subsequent microbial encounters following prolonged engagement of TLRs. This phenomenon, termed endotoxin tolerance (not to be confused with central and peripheral tolerance, which pertain to immune regulation in the adaptive arm of the immune system) is a protective mechanism, evolved to protect the host’s tissues from sustained inflammation\textsuperscript{64}. One of the first observations of this form of tolerance was reported by Paul Beeson in 1946 after he found that administering serial injections of typhoid vaccine to rabbits resulted in progressive protection against the vaccine’s pyrogenicity\textsuperscript{65}. Consistent with this observation, it is now well known that injection of mice with a sublethal dose of LPS protects against a subsequent lethal dose of LPS. Such protection can be largely attributed to cell autonomous endotoxin tolerance in macrophage\textsuperscript{66}. Indeed, suppression of inflammatory activity and decreased responsiveness to repeated encounters with the same PAMP, or in some cases a different PAMP, is one of the primary adaptations underpinning the profound immunoparalysis that ensues following septic shock\textsuperscript{11}. 

Similar to mechanisms of negative regulation of early TLR-driven inflammation, tolerance mechanisms can also be classified as transmembrane and intracellular. Transmembrane tolerance mechanisms include expression of the surface receptors ST2 and TRAILR (TNF-related apoptosis-inducing ligand receptor), and the expression of TLRs themselves. The transmembrane form of ST2, ST2L, is known to suppress TLR signaling by sequestering MyD88 and TIRAP through their TIR domains; however, translocation of ST2L occurs after the early events of TLR activation, such that ST2-deficiency does not affect the response to LPS-induced septic shock but does prevent development of LPS tolerance in vivo. Similarly, TRAILR-deficient cells do not have attenuated TLR signaling acutely during activation by agonists but do exhibit differences at late timepoints.

Expression of some TLRs is also downregulated in response to specific PAMPs, contributing to tolerance. Ubiquitylation of TLR4 and TLR9 by the RING-finger E3 ubiquitin ligase TRIAD3 promotes downregulation of these TLRs by enhanced degradation following LPS and CpG DNA exposure. Anti-inflammatory cytokines such as TGF-β and IL-10 can also impinge on TLR4 expression.

Intracellular mechanisms contributing to acquisition of a tolerant phenotype can be attributed to decreased signaling as a result of changes in surface expression of the receptors described above, as well as to impaired formation and activity of the myddosome (MyD88, TIRAP, and IRAKs) and decreased NF-kB and JNK activation due to negative feedback loops. Strikingly, changes in responsiveness of genes encoding inflammatory cytokines and antimicrobial effector functions in tolerant cells is also upregulated by gene-specific chromatin modifications induced by TLR signaling. Specifically, genes encoding pro-inflammatory cytokines and chemokines lose transcription-activating histone lysine acetylation during prolonged exposure to the TLR4 ligand.
LPS, while genes responsible for antimicrobial effector functions are not silenced by decreased histone acetylation and are in some instances hyperacetylated to promote enhanced expression in re-stimulated tolerant cells\textsuperscript{66}. Since this finding, additional work has strengthened the epigenetic basis of LPS tolerance in macrophages by characterizing the regulation and function of histone acetylation and methylation by chromatin modifying enzymes in the context of inflammation\textsuperscript{75}. However, a complete understanding of the mechanisms underpinning these chromatin modifications and the overall phenotype of endotoxin tolerance remains elusive. Thus, providing clarity to known mechanisms of tolerance, as well as uncovering novel modules of regulation, may inform the design of targeted therapies to relieve immunosuppression in sepsis and improve patient outcome.

**Immunometabolism: a new perspective on the regulation of immunity**

Just as macrophage responsiveness changes over the course of extended stimulation, the field of immunology itself has changed over the span of time humans have been studying host defense. Indeed, from Edward Jenner’s discovery of vaccination at the end of the 18\textsuperscript{th} century to the “Toll Rush” at the turn of the 21\textsuperscript{st} century to the transcriptional profiling of tissue-resident leukocyte subsets at the present, immunology has evolved. Each step forward in the pursuit of knowledge on the subject of host defense leads to an increasing number of questions to fuel further exploration. One such step forward in the last decade was towards understanding the role of metabolism in immune cells. Continued exploration in this area has led to the genesis of a new subfield in immunology, referred to as “immunometabolism.” This relatively new discipline is focused on investigating how shifts in intermediary metabolism support the four tasks of the immune system: recognition, effector functions, regulation, and memory. Although it is still common to find
colleagues, at all levels of experience, who believe that metabolism is a passive network of chemical reactions that simply supplies energy and does not have any direct bearing on specific cellular functions, this belief has been thoroughly debunked by the flood of evidence demonstrating that innate and adaptive immunity depends on dynamic changes in metabolism\textsuperscript{76–82}. Furthermore, an understanding and appreciation of this new underpinning to immunity does not require translation and memorization of the impenetrable charts of metabolic pathways that are commonly presented in textbooks and seminars – although such knowledge is necessary to participate in productive research in immunometabolism – but rather depends only on a modest understanding of the fundamental nature of biochemistry and the overall functions of some major metabolic pathways. Before summarizing metabolic regulation of macrophage activation and function, an overview of these topics is provided.

The opening lines of Josiah Willard Gibbs’ masterwork \textit{On the Equilibrium of Heterogeneous Substances} is a quote from Rudolf Clausius, summarizing the first and second laws of thermodynamics:

\begin{quote}
"Die Energie der Welt ist konstant

Die Entropie der Welt strebt einem Maximum zu."
\end{quote}

- Clausius, 1865

These laws, stating that the energy in the universe is constant and that entropy in the universe tends to a maximum, were the basis for the Gibbs-Helmholtz equation relating the free-energy change in a system ($\Delta G$) to quantities of heat change ($\Delta H$) and entropy change ($\Delta S$) in the system under constant temperature and pressure (\textbf{Eq. 1.1}).
\[ \Delta G = \Delta H - T\Delta S \]  

(Equation 1.1)

This equation, in addition to Walther Nernst’s equation describing the electrochemical behavior of chemical substances participating in oxidation-reduction reactions, paved the way for the systematic discovery and mapping of biochemical reactions in the 20th century. As stated previously, knowledge of individual biochemical reactions is not necessary here, rather these equations provide a conceptual framework within which to think about metabolism. Specifically, one must understand that because the existence of animate objects (organisms) depends on the sum of these biochemical reactions to resist crumbling into complete randomness (\(S\) at a maximum), organisms are bound by the same thermodynamic laws that govern the reactions of inanimate chemicals.

The behavior of heterogeneous substances such as those in a biochemical network depends on the adherence to and utilization of chemical and electrochemical gradients. For example, energy-producing (exergonic; \(\Delta G<0\)) reactions involved in the systematic degradation of nutrients, such as the oxidation of carbohydrates, are coupled to energy-consuming (endergonic; \(\Delta G>0\)) reactions used to build and replenish highly-ordered structures of the body, such as cholesterol and proteins\(^83\). Based on the Gibbs equation, exergonic reactions increase the entropy of a system, while endergonic reactions have the opposite effect. Coupling these two types of reactions keeps the net change in entropy in the system close to zero. The change in free energy in a system is such a powerful way of analyzing the behavior of heterogeneous substances that it can be used as an organizing principle for biochemical networks. Indeed, it is possible to reconstruct experimentally-validated metabolic pathways simply from knowledge of metabolites and enzymes in the system and the Gibbs free energy of the reactions in which they can participate\(^84,85\). In lower organisms
and in some mammalian cells, thermodynamic analysis has also been used to predict metabolic fluxes, optimized to specific cellular functions\textsuperscript{86–89}. Thus, when thinking about central metabolic pathways, the heat and entropy changes occurring over a series of chemical transformations is often enough logic to understand the direction of flux.

In addition to gradients in chemical energy, electrochemical gradients also provide a useful lens through which to view metabolism\textsuperscript{90}. Indeed, electrochemical gradients generated from the separation of charged species across biological surfaces or from the specific spatial orientation or stoichiometry of redox-active substances are used as a motive force to drive myriad processes vital for life. For example, the mitochondrial respiratory chain, also called the electron transport chain (ETC), is responsible for making the majority of ATP in many types of cells, and its function depends on multiple electrochemical gradients\textsuperscript{91–93}. The ETC is comprised of protein complexes situated in the tightly-knit inner mitochondrial membrane and is organized such that electrons stripped from nutrients during oxidative metabolism enter at complexes of the lowest reduction potential (most negative) and flow from complex-to-complex until ultimately reducing oxygen, which has the highest reduction potential (most positive). The potential difference from “negative” to “positive” ends of the ETC is about 1.5 V\textsuperscript{94}, which is equivalent to the nominal charge of a standard alkaline battery! This electrical circuit within the inner mitochondrial membrane is coupled to displacement of protons across the membrane by pumping action of some ETC complexes, establishing a parallel chemical circuit with an electrochemical potential equivalent to 250,000 V/cm. This gradient is sufficient to drive production of ATP through the activity of the F\textsubscript{0}/F\textsubscript{1} ATPase at the end of the respiratory chain\textsuperscript{92}, fueling innumerable energy-consuming biochemical reactions necessary for life.
Beyond these thermodynamic constraints, metabolic networks are also subject to other layers of regulation, including concentrations of metabolites, concentrations of enzymes, and modification status and thus intrinsic activities of enzymes. Although metabolite concentrations can be factored into thermodynamic equations, in most cases application of Le Chatelier’s Principle or the Law of Mass Action is sufficient to address concerns with how levels of reactants and products affect flux\(^94\). These additional layers of regulation are also important for calculating the velocity and directionality (if bidirectional) of reactions through application of Michaelis-Menton kinetic equations; however, this will not be discussed here. Instead, it will be noted that a final fundamental principle of metabolism that is useful for general understanding is that, because the study of living organisms typically involves application of controlled stress(es) in an effort to induce and dissect a specific response, the metabolism of the organism is rarely at true steady-state. As a result, metabolite concentrations are displaced, inducing measurable changes in fluxes through the individual reactions of the organism’s metabolic network. Thus, focusing on metabolites with large displacement from control conditions and study of the reactions in which they participate is a powerful way to gain insight into the specific metabolic requirements for an organism’s response to a particular stress.

Finally, there are numerous mechanisms through which metabolic adaptations underpin specific functions of immune cells. These include meeting bioenergetics demands (supply of ATP for energy-consuming processes), supplying carbon substrates for growth and proliferation or for expanding ER to enhance secretory capacity, producing reactive species like ROS or metabolites that affect signaling, and increasing substrate flux through pathways producing metabolite-derived effector molecules\(^77–80\). Although significant progress has been made towards uncovering and
understanding metabolic instruction of many aspects of immunity, the following section is devoted to summarizing the body of knowledge regarding how metabolic shifts support LPS-induced macrophage activation (classical, or M1 polarization) and effector functions.

**Metabolic regulation of inflammatory macrophage polarization**

In addition to the canonical signal transduction pathways discussed in the previous section of this Introduction, pathogen recognition by TLRs is also coupled to activation of metabolic signaling pathways, enabling coordinate induction of effector functions and the metabolic processes needed to sustain them. Downstream of LPS-TLR4, Akt is activated by TBK1/IKKe and PI3K95. Akt kinases regulate cell survival, metabolism, and cytoskeleton, in part through activation of another metabolic signaling pathway, the mammalian target of rapamycin (mTOR)96,97. The mTOR kinase is found in two complexes in mammals, mTORC1 and mTORC2. Other subunits of these complexes are unique and define the complexes, such as Raptor in mTORC1 and Rictor in mTORC2. mTORC1 links availability of nutrients (especially amino acids) and growth factor signaling to anabolic processes such as macromolecule synthesis and nutrient storage in proliferating cells and tumor cells. In LPS-activated DCs, mTORC1 activity inhibits oxidative metabolism, apparently by stimulating production of NO98, which is a long-appreciated toxin to the ETC99. Importantly, although Akt and mTORC1 are both activated downstream of TLR signaling, Akt activity can be dissociated from mTORC1 activity in this setting95. Relevant metabolic targets of Akt and mTORC1 in control of macrophage activation remain incompletely characterized and represent an important avenue of future investigations. The Akt target HIF-1α has been demonstrated to increase in macrophages following LPS stimulation100, supporting an upregulation of glycolysis, the process by which glucose is incompletely oxidized in the cytosol,
yielding lactate as its final product. HIF-1α is normally an unstable protein but becomes stabilized by LPS-triggered alterations to ETC activity that increases mitochondrial ROS (mtROS) production. Moreover, LPS signaling increases HIF-1α activity by upregulating the PKM2 isoform of the metabolic enzyme pyruvate kinase. In addition to its role in glycolysis, PKM2 is a HIF-1α coactivator, which binds to HIF-1α to enhance its transcriptional activity. Finally, LPS acutely inhibits activation of AMP protein kinase (AMPK), a key regulator of cellular energy homeostasis that stimulates ATP-generating metabolic processes (i.e. oxidation of fatty acids) in response to increasing ADP/ATP ratio, but prolonged LPS exposure leads to enhanced phosphorylation and activation of AMPK due to autocrine/paracrine IL-10 signaling through the IL-10 receptor. Thus, LPS signaling co-opts metabolic machinery to coordinate metabolic shifts, which are described in more detail below.

Early studies demonstrating increased expression of the glucose transporter GLUT1 on M1 macrophages suggested that LPS activation preferentially boosts glucose utilization. Experimental attenuation of glycolysis reduces inflammatory cytokine production and bacterial killing, indicating that glucose utilization in glycolysis is necessary for optimal induction of a pro-inflammatory phenotype. This metabolic switch is driven by LPS-induced activation of Akt to enhance glucose uptake, concomitant with HIF-1α-dependent upregulation of multiple genes encoding glycolytic enzymes. For example, upregulation of PFKFB3, a tissue-specific isoform of PFKFB, drives overall flux through glycolysis, while induction of PDK1, a negative regulator of pyruvate oxidation in the mitochondria, and LDHA, the enzyme that converts pyruvate into lactate, increases aerobic glycolysis. This upregulation of glycolysis at the expense of mitochondrial respiration is referred to as the “Warburg effect” after the German scientist Otto
Warburg, who discovered that cancer cells prefer to generate ATP from glycolysis rather than respiration even in the presence of sufficient oxygen\textsuperscript{111,112}. Glycolytic production of ATP may be important to fuel optimal induction of effector activities and maintain viability in macrophages, given that oxidative metabolism is actually impaired. Glucose oxidation in the mitochondria at very early timepoints before impairment of respiration appears to be important for Ac-CoA and phospholipid synthesis and inflammatory cytokine production in LPS-stimulated DCs and macrophages\textsuperscript{95}. How glucose oxidation and glycolysis are coordinated is incompletely understood but seems to be a pivotal node in control of classical macrophage polarization, as various manipulations that alter this balance perturb the activation phenotype\textsuperscript{108,113}.

In addition to the above changes in glycolysis, TLR signaling also results in reprogramming of the tricarboxylic acid cycle (TCA cycle, or Krebs cycle), the series of oxidative processes in the mitochondrial matrix formulated in the theory of the “citric acid cycle” proposed in 1937 by Hans Krebs and William Johnson. This central pathway in carbon substrate metabolism is necessary for oxidation of glucose-derived pyruvate, fatty acids, and glutamine to generate ATP in the ETC and provide biosynthetic precursors for numerous cellular processes. A series of important experiments in which substrates containing heavy isotopes of carbon were fed to macrophages during LPS stimulation to trace their flux through intermediary metabolism revealed two “breaks” in the TCA cycle of LPS-stimulated macrophages\textsuperscript{114}. The first break regulates the fate of the TCA cycle intermediate citrate, which is normally converted to $\alpha$-ketoglutarate by IDH1. In these macrophages, downregulation of IDH1, but upregulation of immunoresponsive gene 1 (IRG1), an enzyme with decarboxylase activity for citrate-derived aconitase, drives the production of itaconate. By inhibiting the glyoxylate cycle, a variation of the TCA cycle discovered by Krebs’
student Hans Kornberg and found in microorganisms, itaconate has direct microbicidal activity against several pathogens. In addition, itaconate is believed to competitively inhibit succinate dehydrogenase (SDH) in the TCA cycle leading to the second “break”. Because SDH is a subunit of complex II (CII) of the ETC, itaconate production perturbs ETC activity and oxidative metabolism, and has been linked to regulation of mtROS production and inflammatory gene induction. Of note, itaconate has also been demonstrated to activate the anti-inflammatory and antioxidant transcription factor NRF2 via alkylation of the redox-sensing protein KEAP1 (kelch-like ECH-associated protein 1) and through electrophilic stress, both of which may underpin itaconate’s inhibitory effects on inflammatory cytokine production.

As described above, altered carbon flux through the TCA cycle impacts LPS activation. As an additional adaptation to the second break in the TCA cycle, LPS-stimulated macrophages utilize the aspartate-arginosuccinate shunt to re-supply fumarate and also produce arginine (a substrate of iNOS) through a series of reactions involving transformation of aspartate and citrulline via the activity of argininosuccinate synthase (Ass1) and argininosuccinate lyase (Asl). In the context of Mycobacterium infection, macrophages initially import extracellular arginine and export citrulline (a by-product of arginine metabolism by iNOS) but switch to citrulline import for arginine regeneration through this shunt only after extracellular arginine becomes depleted. Such import of extracellular arginine may allow the macrophages to limit T cell responses.

Another key node in glucose utilization, the pentose phosphate pathway (PPP), has also been shown to control classical macrophage activation. The PPP is a glycolytic shunt that produces NADPH for maintaining cellular redox balance and nucleotide synthesis. LPS increases PPP
activity in macrophages by downregulating the sedoheptulose kinase carbohydrate kinase-like protein CARKL, and knockdown or overexpression of CARKL perturbed induction of LPS-inducible genes\textsuperscript{122}. NADPH production is also likely to be important for fueling the activities of NADPH oxidases (NOX) and iNOS, which produce ROS and NO, respectively, and are important components of antimicrobial defense.

More recent studies indicate that glutamine consumption is also enhanced following LPS stimulation and serves to compensate for the first break in the TCA cycle below citrate by supplying α-ketoglutarate in a process called anaplerosis\textsuperscript{114,123}, in which carbons are funneled into the TCA cycle at points other than pyruvate (of note, anaplerosis is another contribution from Kornberg\textsuperscript{124}).

**Mitochondria as hubs in inflammatory activation of macrophages**

It has long been appreciated that stimulation of macrophages and dendritic cells with LPS or LPS+IFN-γ shuts down oxidative metabolism. An underpinning mechanism could be interactions of NO with iron–sulfur clusters in the ETC, since iNOS inhibition rescues oxidative metabolism\textsuperscript{125,126}. In addition, M1 macrophages produce ROS from ETC complex I (CI), which has been linked to enhanced bactericidal activity\textsuperscript{127}. The underlying mechanism involved mitochondria-localized interactions between TRAF6 and ECSIT, a regulator of CI assembly. More recently, a flurry of papers has further underscored the connection between oxidative metabolism, ETC activity, and inflammatory gene induction, thus positioning the mitochondria at the center of LPS activation\textsuperscript{79,82,128}.
The IRG1/itaconate axis appears to play a key role in regulating oxidative metabolism and inflammatory gene induction in M1 macrophages. As discussed above, induction of IRG1 allows for itaconate production and inhibition of SDH\textsuperscript{117}. Addition of exogenous itaconate inhibits oxidative metabolism, while IRG1 deficiency enhances oxidative metabolism\textsuperscript{129}. Importantly, itaconate addition inhibits production of IL-6 and IL-12, while IRG1 deficiency augments their production\textsuperscript{129}, suggesting that the IRG1/itaconate axis serves as a built-in negative feedback that impinges on the ETC to limit excessive inflammatory responses. Another recent study also described ETC alterations during \textit{E. coli} infection, specifically a transient increase in CII activity but a decrease in CI activity\textsuperscript{130}. Increased CII activity was triggered by the activities of NADPH oxidase and the Src kinase Fgr, which phosphorylates the SDHA subunit of CII. Consistent with the other studies referenced in this section, ETC remodeling was linked to inflammatory gene induction\textsuperscript{130}.

How exactly these ETC alterations regulate inflammatory gene induction remains unclear. It has been suggested that CII activity drives reverse electron transport (RET) to potentiate mtROS production and HIF-1α stabilization\textsuperscript{101}. High CII activity in a setting of impaired respiration (i.e. due to elevated NO) leads to over-reduction of the mobile electron carrier ubiquinone (Q), which under normal conditions accepts electrons from CI, II, and glycerol 3-phosphate dehydrogenase (GPD2) and donates to CIII. This over-reduction changes the thermodynamic configuration of the ETC such that the reduction potential of the oxidized/reduced Q pair favors RET from reduced Q (ubiquinol, QH\textsubscript{2}) to CI\textsuperscript{131–133}. Such RET is associated with mtROS production from the Q binding site in CI, which may stabilize HIF-1α to allow for transcriptional induction of its target \textit{Il1b}\textsuperscript{123,134}. In support of this model, mtROS scavengers and pharmacological inhibitors of CII reduce
inflammatory gene induction\textsuperscript{101,129}. Ectopic expression of the mitochondrial alternative oxidase AOX, which should alleviate RET by accepting electrons from over-reduced Q/QH\textsubscript{2}, also recapitulates the phenotype\textsuperscript{101}. However, ETC activities similarly potentiate induction of inflammatory genes not thought to be regulated by HIF-1\(\alpha\) (e.g., \textit{Il6}), so clarification of additional underpinning mechanisms remains an outstanding question.

\textbf{Integration of metabolic signals by chromatin}

A final mode of metabolic regulation of inflammatory macrophage polarization is through gene-specific chromatin modifications. Recall that LPS induces gene-specific changes in promoter region histone acetylation to regulate activation and tolerization\textsuperscript{66,75}. More recent studies have indicated that metabolism impinges on chromatin for transcriptional control of macrophage activation. Cheng et al. showed that priming with the \textit{Candida albicans} product β-glucan enhances the ability of a subsequent LPS challenge to induce genes encoding inflammatory cytokines. This process is dependent on Akt and mTORC1 as well as glycolytic activity and correlates with chromatin changes at the promoters of these genes\textsuperscript{135}. There is also evidence macrophages polarized to an anti-inflammatory (M2) phenotype by the Th2 cytokine IL-4 also rewire glucose metabolism in an Akt-mTORC1-dependent manner to supply acetyl-CoA (Ac-CoA) for histone acetylation and activation of a subset of IL-4-inducible genes\textsuperscript{136}. Thus, consistent with observations in other cell types, shifts in macrophage metabolism can regulate transcriptional responses through modulation of substrates used for chromatin modifications\textsuperscript{137,138}. Elucidating new regulatory modules and further characterizing known pathways in macrophage metabolism that are affected downstream of pathogen recognition will illuminate novel targets for therapeutic intervention to manipulate host responses to microbial infection.
Thesis summary

This thesis reports on several novel insights into the metabolic underpinnings of macrophage inflammatory responses, specifically at the levels of glucose oxidation and mitochondrial respiration. In Chapter 2, we describe histone acetylation as a fundamental mechanism by which glucose metabolism fuels inflammatory gene induction and underpins inflammatory gene suppression during LPS activation and tolerance, respectively. The Ac-CoA-producing enzyme ATP-citrate lyase (ACLY) and the histone acetyltransferase p300 are implicated in this integration of enhanced glucose metabolism with increased histone acetylation. Furthermore, we provide an important update and revision to the current paradigm of how mitochondrial respiration supports inflammatory gene induction, demonstrating that 1) ETC activity critically underpins induction and suppression of inflammatory genes via regulation of carbon flux through the TCA cycle to supply substrate to the ACLY-p300 axis for histone acetylation, and 2) that the glycerol phosphate shuttle and its key regulatory enzyme GPD2, a component of the ETC seldom included in textbook schemes of the respiratory chain, is a novel LPS-inducible module in macrophage metabolism that drives enhanced glucose oxidation and thus stands at the epicenter of the metabolic regulation of macrophage inflammatory responses *in vitro* and *in vivo*. Thus we propose a new paradigm for how glucose utilization underpins M1 macrophage activation and function, defining a model in which GPD2 and the glycerol phosphate shuttle integrate the extent of microbial stimulation with glucose oxidation to balance the beneficial and detrimental effects of the inflammatory response.

In Chapter 3, additional experiments that extend and further support this model are reported, including 1) implication of Akt-dependent signaling in regulating LPS-inducible changes in respiration and inflammatory gene induction such that pharmacological inhibition of Akt recapitulates the effects of pharmacological and genetic inhibition of glucose oxidation on
macrophage LPS activation and tolerance, and 2) discovery of a subset of LPS-inducible genes that display enhanced Akt-dependent expression in tolerant, restimulated macrophages. Induction of these primed (P) genes is supported by the activity of Ac-CoA synthetase short-chain family member 2 (ACSS2), which produces Ac-CoA from acetate, serving as an alternative pathway fueling histone acetylation in the glucose-oxidation-deficient tolerant state. Finally, Chapter 4 includes further discussion of results and future directions.
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Chapter 2

Glycerol phosphate shuttle enzyme GPD2 drives glucose oxidation to regulate macrophage inflammatory responses

This chapter is a reproduction of a published manuscript with minor modifications, including incorporation of unpublished data.

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ABSTRACT

Macrophages are activated during microbial infection to coordinate inflammatory responses and host defense. Here we show that in macrophages activated by bacterial lipopolysaccharide (LPS), mitochondrial glycerol 3-phosphate dehydrogenase (GPD2) regulates glucose oxidation to drive inflammatory responses. GPD2, a component of the glycerol phosphate shuttle (GPS), boosts glucose oxidation to fuel acetyl-CoA production, histone acetylation and inflammatory gene induction. While acute LPS exposure drives macrophage activation, prolonged exposure triggers entry into LPS tolerance, where macrophages orchestrate immunosuppression to limit the detrimental effects of sustained inflammation. We find that the shift in the inflammatory response is modulated by GPD2, which coordinates a shutdown of oxidative metabolism that limits acetyl-CoA availability for histone acetylation at inflammatory genes, thus contributing to suppression of inflammatory responses. Therefore, GPD2 and the GPS integrate the extent of microbial stimulation with glucose oxidation to balance the beneficial and detrimental effects of the inflammatory response.
INTRODUCTION

Microbial infection elicits an inflammatory response characterized by activation of immune cells and elevated local and circulating pro-inflammatory cytokines (e.g. IL-6 and IL-1β) and enhanced antimicrobial activity. Macrophages are key regulators of innate immunity and inflammation, coupling recognition of infection by detection of Pathogen-Associated Molecular Patterns (PAMPs) to induction of inflammatory responses. For example, upon detection of the gram-negative bacterial component lipopolysaccharide (LPS), macrophages are activated (hereafter LPS activation) to coordinate the induction of innate and adaptive immune responses by producing chemoattractants and inflammatory cytokines. However, in response to overwhelming infection or protracted exposure to LPS, activated macrophages shift to a refractory or tolerant state (hereafter LPS tolerance)^1,^2^_. Tolerant macrophages mount a blunted response to further LPS challenge, and produce markedly lower levels of inflammatory cytokines, contributing to the induction of whole-body immunosuppression. Such immunosuppression limits the detrimental effects of sustained inflammation, which otherwise damages multiple organ systems and leads to mortality^3_. Therefore, acute microbial infection elicits an inflammatory response that confers host defense, but persistent and overwhelming microbial infection triggers inflammation followed by a protracted period of immunosuppression as a protective mechanism to mitigate multiorgan dysfunction^1,^2,^3_.

Cellular metabolism is increasingly appreciated for its role in regulating macrophage activation and functions as well as innate immunity and inflammation. Cellular metabolism interfaces with signal transduction and other regulatory processes, and genetic and pharmacological modulation of metabolism can influence macrophage activation and functions^4,^5,^6_. Many such studies have
used macrophage activation (or polarization) by polarizing signals like LPS and the cytokine IL-4 as a model to elucidate fundamental mechanisms by which metabolism regulates macrophage activation. One paradigm that has emerged is that in macrophages activated by IL-4, which orchestrate Type 2 inflammatory responses like tissue repair and fibrosis, oxidative metabolism is increased to boost so-called M2 activation\(^4,6,7,8\). Increased oxidative metabolism upregulates the production of citrate, a TCA cycle intermediate, which is used by the enzyme ATP-citrate lyase (ACLY) to generate a nucleocytosolic pool of acetyl-CoA (Ac-CoA). Because Ac-CoA is the metabolic substrate for histone acetylation, ACLY-dependent Ac-CoA production links increased oxidative metabolism to enhanced expression of IL-4-inducible genes during M2 activation\(^9\). In addition, increased oxidative metabolism supports the production of another TCA intermediate alpha-ketoglutarate (AKG), a cofactor for the histone demethylase Jmjd3, to regulate histone demethylation and fuel IL-4-inducible gene expression\(^10\). It is noteworthy that in addition to M2 activation, increased oxidative metabolism is a cornerstone of cellular activation, differentiation, and proliferation in many contexts\(^11\). In contrast, a metabolic hallmark of LPS-stimulated macrophages is a robust shutdown of oxidative metabolism\(^4,5,12\). Such shutdown appears to be mediated by multiple mechanisms, including induction of iNOS and production of nitric oxide (NO), which modifies multiple complexes in the electron transport chain (ETC)\(^13,14\); induction of IRG1 and production of itaconate, a metabolite that inhibits Complex II of the ETC\(^15\); and induction of reverse electron transport (RET), which counters oxidative phosphorylation\(^16\). In addition to respiratory shutdown, another metabolic hallmark of LPS-stimulated macrophages is upregulation of aerobic glycolysis, the process by which glucose-derived pyruvate is shunted away from mitochondrial oxidative metabolism towards the production of lactate\(^4,5,8,12,17\). It has been proposed that induction of aerobic glycolysis, at the expense of mitochondrial glucose oxidation,
allows for rapid ATP production during infection by fast-replicating microbes. Beyond this bioenergetic consideration, however, it is unclear how respiration shutdown and aerobic glycolysis induction contribute to the inflammatory responses of LPS-stimulated macrophages\textsuperscript{4,5}.

Here we show that contrary to the paradigm that LPS-stimulated macrophages drive aerobic glycolysis and shut down respiration, macrophages acutely exposed to LPS upregulate glucose oxidation in the mitochondria. Glucose oxidation supports production of Ac-CoA, thus increasing substrate availability for histone acetylation at the inflammatory genes \textit{Il6} and \textit{Il1b}. A key regulator of such glucose oxidation is GPD2, mitochondrial glycerol phosphate dehydrogenase, a rate-limiting enzyme in the glycerol 3-phosphate shuttle (GPS). LPS signaling increases GPD2 activity and flux through the GPS, and in macrophages deficient for GPD2, glucose oxidation, histone acetylation, and \textit{Il6} and \textit{Il1b} gene induction in response to acute LPS exposure is attenuated. Importantly, we show that there is a metabolic shift from increased to decreased respiration over the course of LPS exposure as macrophages transition from activation to tolerance, and that in tolerant macrophages, decreased respiration limits the ability of glucose oxidation to support Ac-CoA production thus contributing to the inability to induce \textit{Il6} and \textit{Il1b}. Moreover, the shift from increased to decreased oxidative metabolism is regulated by GPD2-mediated glucose oxidation, since block of glucose utilization or GPD2 deficiency prevents this shift, leading to rescue of histone acetylation and \textit{Il6} and \textit{Il1b} induction. We propose that GPD2 boosts oxidative metabolism to drive inflammatory gene induction during LPS activation but suppresses oxidative metabolism to contribute to inhibition of inflammatory gene induction during LPS tolerance. Therefore, GPD2 and the GPS appear to couple the duration and extent of microbial stimulation to the balance of inflammatory response induction and suppression.
RESULTS

ACLY supports histone acetylation and inflammatory gene induction during LPS activation

In a recent study, we proposed that one mechanism by which increased oxidative metabolism supports M2 activation is via enhanced production of Ac-CoA, thus bolstering histone acetylation at IL-4-inducible genes. An important regulator of this process is ACLY, an enzyme that produces a nucleocytoplasmic pool of Ac-CoA that can be used for histone acetylation\(^9\). We started the current study by asking about the role of ACLY in macrophages acutely exposed to LPS (hereafter LPS-activated macrophages).

We found that LPS stimulation of bone marrow derived macrophages (BMDMs) rapidly increased ACLY S455 phosphorylation, an activating phosphorylation\(^1^8\), indicating that LPS signaling enhanced ACLY activation (Fig. 2.1a). Next, we asked how ACLY activity influenced Il6 and Il1b, prototypes of the LPS-inducible inflammatory response, during LPS activation. In this and all other experiments examining LPS activation in our study, all parameters of LPS activation are assessed between 0-3 hours after LPS stimulation. We found that treatment with the ACLY inhibitor SB-204990 attenuated histone acetylation at Il6 and Il1b gene promoters (Fig. 2.1b) and impaired Il6 and Il1b gene induction (Fig. 2.1c).
Figure 2.1 ACLY activity supports inflammatory gene induction in LPS-activated macrophages. BMDMs were stimulated with LPS for 0-3 hours with or without ACLY inhibitor SB 204990 (ACLYi) pretreatment, followed by immunoblot analysis of ACLY phosphorylation at Ser455 (a), ChIP-qPCR analysis of histone acetylation at the *Il6* and *Il1b* promoters (b), and qPCR analysis of *Il6* and *Il1b* gene expression (c). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (c) or triplicates (b)).

Two structurally distinct ACLY inhibitors similarly reduced LPS-inducible histone acetylation and expression of *Il6* and *Il1b* (Supplementary Fig. 2.1a,b), suggesting specificity in the effects of the ACLY inhibitors. Consistent with the idea that histone acetylation and histone acetyltransferases including CBP/p300 in particular support LPS-inducible gene expression\(^{19,20,21,22,23}\), inhibition of p300 with the selective inhibitor C646 reduced histone acetylation and induction of *Il6* and *Il1b* (Supplementary Fig. 2.1c,d). Together these findings support the idea that ACLY activity fuels histone acetylation and inflammatory gene induction in LPS-activated macrophages.
Glucose oxidation is enhanced during LPS activation to fuel histone acetylation and inflammatory gene induction in macrophages

ACLY-dependent Ac-CoA production depends on availability of the TCA cycle intermediate citrate and oxidative metabolism, therefore the data from our ACLY analysis was in apparent conflict with the paradigm that LPS-stimulated macrophages shut down respiration. By measuring oxygen consumption, we found that acute LPS exposure triggered a small but significant and highly-reproducible increase in mitochondrial oxidative metabolism (Fig. 2.2). While longer LPS exposure led to a progressive and sustained decrease in respiration, the early increase was transient and likely explains the many reports that conclude that LPS stimulated macrophages shut down respiration (Supplementary Fig. 2.2a). Because LPS stimulation is well-known to enhance glucose utilization (Supplementary Fig. 2.2b and 4), we asked if the early increase in oxidative metabolism could be fueled by glucose oxidation. Indeed, treatment with the glucose utilization inhibitor 2-deoxyglucose (2DG) abrogated the early increase in oxidative metabolism (Fig. 2.2).

Figure 2.2 LPS activation increases glucose oxidation in macrophages. Mitochondrial respiration, determined by oxygen consumption rate (OCR), in BMDMs stimulated with LPS for 1h +/- 2DG pretreatment. Basal and maximal respiration were calculated by subtraction of non-mitochondrial respiration (OCR post-Rot/AA) from mean OCR pre-oligomycin and post-FCCP injection, respectively. *p ≤0.05, **p ≤0.01, ***p ≤0.001 (Student’s t-test). Data are from one experiment representative of ten experiments (mean and s.e.m. of octuplets).
Glucose oxidation supports inflammatory gene induction in LPS-activated macrophages by providing carbon substrate for histone acetylation. BMDMs were stimulated with LPS for 0-3 h, +/- 2DG pretreatment, followed by analysis of the following parameters. $^{13}$C$_6$-glucose tracing into citrate-isocitrate (a) and Ac-CoA (b) during LPS activation presented as abundance of m+2 isotopologue relative to all other isotopologues. (c) Incorporation of carbons from $^{14}$C$_6$-glucose into histones in naïve and LPS-activated BMDMs. (d) Promoter region histone acetylation and (e) expression of Il6 and Il1b and (f) IL-6 cytokine production during LPS activation +/- 2DG pretreatment. *p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001 (Student’s t-test). Data are from one experiment representative of three (a-c) or four (d-f) experiments (mean and s.e.m. of duplicates (e,f), triplicates (d), or quadruplicates (a,b)).

To further explore the possibility that glucose oxidation supports Ac-CoA production and inflammatory gene induction in LPS-activated macrophages, we turned to glucose isotope tracing experiments using uniformly $^{13}$C-labeled glucose. Consistent with this possibility, we found that acute LPS stimulation enhanced labeling of glucose-derived carbons into citrate/isocitrate (Fig. 2.3a). Furthermore, we observed a robust increase in labeling of (m+2) Ac-CoA, indicating that glucose oxidation fueled Ac-CoA production during LPS activation (Fig. 2.3b). Tracing glucose
carbons all the way into histones, we were also able to demonstrate increased labeling of histones in LPS-activated macrophages (Fig. 2.3c). Such glucose metabolism supported histone acetylation at the promoters of Il6 and Il1b, since 2DG treatment attenuated LPS-inducible histone acetylation at these promoters (Fig. 2.3d). Correlating with effects on promoter histone acetylation, 2DG treatment attenuated LPS-inducible expression of Il6 and Il1b (Fig. 2.3e). Finally, 2DG treatment reduced production of IL-6 cytokine in LPS-activated macrophages (Fig. 2.3f), while production of mature IL-1β protein in BMDMs requires inflammasome activation\textsuperscript{24} and was not further examined. Incubation of BMDMs in glucose free media also led to diminished histone acetylation and induction of Il6 and Il1b, although the effects were more modest compared to 2DG treatment likely because glycogen stores are being tapped to fuel glycolysis (Supplementary Fig. 2.3).

Together the mitochondrial oxygen consumption, glucose tracing and functional analyses suggest that Ac-CoA availability can be limiting for histone acetylation and inflammatory gene induction during LPS activation, and that LPS stimulation boosts glucose oxidation leading to increased Ac-CoA availability for supporting histone acetylation and inflammatory gene induction.

**GPD2 regulates glucose utilization and oxidation in LPS-activated macrophages**

In the cytosolic phase of glucose oxidation, glucose metabolism is coupled to NADH production (at the step catalyzed by GAPDH), which could slow down glucose oxidation if cytosolic NAD$^+$ is not regenerated. The malate-aspartate shuttle (MAS) is thought to be ubiquitously expressed and deployed during glucose oxidation to regenerate cytosolic NAD$^+$ while transferring glucose-derived electrons, in the form of NADH, to Complex I of the ETC\textsuperscript{25}. However, the kinetics by which glucose carbons label histones in our experiment (1.5h; see Fig. 2.3c), relative to steady-state histone labeling by the relevant carbon substrate in other contexts (~24h)\textsuperscript{26, 27}, suggested an
alternative mechanism for rapidly mobilizing glucose oxidation in LPS-activated macrophages. Using metabolomics, we identified the glycerol phosphate shuttle (GPS) as a top metabolic pathway regulated during LPS activation (Fig. 2.4a). The GPS is a glycolytic shunt that delivers glucose-derived electrons to the ETC. In this shuttle, the NADH produced by GAPDH is used to reduce dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G3P) in a reaction catalyzed by cytoplasmic glycerol 3-phosphate dehydrogenase (cGPDH, or GPD1); G3P then serves as a source of electrons for the ETC through the activity of mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH, or GPD2). GPD2 is a non-canonical component of the ETC that couples oxidation of G3P back to DHAP with reduction of its FAD cofactor to FADH₂, from which electrons flow downward through the ETC to drive mitochondrial respiration (Fig. 2.4b and Supplementary Fig. 2.4).

Figure 2.4 LPS activation enhances glycerol phosphate shuttle activity in macrophages. (a) Global steady-state metabolite profiling of unstimulated and LPS-stimulated BMDMs, with 10 most-significantly enhanced pathways presented, ranked by p value, and pathways of highest interest in red text. (b) Schematic depicting the spatial and biochemical position of the GPS and GPD2 at the nexus between glycolysis and electron transport. (c) Expression of GPS enzymes Gpd1 and Gpd2 in BMDMs +/- 1.5 h LPS stimulation. *p ≤ 0.05, **p ≤ 0.01 (Student’s t-test). Data are from one experiment representative of one (a) or three (c) experiments (mean and s.e.m. of duplicates (c) or quadruplicates (a)).
Like the MAS, the GPS regenerates cytosolic NAD\(^+\) to maintain cytosolic redox balance during glucose oxidation. Unlike the MAS, GPS activity is restricted to a limited number of tissues because GPD2 is expressed in a tissue-specific manner\(^{28,29}\). The physiological role of the GPS is poorly understood, but it has been proposed that GPD2/GPS activity allows for high rates of glucose oxidation, given that GPD2 activity enhances glucose-stimulated insulin secretion in pancreatic β-cells\(^{29,30}\). Nothing is known of GPD2 in macrophages except that its biochemical activity is increased after microbial infection\(^{31}\).

Consistent with the identification of the GPS in our metabolomics analysis, we found that BMDMs expressed GPD2 and that LPS stimulation further augmented GPD2 transcript and protein, while transcript levels of GPD1 were very modestly affected by LPS stimulation (Fig. 2.4c and Supplementary Fig. 2.5a). Furthermore, LPS stimulation increased GPD2 activity, as assessed by Seahorse extracellular flux analysis of G3P-driven respiration (Supplementary Fig. 2.5b and see Discussion). Using Crispr-Cas9 genome editing, we generated mice lacking Gpd2 (Fig. 2.5a). GPD2 KO mice are viable, fertile, and appear normal. BMDMs from GPD2 KO mice develop normally as indicated by CD11b and F4/80 expression (Fig. 2.5b). However, GPD2 KO BMDMs lack GPD2 protein (Fig. 2.5c) and activity, as assessed by G3P-driven respiration (Fig. 2.5d).
Figure 2.5 Generation of GPD2 KO mice for studying the role of glycerol phosphate shuttle activity in macrophage inflammatory responses. (a) Guide RNA (gRNA) targeted to exon 6 in Gpd2 to generate whole-body GPD2 KO mice using CRISPR-Cas9 genome editing. (b) FACS analysis of F4/80 and CD11b expression on BMDMs from WT and GPD2 KO mice. Numbers indicate percent of cells in gate (F4/80^+CD11b^+). (c) Immunoblot analysis of GPD2 in naïve WT and GPD2 KO BMDMs. Bar graph represents fold change in OCR after addition of G3P, ADP, and rotenone (Rot). ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment representative of one (c) or three (d) experiments (mean and s.e.m. of sextuplets (d)).

Using glucose tracing experiments, we found that acute LPS stimulation of WT BMDMs led to a dramatic increase in the abundance of total and (m+3) G3P, indicative of enhanced flux through the GPS. In GPD2 KO BMDMs, abundance of total and (m+3) G3P was further increased, consistent with accumulation of the GPD2 substrate in the absence of GPD2 activity (Fig. 2.6a). We also found that the early, 2DG-sensitive increase in oxidative metabolism (Fig. 2.2) was diminished in GPD2 KO BMDMs (Fig. 2.6b), indicating a role for GPD2 in mediating glucose oxidation in LPS-activated macrophages. Importantly, the well-established ability of LPS to
upregulate glucose uptake and utilization was impaired in GPD2 KO BMDMs (Fig. 2.6c), indicating that GPS activity is rate-limiting for glucose utilization in LPS-activated macrophages. In line with reduced glucose utilization and oxidation, GPD2 KO BMDMs displayed less LPS-inducible glucose labeling into many glycolytic and TCA intermediates (Supplementary Fig. 2.6) and attenuated aerobic glycolysis (Fig. 2.6d). Taken together, our metabolic analyses of GPD2 KO BMDMs identify the GPS as a critical regulator of glucose utilization and oxidation in LPS-activated macrophages.

**Figure 2.6** The GPS enzyme GPD2 regulates glucose oxidation in LPS-activated macrophages. Wild-type (WT) and GPD2 KO BMDMs were stimulated with LPS for 0-3 h, followed by analysis of the following parameters. (a) $^{13}$C$_6$-glucose tracing into glycerol 3-phosphate (presented as peak areas for each isotopologue) in WT and GPD2 KO BMDMs. (b) Basal and maximal mitochondrial respiration, and (c) uptake of $^3$H-deoxy-D-glucose in WT and GPD2 KO BMDMs. (d) Extracellular acidification rate (ECAR), indicative of aerobic glycolysis, in naïve and LPS-activated WT and GPD2 KO BMDMs. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ (Student’s t-test). Data are from one experiment representative of three (a,c,d) or four (b) experiments (mean and s.e.m. of triplicates (a,c) or sextuplets (b,d)).
GPD2-mediated glucose oxidation fuels inflammatory gene induction during LPS activation

We next sought to determine how reduced glucose utilization and oxidation in GPD2 KO BMDMs affects LPS activation. We found that in GPD2 KO BMDMs, LPS-inducible labeling of glucose carbons into citrate (Fig. 2.7a) and Ac-CoA was reduced (Fig. 2.7b). LPS-inducible histone acetylation at the Il6 and Il1b promoters was also attenuated (Fig. 2.7c), leading to diminished Il6 and Il1b gene induction (Fig. 2.7d). Finally, GPD2 KO BMDMs produced reduced levels of IL-6 (Fig. 2.7e). Collectively, our data indicates that GPS activity boosts glucose oxidation, leading to enhanced carbon substrate availability for histone acetylation and increased inflammatory gene induction (Supplementary Fig. 2.7).
Figure 2.7 GPD2 activity controls inflammatory gene induction in LPS-activated macrophages by regulating Ac-CoA production and histone acetylation. WT and GPD2 KO BMDMs were stimulated with LPS for 0-3 h, followed by analysis of the following parameters. (a,b) $^{13}$C$_6$-glucose tracing into (a) citrate and (b) Ac-CoA in WT and GPD2 KO BMDMs, presented as LPS-induced fold change in percent m+2 enrichment. (c) Promoter region histone acetylation and (d) expression of Il6 and Il1b and (e) IL-6 cytokine production in WT and GPD2 KO BMDMs. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ (Student’s t-test). Data are from one experiment representative of three (a,b) or four (c-e) experiments (mean and s.e.m. of duplicates (d,e), triplicates (a,c), or quadruplicates (b)).

Glucose utilization drives shutdown of respiration and suppression of inflammatory gene induction in LPS tolerant macrophages

While the data above indicated that the early increase in glucose oxidation fuels inflammatory gene induction after acute LPS exposure, what could be the rationale and relevance of the shift to decreased oxidative metabolism during prolonged exposure (Supplementary Fig. 2.2a)? Importantly, the inflammatory response is well established to shift over the course of LPS exposure and microbial infection, in vitro and in vivo. Acute LPS stimulation and microbial infection trigger induction of inflammation responses, while prolonged LPS stimulation and microbial infection drive entry into a state of immunosuppression. Therefore, we hypothesized that consistent with the role of increased oxidative metabolism in supporting inflammatory gene induction during LPS activation, the decreased oxidative metabolism in LPS tolerant macrophages could limit Ac-CoA availability for histone acetylation, thus contributing to inflammatory gene suppression.

This hypothesis was tested using a well-established model of LPS tolerance, where an extended exposure to LPS triggered tolerance as indicated by the inability to upregulate Il6 and Il1b after LPS rechallenge (Fig. 2.8 and 1-2). In contrast to LPS activation which was examined at early time points (0-3 h), LPS tolerance throughout this study was examined at late time points, 12-28 h after LPS stimulation.
Figure 2.8 Naïve and LPS tolerant macrophages exhibit profound differences in responsiveness to LPS stimulation. (a) Schematic of workflow for in vitro LPS tolerance experiments. BMDMs were left unstimulated (naïve, N) or stimulated with LPS for 24h to induce LPS tolerance (tolerance, T). LPS responsiveness was assessed by stimulating naïve (N+L) or re-stimulating tolerant (T+L) BMDMs with LPS. In the T+L+2DG condition, 2DG was added during tolerance induction and washed out prior to LPS restimulation in the absence of inhibitor. Naïve and Tolerant cells were collected as baseline controls (N and T). (b) Representative induction of Il6 and Il1b genes in BMDMs treated according to (a).

We found that in contrast to naïve macrophages where LPS exposure drove glucose conversion into citrate and Ac-CoA, in tolerant macrophages LPS exposure failed to drive glucose conversion into citrate and Ac-CoA (Fig. 2.9a,b). Therefore, tolerance-associated shutdown of respiration was incompatible with LPS-inducible glucose oxidation and Ac-CoA production.

Figure 2.9 Glucose utilization impairs respiration in tolerant macrophages. $^{13}$C$_6$-glucose tracing into citrate-isocitrate (a) and Ac-CoA (b) in N, N+L, T, and T+L conditions. Incorporation of labeled-glucose-derived carbons presented as abundance of m+2 isotopologue relative to all other isotopologues. (c) Maximal respiration after 12h LPS stimulation +/- 2DG pretreatment. *p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of quadruplicates (a,b) or octuplets (c)).
Serendipitously, we found that tolerance-associated respiration shutdown was regulated by glucose utilization. 2DG treatment during LPS tolerance induction led to a striking although incomplete rescue of respiration, indicating that glucose utilization was necessary for optimal inhibition of oxidative metabolism (Fig. 2.9c). Furthermore, 2DG-mediated respiration rescue was associated with a partial recovery of histone acetylation and inflammatory gene induction in tolerant macrophages. While LPS stimulation of naïve and tolerant BMDMs led to robust and weak increases in histone acetylation at the Il6 and Il1b promoters respectively (comparing N and N+L with T and T+L), 2DG treatment during tolerance induction partially restored histone acetylation at the Il6 and Il1b promoters (comparing T, T+L and T+L+2DG) (Fig. 2.10a). Moreover, 2DG treatment during tolerance induction led to recovery of Il6 and Il1b induction (Fig. 2.10b) and IL-6 production (Fig. 2.10c).

Figure 2.10 Glucose utilization limits inflammatory gene induction in tolerant macrophages. (a) Promoter region histone acetylation and (b) expression of Il6 and Il1b and (c) IL-6 cytokine production in N, N+L, T, and T+L+2DG conditions. *p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001 (Student’s t-test). Data are from one experiment representative of three (a) or four (b,c) experiments (mean and s.e.m. of duplicates (b,c) or triplicates (a)).
Of note, such effects of 2DG treatment could be disassociated from global effects on LPS signaling. NF-κB and IRF3 are targets of the two major branches of LPS signaling and master regulators of the LPS-inducible transcriptional response. They are robustly activated by LPS signaling in naïve macrophages and poorly activated in tolerant macrophages, as indicated by IκBα degradation and resynthesis and IRF3 activating phosphorylation respectively (Fig. 2.11a,b). Importantly, 2DG treatment during tolerance induction had marginal effects on NF-κB and IRF3 activation in tolerant macrophages (Fig. 2.11a,b), indicating that the effects of 2DG in recovering Il6 and Il1b induction may not be due to general perturbation of LPS signaling.

Figure 2.11 Glucose utilization impairs respiration and limits inflammatory gene induction in tolerant macrophages without affecting TLR signaling. BMDMs were left unstimulated (N) or tolerized (T) by 24h LPS treatment +/- 2DG pretreatment followed by washout of inhibitor and analysis of LPS-inducible IκBα degradation (a) and IRF3 phosphorylation (b). Data are from one experiment representative of three experiments.

Rather, our data is more consistent with glucose utilization regulating oxidative metabolism to influence inflammatory gene induction. Oxidative metabolism first increases and then decreases over the course of LPS exposure, supporting an LPS-stimulated increased in glucose oxidation and Ac-CoA production during activation but not during tolerance. This shift in oxidative metabolism appears to be modulated by glucose utilization, as 2DG treatment ablates both the early increase and the delayed decrease of respiration. Furthermore, respiration shutdown appears to contribute to the inability of LPS to stimulate histone acetylation and inflammatory gene induction in tolerant
macrophages, since 2DG treatment restores oxidative metabolism and LPS-inducible histone acetylation and inflammatory gene induction to tolerant macrophages.

**GPD2 activity drives suppression of respiration and inflammatory genes during tolerance**

To more rigorously test the role of glucose utilization—and in particular glucose oxidation—in influencing LPS tolerance induction, we turned to GPD2 KO BMDMs. We found that the delayed inhibition of oxidative metabolism was attenuated in GPD2 KO BMDMs, indicating that GPD2 activity was necessary for optimal respiration shutdown (Fig. 2.12a). Tolerance-associated defects in LPS-inducible histone acetylation and inflammatory gene induction were also partially rescued by GPD2 deficiency. While LPS stimulation poorly increased *Il6* and *Il1b* histone acetylation in tolerant WT BMDMs, tolerant GPD2 KO BMDMs displayed enhanced LPS-inducible histone acetylation (Fig. 2.12b). Correlating with the differences in histone acetylation, tolerant GPD2 BMDMs displayed enhanced LPS-inducible expression of *Il6* and *Il1b* (Fig. 2.12c) and production of IL-6 (Fig. 2.12d), indicating that GPD2 activity was needed for optimal tolerance-associated suppression of inflammatory responses.

We also asked if GPD2 influences LPS signaling by examining activation of NF-κB and IRF3. Importantly, GPD2 deficiency led to very modest effects on activation of NF-κB and IRF3 in either the activation or tolerance conditions (Fig. 2.12e,f). Therefore, GPD2 does not appear to exert broad effects on LPS signaling to influence inflammatory gene induction during LPS activation and tolerance.
Figure 2.12 GPD2 activity drives suppression of inflammatory responses in tolerant macrophages. (a) Basal and maximal respiration after 12h LPS stimulation in WT and GPD2 KO BMDMs. Promoter histone acetylation (b), Il6 and Il1b gene expression (c), and IL-6 cytokine production (d) in WT and GPD2 KO BMDMs in the N, N+L, T, and T+L conditions. Immunoblot analysis of LPS-inducible IκBα degradation (e) and IRF3 phosphorylation (f) in naïve and tolerant WT and GPD2 KO BMDMs. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (c,d), triplicates (b), octuplets (a)).
We considered alternative mechanisms by which GPD2-mediated glucose oxidation could contribute to suppression of the inflammatory response in tolerant macrophages. Because 2DG treatment during tolerance induction protected against respiration shutdown and inflammatory gene suppression, we hypothesized that GPD2-mediated glucose oxidation could regulate a shift from increased to decreased oxidative metabolism that contributes to a shift from inflammatory gene induction to suppression. In LPS-stimulated macrophages, multiple factors are thought to modulate respiration shutdown including induction of iNOS, IRG1, and reverse electron transport (RET)\textsuperscript{13, 14, 15, 16}. However, GPD2 deficiency had little effect on Nos2 and Irg1 expression during LPS stimulation (data not shown), therefore we considered a role in induction of RET.

Intriguingly, the etiology and regulation of RET intimated a role in GPD2-mediated regulation of inflammatory responses. In contrast to forward electron transport (FET), which is the normal operation of the ETC, RET is a consequence of overwhelming electron influx into the ETC leading to saturation of electron transport capacity, creating thermodynamic conditions favoring reverse electron flow\textsuperscript{32}. We considered that GPD2-driven FET would initially boost oxidative metabolism to support increased Ac-CoA production and histone acetylation during activation; however sustained GPD2 activity during prolonged LPS exposure would overwhelm forward electron transport capacity leading to RET, thus limiting TCA cycling, Ac-CoA production, and inflammatory gene induction (\textbf{Supplementary Fig. 2.7}). To test this hypothesis, we examined RET using two complementary approaches. RET through CI leads to increased production of superoxide that is sensitive to the CI inhibitor rotenone, in contrast to FET where rotenone enhances superoxide production (\textsuperscript{33, 34} and \textbf{Fig. 2.13}). Another consequence of RET is attenuation of NADH oxidation to NAD\textsuperscript{+} at CI (compared to robust oxidation during FET) (\textsuperscript{32} and \textbf{Fig. 2.13}).
Figure 2.13 Schematic depicting FET and RET during LPS activation and tolerance. During acute LPS exposure (LPS activation), electrons from oxidation of metabolic substrates (i.e. glucose), flow forward through the ETC (green dotted line), creating a proton motive force for ATP production and also returning electron acceptor molecules (i.e. NAD\(^+\)) for continued oxidation of metabolic substrates. LPS-induced GPD2 activity initially boosts forward electron transport (FET; left) to support an increase in glucose oxidation for acetyl-CoA (Ac-CoA) synthesis and induction of inflammatory genes by enhanced histone acetylation. However, sustained GPD2 activity may overwhelm the ubiquinone pool (Q), the common sink for electrons from CI, CII, and GPD2, leading to a thermodynamic environment that permits electron backflow (red dotted line). Such reverse electron transport (RET) decreases return of NAD\(^+\) molecules to the TCA cycle, impairing glucose oxidation for Ac-CoA synthesis and histone acetylation at inflammatory genes. Therefore, we propose that GPD2 activity turns the ETC into a rheostat for inflammatory gene induction in BMDMs, linking the duration of LPS exposure to the directionality of electron transport to control glucose oxidation and balance induction and suppression of inflammatory responses.

First, we found that while rotenone treatment increased superoxide levels in naïve WT BMDMs as expected, it decreased superoxide levels in tolerant WT BMDMs indicative of RET after prolonged LPS exposure (Fig. 2.14a,b). Importantly, GPD2 deficiency diminished the ability of rotenone to reduce superoxide levels during tolerance, indicating that GPD2 activity contributed to tolerance-associated RET (Fig. 2.14a,b). The effects of 2DG treatment were even more striking, allowing rotenone to stimulate superoxide levels indicating complete rescue of tolerance-associated RET (Fig. 2.14a,b). Second, we used an assay based on detection of NAD(P)H autofluorescence in real-time, where addition of rotenone acutely blocks CI activity to reveal NADH oxidation at CI\(^{35}\). A robust increase in NADH levels after rotenone treatment is indicative
of FET at CI, while a modest increase is indicative of diminished FET and/or enhanced RET, since the net NADH level is determined by the relative magnitudes of opposing FET and RET.

**Figure 2.14** GPD2 activity drives induction of reverse electron transport (RET) in tolerant macrophages. (a,b) WT or GPD2 KO BMDMs were left unstimulated or stimulated 12h with LPS +/- 2DG; in some conditions, rotenone was included in the final 3h of culture (+Rot) to assess sensitivity of mitochondrial superoxide production to CI inhibition. (a), representative experiment showing mitochondrial superoxide production as measured by MitoSox mean fluorescence intensity (MFI); (b), data compiled from three representative experiments, with bar graph representing percent change in MitoSox MFI after Rot treatment relative to no Rot. (c) Real-time NAD(P)H autofluorescence in WT BMDMs unstimulated (Naive) or stimulated with LPS for 12h (LPS) before and after treatment with rotenone (Rot). (d) WT or GPD2 KO BMDMs were left unstimulated or stimulated 12 h with LPS +/- 2DG, followed by analysis of change in NAD(P)H autofluorescence after addition of Rot. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from three experiments (b), two experiments (c,d), or one experiment representative of three (a) experiments (mean and s.e.m. of duplicates (a,d) or measurements from ≥100 cells per condition at each time during assay (c)).

We found that in WT macrophages, rotenone differentially modulated NADH levels in naïve and tolerant conditions, robustly enhancing NADH levels in the naïve state indicative of robust FET while modestly enhancing NADH levels in the tolerant state indicative of reduced FET and/or increased RET (**Fig. 2.14c,d**). Importantly, rotenone did not differentially modulate NADH levels
between the naïve and tolerant states in GPD2 KO BMDMs or 2DG-treated WT BMDMs (Fig. 2.14d), indicating that GPD2 deficiency or 2DG treatment mitigated reduction of FET and/or induction of RET during tolerance. An orthogonal approach to measure NAD\(^+\) and NADH levels also indicated a reduction of the NAD\(^+\)/NADH ratio during LPS tolerance consistent with RET induction, and attenuation by GPD2 deficiency indicative of a role for GPD2 in tolerance-associated RET induction (Supplementary Fig. 2.8). Reversal of RET-induced redox imbalance by treatment with the NAD\(^+\) precursor nicotinamide (NAM) rescued LPS-inducible histone acetylation at the \(I\ell6\) and \(I\ell1b\) promoters in tolerant WT BMDMs (Supplementary Fig. 2.9a), leading to complete rescue of \(I\ell6\) and \(I\ell1b\) gene induction (Supplementary Fig. 2.9b). Such rescue by NAM suggests that RET may contribute to suppression of inflammatory gene induction in tolerant macrophages by driving accumulation of NADH at the expense of NAD\(^+\) return to the TCA cycle, restricting substrate oxidation and thus limiting Ac-CoA production for histone acetylation and inflammatory gene induction.

Together these findings allow us to propose a model whereby GPS activity uses the ETC as a rheostat to dynamically influence inflammatory gene induction and suppression. During acute LPS exposure, increased GPS activity initially increases FET to boost oxidative metabolism and Ac-CoA production, leading to enhanced histone acetylation and inflammatory gene induction. However, sustained GPS activity during prolonged LPS exposure eventually overwhelms the ETC to trigger RET and redox imbalance (i.e., imbalance of the NAD\(^+\)/NADH ratio), leading to reduced Ac-CoA production that limits histone acetylation for inflammatory gene induction in tolerant macrophages.
**GPD2 activity suppresses inflammatory responses during septic shock**

Because GPD2 suppresses inflammatory responses in an *in vitro* model of LPS tolerance (Fig. 2.12c,d), we asked if GPD2 could similarly regulate inflammatory response suppression *in vivo*. We used an established model of *in vivo* LPS tolerance, in which a sub-lethal dose of LPS protects against septic shock triggered by a subsequent, lethal dose of LPS (Fig. 2.15a). In contrast to mice that did not receive the sub-lethal LPS pretreatment, mice that received the pretreatment profoundly suppressed IL-6 production during septic shock, as expected. Importantly, such suppression was attenuated in GPD2 KO mice leading to enhanced IL-6 levels (Fig. 2.15b). Since GPD2 is expressed at high levels in only a few tissue and cell types (including brown adipose tissue (BAT), pancreas, and brain in addition to macrophages)\(^{28,29}\), increased IL-6 production was likely due to cell-autonomous GPD2 deficiency in macrophages and related myeloid cells, and GPD2 deficiency did not affect some clinical parameters such as drop in blood glucose levels and lymphocyte numbers (*Supplementary Fig. 2.10*). Importantly, the drop in body temperature characteristic of septic shock was exaggerated in GPD2 KO mice (Fig. 2.15b). Finally, WT mice receiving sublethal LPS pretreatment survived subsequent lethal LPS challenge while GPD2 KO mice succumbed, demonstrating a profound defect in LPS tolerance in the absence of GPD2 (Fig. 2.15c). Collectively the data indicates that GPD2 activity suppresses inflammatory responses to protect against systemic physiological derangement multiorgan dysfunction and mortality during septic shock.
Figure 2.15 GPD2 activity supports LPS tolerance in vivo. (a) In vivo LPS tolerance protocol. WT and GPD2 KO mice were injected with a sublethal dose of LPS (3 mg/kg) or vehicle (saline) followed 24 h later by a lethal dose of LPS (30 mg/kg IP). (b) Serum levels of IL-6 assessed after lethal LPS challenge. \( p=0.0155 \) (n=9) and \( p=0.0237 \) (n=10) respectively for mice without or with sublethal LPS pretreatment; 2way ANOVA with Sidak’s multiple comparisons test. (c) Body temperature after lethal LPS challenge. (d) Survival of mice after lethal LPS challenge in the in vivo LPS tolerance protocol. \( p =0.0012 \); WT n=20, KO n=19. *\( p \leq 0.05 \), **\( p \leq 0.01 \) (Student’s t-test). Data are from two experiments (mean and s.d. shown).
DISCUSSION

In this study, we make several findings regarding the role of glucose utilization in controlling the fate of macrophages exposed to LPS. First, although glucose oxidation has been reported in LPS-stimulated dendritic cells in a limited number of studies\textsuperscript{14,36}, the paradigm is that LPS-stimulated macrophages divert glucose to aerobic glycolysis and shut down oxidative metabolism. Importantly, how such glucose utilization influences macrophage responses to LPS remains not well understood. Using detailed glucose tracing experiments, we demonstrate that in LPS-activated macrophages, oxidative metabolism is increased to fuel Ac-CoA production and provide carbon substrate for histone acetylation at the \textit{Il6} and \textit{Il1b} promoters. Conversely, 2DG treatment attenuates oxidative metabolism, histone acetylation, and \textit{Il6} and \textit{Il1b} induction. These findings define a new role for glucose utilization in LPS-stimulated macrophages—namely glucose oxidation—as well as an underpinning basis by which such glucose utilization drives LPS activation (Supplementary Fig. 2.7). We also identify the Ac-CoA producing enzyme ACLY as a regulator of this process, showing that its LPS-inducible activity supports histone acetylation and inflammatory gene induction during LPS activation.

We further identify GPD2 and the GPS as key and hitherto unknown regulators of glucose oxidation in LPS-stimulated macrophages. GPD2 is expressed in a tissue-restricted fashion, but is expressed at high levels in macrophages and further upregulated by LPS stimulation. LPS stimulation dramatically increases flux through the GPS (Fig. 2.6a). This is likely due to multiple factors, including increased glucose utilization stimulating substrate delivery to the GPS, as well as increased GPD2 expression. We also considered the possibility that LPS stimulation could
increase the intrinsic activity of GPD2 to enhance GPS flux, since GPD2 contains two EF hands that are thought to be activated by Ca\(^{2+}\) signaling and LPS stimulation mobilizes Ca\(^{2+}\) signaling\(^{20, 37}\). In our experiments, LPS stimulation increased intrinsic GPD2 activity, but only at late time points (Supplementary Fig. 2.5b). One possibility is that this assay is not robust and/or sensitive enough to detect a smaller increase in GPD2 activity acutely after LPS stimulation. Alternatively, LPS-inducible intrinsic GPD2 activity drives GPS activity during tolerance induction rather than LPS activation, and the latter is driven mainly by increased glucose utilization and substrate flux. In any case, our data indicates that LPS stimulation mobilizes the GPS to bolster inflammatory responses during LPS activation, such that GPD2 deficiency attenuates LPS-inducible glucose oxidation and reduces Ac-CoA production, histone acetylation, and \(ll6\) and \(ll1b\) induction. Of note, the effects of GPD2 deficiency are not quite as pronounced as that of 2DG treatment, which may reflect the ability of glucose utilization to drive inflammatory gene induction independent of GPD2-mediated glucose oxidation, and/or compensatory adaptations in the genetic model due to constitutive loss of GPD2 activity. Complementing our analysis of GPD2 KO BMDMs, acute inhibition of GPD2 activity with a pharmacological inhibitor recapitulates our key findings in the genetic model, including effects on respiration, histone acetylation and inflammatory gene induction (Supplementary Fig. 2.11). Together these findings identify the GPS as a critical regulator of glucose oxidation and inflammatory gene induction in LPS-activated macrophages. Given the primacy of glucose utilization in LPS-stimulated macrophages, future studies to examine the GPS in this context are warranted.

Third, although respiration shutdown is a metabolic hallmark of LPS-stimulated macrophages, its role if any in modulating macrophage responses to LPS had been unclear\(^4, 5, 38\). Here we show that
respiration shutdown occurs only at delayed time points after LPS stimulation, after the initial increase in oxidative metabolism. Such shutdown limits the oxidation of glucose (and of other carbon substrates), thus reducing Ac-CoA production and histone acetylation to reinforce inflammatory gene suppression in tolerant macrophages. Little is known of the metabolic underpinnings of LPS tolerance, and we propose that shutdown of oxidative metabolism is central to metabolic reprogramming during LPS tolerance. The other metabolic hallmark of LPS-stimulated macrophages, induction of aerobic glycolysis, is presumably a compensatory mechanism to support cellular bioenergetics (i.e., ATP production) in the face of respiration shutdown.

Fourth, GPD2 and the GPS are important regulators of tolerance-associated respiration shutdown, since inhibiting glucose utilization or GPD2 deficiency is sufficient for partial restoration of oxidative metabolism. This leads to a partial recovery of histone acetylation and inflammatory gene induction in tolerant macrophages, as well as increased IL-6 production and mortality in a mouse model of septic shock. We propose a model in which GPS activity influences oxidative metabolism to modulate a shift from inflammatory response induction to suppression. During acute LPS exposure, GPS activity drives FET and Ac-CoA production to enhance histone acetylation and inflammatory gene induction, but during prolonged LPS exposure, sustained GPS activity eventually saturates electron transport capacity to trigger RET and limit Ac-CoA production for histone acetylation and inflammatory gene induction (Supplementary Fig. 2.12). In support of this model, we found that rotenone-sensitive superoxide and NADH production increases in the tolerant state indicative of RET induction, and that these events are impaired in GPD2 deficiency and upon 2DG treatment. Our findings are the first to describe a physiological role for GPD2-
driven RET, since GPD2-mediated oxidation of G3P has been shown to induce RET only in isolated mitochondria.

Note that in our model for GPS-mediated regulation of inflammatory responses, GPS activity triggers RET as a consequence of its initial boost of FET. Such GPS activity allows the GPS to integrate the duration of LPS exposure with ETC activity, supporting inflammatory response induction after acute microbial exposure but contributing to inflammatory response suppression after prolonged microbial exposure (Supplementary Fig. 2.12). Inflammation confers host defense at the expense of tissue damage and must be carefully regulated to balance its beneficial and detrimental effects, and GPS activity, by integrating the extent of microbial exposure to inflammatory response induction versus suppression, may provide one such balancing mechanism.

As discussed above, our detailed metabolic analyses implicate histone acetylation at inflammatory genes as a key target by which GPD2 regulates inflammatory gene expression. Complementing the metabolic analyses, our analysis of LPS signaling indicates no broad effects of 2DG treatment or GPD2 deficiency on NF-kB and IRF3 activation; furthermore, our RNA-seq analysis of LPS-activated WT and GPD2 KO BMDMs indicates that GPD2 regulates a small subset of LPS-inducible genes (data not shown). In this regard two points are worthy of discussion. First, while GPD2 does not globally modulate LPS signaling or LPS activation, GPD2 activity could impinge on specific aspects of LPS signaling that contribute to its regulation of inflammatory gene expression. For example, GPD2-mediated glucose oxidation could influence the expression or activity of specific signaling components or transcription factors, via effects on promoter histone acetylation and protein acetylation respectively. Such a role for GPD2 would be non-exclusive
with the one that we propose here—modulation of histone acetylation at inflammatory genes—and could be investigated in future studies. Second, how GPD2-mediated glucose oxidation and Ac-CoA production can specifically modulate histone acetylation and the expression of a subset of LPS-inducible genes remains unclear. One possibility is that ACLY is recruited to chromatin, producing a local pool of Ac-CoA that regulates histone acetylation in a gene-specific manner. In support of this possibility, ACLY can be found in the nucleus\(^\text{39}\), and precedence for such gene-specific association and regulation has been established by the metabolic machinery that produces S-adenosylmethionine, the substrate for histone and DNA methylation\(^\text{40}\).

One paradigm that has emerged from the macrophage metabolism field is that LPS-stimulated macrophages shut down oxidative metabolism while M2 macrophages increase oxidative metabolism. Our findings indicate that increased oxidative metabolism is a common feature of LPS-activated and M2 macrophages, and that one consequence of this process is provision of A-CoA for histone acetylation at inducible genes. While a role for histone acetylation in bolstering gene induction has long been appreciated, a seminal study showed that in tumor cells, Ac-CoA availability can be limiting for histone acetylation thus influencing gene expression\(^\text{39}\). A similar principle appears to be true in macrophages, thus allowing levels of carbon substrates like glucose to modulate the transcriptional basis of macrophage activation. Furthermore, because Ac-CoA levels can be limiting, polarizing signals like LPS and IL-4 mobilize oxidative metabolism, by impinging on key regulators like GPD2, to drive optimal gene induction and macrophage activation. In the case of LPS-stimulated macrophages, this process is further exploited by LPS signaling to eventually influence respiration shutdown and inflammatory gene suppression.
It is noteworthy that LPS-stimulated macrophages upregulate flux through the GPS by increasing GPD2 expression and activity, given that macrophages can deploy the malate/aspartate shuttle to maintain redox balance during glucose oxidation\textsuperscript{41}. We propose that GPD2/GPS-mediated glucose oxidation has a unique, non-redundant role in optimal control of inflammatory gene induction and suppression. In particular, because GPD2 is situated in the ETC directly bridging cytosolic glucose metabolism with ETC flux, GPS activity may allow for very robust glucose oxidation. Indeed, available evidence in the literature\textsuperscript{30} and the rapid kinetics of our glucose labeling experiments support this notion. We propose that in LPS-stimulated macrophages, rapid and robust mobilization of glucose oxidation drives ETC flux and inflammatory gene expression, and in the event of sustained LPS exposure, contributes to RET induction. In contrast, the malate-aspartate shuttle may support more modest rates of glucose oxidation that does not allow for a comparable increase in ETC activity, and would be fundamentally incompatible with RET induction (given that NADH derived from this shuttle is delivered to Complex I). Therefore, deployment of the GPS in the high metabolic state of LPS-stimulated macrophages seems to enable appropriate modulation of inflammatory response induction and suppression. We propose that the GPS is a hitherto unappreciated and unique regulator of LPS responses, using the ETC as a rheostat to couple duration of LPS exposure to ETC directionality to modulate a balance between the induction and suppression of inflammatory responses.
MATERIALS AND METHODS

BMDM culture. BMDMs from male and female mice were differentiated and cultured as described previously. For LPS activation (0-3h post-LPS), BMDMs were stimulated with 100 ng/mL LPS (LPS Ultra-Pure, Sigma-Aldrich). All of the metabolic parameters associated with LPS activation, including glucose uptake and oxidation, glucose tracing into TCA intermediates and Ac-CoA, ACLY phosphorylation, glucose labeling into histones, and promoter histone acetylation, were assessed at early time points after LPS stimulation (0-3 h), while the downstream consequences of such metabolic events, i.e., inflammatory gene induction and cytokine production, were assessed at 3 h after LPS stimulation. For LPS tolerance (12-28h post-LPS), BMDMs were challenged with 100 ng/mL LPS for 24h to induce tolerance (T), washed, and then rechallenged with 10 ng/mL LPS (T+L) for the desired amount of time. In such experiments, naïve BMDMs were incubated in media for 24h (N), washed, and challenged with 10 ng/mL LPS (N+L) to provide responsive-cell controls. Pharmacological inhibition of ACLY (ACLYi, 80-160 µM; SB-204990, Tocris, United Kingdom), glucose utilization (2DG, 5-10 mM; 2-deoxy-glucose, Sigma-Aldrich), and GPD2 (GPD2i, 300 µM; iGP-1, Calbiochem) was achieved by pretreating BMDMs for 1h prior to LPS activation. For rescue of tolerance, BMDMs were pretreated for 1h prior to activation, but drugs were not added back after wash before restimulation. Additional drugs include the ACLY inhibitors BMS-303141 and Medica16, the p300 inhibitor C646, and the glutamine and fatty acid oxidation inhibitors BPTES and Etomoxir.
**Seahorse assays.** Basal and LPS-induced changes in oxygen consumption (OCR) and extracellular acidification (ECAR) rates were measured with a Seahorse XF96 Extracellular Flux Analyzer (Agilent). For experiments in intact cells, assays were performed in Seahorse XF Assay Medium supplemented with glucose (11 mM) and adjusted to pH 7.4 with NaOH. Mix-wait-measure durations were set to 3, 2, and 3 minutes, respectively. For the MitoStress Test, oligomycin, FCCP, and rotenone/antimycin A were sequentially injected to achieve final concentrations of 1, 1.5, and 2 µM. Glycolysis Tests were performed in unsupplemented XF Assay Medium, and glucose, oligomycin, and 2DG were sequentially injected to achieve final concentrations of 11 mM, 1 µM, and 500 mM, respectively. Non-mitochondrial OCR was subtracted from the mean pre-oligomycin OCR and the mean post-FCCP OCR to calculate Basal and Maximal mitochondrial respiration, respectively. For experiments in permeabilized cells, assays were performed in mannose sucrose buffer supplemented with 0.1% BSA and adjusted to pH 7.2 with KOH (MAS-BSA Buffer). Cells were permeabilized by injection of perfringolysin O (PMP reagent, Agilent) in the XF96 Analyzer, and specific mitochondrial enzyme activities were determined by measuring State 3 respiration in the presence of substrate/inhibitors/ADP, followed by sequential injection of oligomycin and antimycin A. GPD2 activity was measured as the change in OCR induced by injection of 10 mM sn-glycerol 3-phosphate (G3P, Sigma-Aldrich), 1 mM ADP, and 2 uM Rotenone. After each experiment, OCR and ECAR values were normalized to DNA content in each well by Hoechst 33342 staining (Life Technologies, Carlsbad, CA).
Steady-state metabolomics and relative metabolic flux. For steady-state analysis of metabolic networks, BMDMs were stimulated for the desired period of time and then lysed and polar metabolites extracted using 80:20 methanol:water (HPLC-grade, Sigma-Aldrich). Extracts were dried under nitrogen gas followed by LC-MS (Beth Israel Deaconess Medical Center Mass Spectrometry Facility) to monitor targeted metabolites from a library of 300 species, including those involved in glycolysis, the glycerol 3-phosphate shuttle, and the TCA cycle. MS peak areas were normalized to median and sample protein concentrations, and LPS-induced fold-change data was run through the Metaboanalyst 2.0 web-based pipeline to visualize metabolic pathway enrichment. For relative metabolic flux analysis, naïve and LPS-stimulated BMDMs were washed with glucose-free medium and then labeled with 2 g/L $^{13}$C$_6$-glucose (Cambridge Isotope Laboratories) in complete medium (10% dialyzed FBS) for 30 min. Metabolites were extracted, dried, and run as in$^{44}$. Normalized peak data was analyzed for percent labeling of glucose into each possible isotopologue of species in glycolysis, the glycerol 3-phosphate shuttle, and the TCA cycle.

Acyl-CoA mass spectrometry. Naïve and LPS-stimulated BMDMs were washed with glucose-free medium and then labeled with 2 g/L $^{13}$C$_6$-glucose (Cambridge Isotope Laboratories) in complete medium (10% dialyzed FBS) for 30 min. Cells were then lysed in 800 uL ice-cold 10% trichloroacetic acid and extracted as previously described$^{45}$. Analysis by LC-high resolution MS was conducted on an Ultimate 3000 UHPLC coupled to a Q Extractive Plus operating in the positive ion mode as previously described$^{46}$. Isotopologue analysis was conducted using FluxFix$^{47}$. 
**Glucose tracing into histones.** Glucose tracing into histones was done following an established protocol\(^48\). In the last 1.5h of stimulation, BMDMs were labeled with \(^{14}\text{C}_6\)-glucose followed by acid extraction of acetylated histones using a method previously described\(^27\). Radiolabel incorporation was assessed by scintillation counting and normalized to total protein concentration.

**Glucose uptake.** Basal and LPS-inducible glucose uptake in BMDMs was measured using \(^3\text{H}\)-deoxy-D-glucose (2-\(^3\text{H}[G]\)) (PerkinElmer) in KRBH buffer as described previously\(^9\). Counts per minute (cpm) values were normalized to protein concentration in each sample.

**NAD\(^+\)/NADH analyses.** For NADH autofluorescence, BMDMs were excited at 340/26 nm on a Nikon Eclipse Ti-S microscope. The emitted signal was collected using a 460/80 nm bandpass filter. Autofluorescence was analyzed with Fiji image processing software. Measurements of the NAD\(^+\)/NADH ratio were done using a colorimetric kit (Biovision) according to the manufacturer's protocol.

**Chromatin immunoprecipitation.** Acetylation at inflammatory gene promoters was measured by chromatin immunoprecipitation (ChIP) assay as described previously\(^9\). Promoters are defined as the region between -500 bp and the transcription start site. Antibodies used were as follows: acetylated H3 (Millipore 06–599), acetylated H3K27 (Abcam ab4729), acetylated H4 (Millipore 06–866), and IgG (Santa Cruz, Dallas, TX, SC-2027). Fold enrichment was calculated as ChIP signals normalized to input.
**Gene expression.** RNA was isolated using RNA-Bee (Tel-Test) according to the manufacturer’s protocol. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCRs were run on a Bio-Rad C1000 Thermalcycler, and expression of target genes was calculated by the ddCT method using CFX Manager Software (Bio-Rad). Relative expression values for target genes were calculated by normalization to expression of hypoxanthine phosphoribosyltransferase (HPRT).

**Generation of GPD2 KO mice.** GPD2 KO mice were generated on the C57BL/6J background using a gRNA targeting exon 6 of mGPD2 (oligo 1: CACCGCGTACCGTCATAGTAGACAA, oligo 2: CGCATGGCAGTATCATCTGTTCAAA). gRNA and Cas9 RNA were injected into eggs followed by implantation into recipient female mice at the BWH Transgenic Core. Mice were backcrossed two times to C57BL/6 mice. BMDMs from two independently generated lines were used with similar results.

**Mice.** Wild-type C57BL/6J mice (The Jackson Laboratory) and whole-body Gpd2-/- (KO) mice were maintained under specific pathogen-free conditions at the Harvard T.H. Chan School of Public Health in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Male and female mice were used for in vivo LPS tolerance experiments and as a source of bone marrow for BMDM culture between 6 and 12 weeks of age. All protocols were approved by the IACUC of Harvard Medical School. In the in vivo LPS tolerance experiments, WT and GPD2 KO mice were injected with vehicle (saline) or 3 mg/kg LPS via intraperitoneal injection (IP) followed by 30 mg/kg LPS IP 24h later. Serum IL-6 and internal body temperature (TH-5 Thermalert Clinical Monitoring Thermometer, Physitemp) were measured for 6 h after lethal LPS
challenge. Survival of WT and GPD2 KO mice that received both challenges was recorded as an indication of differential endotoxin tolerance in vivo. Blood glucose and lactate levels in septic mice were measured using standard glucose and lactate meters (Clarity BG1000, Clarity Diagnostics; Lactate Plus, Nova Biomedical). Numbers of circulating lymphocytes and monocytes were obtained using a Hemavet 950 (Drew Scientific).

**Immunoblot and enzyme-linked immunosorbent assays.** For immunoblotting, BMDMs were lysed in 1% NP-40 buffer or RIPA buffer, and protein concentration was determined using the Bradford method or micro BCA assay (Pierce). Primary antibodies used were anti-phospho-ACLY(S455) and anti-ACLY (1:1000, Cell Signaling), anti-GPD2 (1:800, ProteinTech), and anti-a-Tubulin (1:5000, Sigma-Aldrich). For ELISA, cell culture supernatants and mouse whole blood were spun down (5 min at 400 g and 20 min at 13,000 g, respectively), diluted appropriately in 5% BSA, and assayed for IL-6 using BioLegend Standard ELISA kits according to the manufacturer’s protocols. For in vitro LPS tolerance experiments, cell culture supernatants in the T and T+L conditions were collected after LPS washout and rest or restimulation for 4h, allowing for measurement of IL-6 produced during tolerance rather than activation.

**Flow cytometry.** Efficiency of macrophage differentiation from bone-marrow-derived progenitors was determined by staining D7 BMDMs with anti-F4/80 (BM8) and anti-CD11b (M1/70) antibodies (BD Bioscience) for 30 min at 4°C in FACS buffer (2% FCS), followed by washes and fixation in 2% ultrapure paraformaldehyde. Mitochondrial superoxide was measured by MitoSox (Life Technologies) staining according to the manufacturer's protocol. Briefly, BMDMs were left unstimulated or stimulated with LPS for the indicated times and then labeled
with 2.5 uM MitoSox Red in the final 15 minutes of treatment, followed by washout and no
fixation. Mean fluorescence intensity of MitoSox in the PE channel was measured to determine
levels of mitochondrial superoxide at the time of labeling. All samples were acquired on a BD
FACS Fortessa flow cytometer, and FlowJo software (v10.4) was used to analyze data.

**Statistical analysis.** Statistical significance was determined by a two-tailed Student’s $t$-test or
2way ANOVA with Sidak’s multiple comparisons test using Prism software (GraphPad). *, **, 
***, and **** in figures denote $p$ value $\leq 0.05$, 0.01, 0.001, and 0.0001, respectively.

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**Competing Interests**

The authors declare no competing interests.
Supplementary Figure 2.1 ATP-citrate lyase (ACLY) and p300 activity are necessary for supporting pro-inflammatory gene induction during LPS activation in BMDMs. *Il6* and *Il1b* promoter region histone acetylation (a,d) and gene expression (b,c) in BMDMs unstimulated (Naive) or stimulated with LPS (LPS) in the presence or absence of the ACLY inhibitors BMS 303141 (LPS+BMS) or Medica16 (LPS+Medica16) or the p300 inhibitor C646. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (b,c) or triplicates (a,d)).
Supplementary Figure 2.2 LPS exposure modulates mitochondrial respiration and glucose uptake as a function of time. (a) Oxygen consumption rate (OCR) was measured using Seahorse Extracellular Flux Analysis on BMDMs unstimulated (Naive) or stimulated with LPS for the indicated times. Maximal OCR was calculated as the mean uncoupled OCR after FCCP injection, minus the non-mitochondrial OCR remaining after rotenone/antimycin A (Rot/AA) injection. Acute LPS exposure induces a transient but highly-reproducible increase in maximal respiration, followed by a gradual and sustained shutdown during prolonged LPS exposure. This dynamic change in respiration in BMDMs stimulated with LPS traces the metabolic shift underpinning the transition from activation to tolerance. (b) Glucose uptake in naïve and LPS-activated BMDMs measured by 3H-deoxy-D-glucose uptake (cpm = counts/min). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment representative of three (b) or ten (a) experiments (mean and s.e.m. of duplicates (b) or octuplets (a)).

Supplementary Figure 2.3 Glucose starvation attenuates pro-inflammatory gene induction and promoter region histone acetylation in LPS-activated BMDMs. Expression (a) and promoter region histone acetylation (b) of Il6 and Il1b in naïve and LPS-activated BMDMs pre-incubated in glucose-free media for 24h. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (a) or triplicates (b)).
Supplementary Figure 2.4 Depiction of the spatial and biochemical position of GPD2 at the nexus between glycolysis and electron transport. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) generates NADH from oxidation of glucose in the cytosol, supplying electrons for reduction of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G3P) by cytosolic glycerol 3-phosphate dehydrogenase (GPD1). Electrons from G3P are then passed directly to the electron transport chain (ETC) through the FAD cofactor of mitochondrial glycerol 3-phosphate dehydrogenase (GPD2), reducing ubiquinone (Q) to ubiquinol (QH$_2$) and driving the proton motive force through the remaining parts of the ETC to fuel oxidative phosphorylation. As the glycerol 3-phosphate shuttle (GAPDH-GPD1-GPD2) directly links glycolysis to the ETC and resupplies NAD$^+$ to the former, increased GPD2 activity serves as a metabolic node by which the ETC may regulate the rate of glucose utilization. LPS activation increases GPD2 expression, permitting the acquisition of a high metabolic state characterized by increased glucose oxidation and G3P-drive mitochondrial respiration.
Supplementary Figure 2.5 LPS activation increases expression and activity of GPD2 in BMDMs. (a) Expression and (b) activity of GPD2 in naïve and LPS-activated WT and GPD2 KO BMDMs, measured by Western blot and Seahorse. Activity was calculated as the oxygen consumption rate (OCR) in permeabilized BMDMs after injection of G3P, ADP, and rotenone relative to naïve.
Supplementary Figure 2.6 GPD2 regulates glucose flux through glycolysis and the TCA cycle. $^{12}$C$_6$-glucose tracing into intermediates of glycolysis and TCA cycle in WT and GPD2 KO BMDCMs during LPS activation, presented as LPS-induced fold change in percent m+6 (1,2), m+3 (3-7), or m+2 (8-11) enrichment. Data are from one experiment representative of two experiments (mean and s.e.m. of triplicates).
Supplementary Figure 2.7 Model integrating GPD2-regulated mitochondrial oxidative metabolism with LPS activation. GPD2-dependent transport of electrons from the glycerol phosphate shuttle (GPS) through the ETC is upregulated by LPS activation to support an increase in glucose-derived carbon flux through glycolysis and the TCA cycle, providing increased availability of citrate and Ac-CoA. Coupled with an LPS-induced increase in ACLY activity, enhanced production of Ac-CoA drives histone acetylation and induction of inflammatory genes like *Il6* and *Il1b*. As oxidation of carbon substrates in the TCA cycle depends on the availability of electron acceptors such as NAD⁺, the cyclic reduction and oxidation of these molecules serves as an important link between oxidative metabolism in the TCA cycle and electron transport chain (ETC) activity.

Supplementary Figure 2.8 Prolonged LPS stimulation disrupts forward electron transport, leading to a shift in mitochondrial redox balance. NAD⁺/NADH ratio in WT and GPD2 KO BMDCMs unstimulated (Naïve) or stimulated with LPS for 12h (LPS). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates).
Supplementary Figure 2.9 Nicotinamide (NAM) reverses RET-redox imbalance to rescue pro-inflammatory gene induction and promoter region histone acetylation in tolerant BMDMs. Promoter region histone acetylation (a) and expression (b) of *Il6* and *Il1b* in naïve (N), LPS activated (N+L), tolerant (T), and tolerant rechallenged BMDMs +/- NAM during tolerization (T+L+NAM). *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (b) or triplicates (a)).

Supplementary Figure 2.10 GPD2 deficiency does not affect some clinical parameters altered during sepsis. Blood glucose (a) and lactate (b), which decreased and modestly increased during sepsis, were not dependent on the presence of GPD2. WT and GPD2 KO mice experienced equal changes in levels of circulating lymphocytes (c) and monocytes (d) during sepsis.
Pharmacological inhibition of GPD2 modulates LPS activation and tolerance. Inhibiting the increase in GPD2 activity induced by LPS activation (0-3h LPS) using iGP-1 (GPD2i) attenuated enhanced respiration (a), histone acetylation at Il6 and Il1b promoters (b), and expression of Il6 and Il1b (c). GPD2 inhibition partially protected against respiration shutdown during prolonged LPS exposure (12-24h LPS) (d). Cotreatment with GPD2i during induction of tolerance, followed by restimulation in the absence of inhibitor (T+L+GPD2i) demonstrated rescue of promoter histone acetylation (e) and expression of Il6 and Il1b (f).
Supplementary Figure 2.12 Model of the metabolic adaptations underpinning the biphasic functional response to LPS exposure. Duration of LPS exposure regulates the transition from inflammatory gene induction to suppression (blue histogram) in macrophages. Acute LPS exposure, or activation, induces an increase in GPD2-dependent oxidative metabolism, which enhances the production of Ac-CoA from glucose to fuel histone acetylation and thus promote expression of the inflammatory genes Il6 and Il1b. Prolonged LPS exposure produces a state of tolerance in which induction Il6 and Il1b expression is attenuated due to decreased respiration limiting flux of glucose through the TCA cycle into Ac-CoA for histone acetylation.
REFERENCES


Additional metabolic modules regulating macrophage responses to microbial encounter: roles for Akt and nucleocytosolic acetyl-CoA synthetase ACSS2

This chapter is a collection of unpublished data. It is presented here as a completed work to facilitate understanding and serve as a reference for further investigation.

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P.K.L. designed and performed experiments and analyzed data. P.K.L. prepared figures. T.H. supervised the project, including experimental design and data analysis. J.J., Y.K., and I.A. helped with gene expression, RNA-seq analysis, and LPS dose immunoblots, respectively.
ABSTRACT

Macrophages are activated during microbial infection to coordinate inflammatory responses and host defense. Here we show that in macrophages activated by bacterial lipopolysaccharide (LPS), the serine/threonine kinase Akt regulates glucose oxidation driven by mitochondrial glycerol 3-phosphate dehydrogenase (GPD2) to control histone acetylation and inflammatory gene induction. While acute LPS exposure drives macrophage activation, prolonged exposure triggers entry into LPS tolerance, where macrophages orchestrate immunosuppression to limit the detrimental effects of sustained inflammation. We find that Akt couples the strength and duration of LPS exposure to a shutdown of oxidative metabolism, limiting histone acetylation at inflammatory genes and thus contributing to suppression of inflammatory responses. In LPS tolerant macrophages re-exposed to LPS, Akt also regulates the induction of a subset of highly-responsive, primed (P) genes, important for anti-microbial activity and mitochondrial respiration. Such induction of P genes is fueled by acetate-derived Ac-CoA production catalyzed by nucleocytosolic acetyl-CoA synthetase (ACSS2) rather than citrate-derived Ac-CoA from GPD2-dependent glucose oxidation and ATP-citrate lyase (ACLY) activity. Therefore, Akt and ACSS2 act as additional regulatory modules integrating TLR signaling with shifts in macrophage metabolism to induce gene-specific responses to microbial encounter.
INTRODUCTION

Macrophages, innate immune cells of the myeloid lineage, are the cornerstone of the host’s response to microbial encounter. Expression of a repertoire of pathogen recognition receptors (PRRs), enables macrophages to sense invading microbes through detection of various pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) from gram-negative bacteria. Recognition of LPS by the PRR Toll-like receptor 4 (TLR4) polarizes macrophages to an inflammatory phenotype, characterized by upregulation of pro-inflammatory cytokine and chemokine production, expression of co-stimulatory molecules to enhance adaptive immunity, and increased phagocytosis and killing of bacteria. Although this robust response is critical for conquering infection, over-exuberant inflammatory responses result in tissue damage that may become fatal to the host, as observed in patients with multi-organ dysfunction as a consequence of systemic inflammation during septic shock\(^1\)–\(^3\). As a mechanism of protection against inflammation-mediated organ damage, macrophages exposed to LPS for a prolonged period shift into a state of reduced responsiveness, or tolerance, to further activation\(^4\)\(^,\)\(^5\). Such tolerance contributes to the whole-body immunosuppression observed in patients that survive septic shock, mitigating multi-organ dysfunction\(^6\)\(^,\)\(^7\).

In recent years, metabolism has come to be appreciated as an important layer of regulation in host defense\(^8\)–\(^11\). Indeed, specific metabolic shifts within innate and adaptive immune cells have been shown to influence each of the four tasks of the immune system: recognition, effector functions, regulation, and memory. Mechanisms underpinning such regulation are context-dependent and range from integration with signaling to gene-specific chromatin modifications. The current
paradigm for metabolic instruction of inflammatory macrophage activation is that shutdown of oxidative phosphorylation and induction of aerobic glycolysis are important adaptations coupling TLR signaling with enhanced inflammatory cytokine production\textsuperscript{9,12}. In our previous study, we revised this conceptual framework, by demonstrating that oxidative metabolism actually supports induction of macrophage inflammatory responses, such that impairment drives inflammatory gene suppression and LPS tolerance (see \textbf{Chapter 2}). Specifically, we reported that the glycerol phosphate shuttle (GPS), a glycolytic shunt, is a novel LPS-inducible metabolic module that boosts glucose oxidation to drive inflammatory gene induction during LPS activation but suppresses oxidative metabolism to contribute to inhibition of inflammatory gene induction during LPS tolerance. Consistent with the well-established link between oxidative metabolism and regulation of transcriptional responses\textsuperscript{13}, we identified availability of nucleoeytosolic acetyl-CoA (Ac-CoA) as a node coupling glucose oxidation to histone acetylation at the inflammatory genes \textit{Il6} and \textit{Il1b}. Thus, the model proposed in our previous study provides much needed clarity to the relationship between glucose metabolism and induction of macrophage inflammatory responses; however, the nexus between TLR signaling and glucose oxidation remains incompletely understood. Additionally, metabolic regulation of other macrophage effector functions in the context of bacterial infection has not been well-investigated and thus constitutes a largely untapped and important solution space.

Here we show that the serine/threonine kinase Akt (protein kinase B, PKB), a known target downstream of PI3K\textsuperscript{14} and TBK1/IKK\epsilon\textsuperscript{15}, integrates glucose oxidation with the strength and duration of LPS stimulation to control the amplitude and duration of inflammatory gene induction. We also identify a subset of genes, constituting 8\% of the LPS-inducible transcriptome picked up
by our analysis, that become hyper-responsive following prolonged LPS exposure, in contrast with the hypo-responsiveness of inflammatory genes that is characteristic of the LPS tolerant state. These primed genes, or “P genes,” encode molecules important for biological processes distinct from tolerant genes, including phagocytosis, mitochondrial ATP production, and ion transport and also show regulation by Akt. Moreover, consistent with the shift from increased to decreased oxidative metabolism over the course of LPS exposure as macrophages transition from activation to tolerance, P genes are not directly supported by glucose oxidation since block of glucose utilization or attenuation of citrate conversion to Ac-CoA by inhibition of ATP-citrate lyase (ACLY) does not abolish induction of P genes in tolerant bone marrow derived macrophages (BMDMs). Instead, we show that induction of P genes is supported by acetate-derived Ac-CoA production catalyzed by nucleocytosolic acetyl-CoA synthetase (AceCS1, or ACSS2) and highlight histone deacetylation as a source of acetate fueling this process. Although such regulation is comparable to observations in other contexts of impaired Ac-CoA production from glucose oxidation\(^1\)\(^6\),\(^1\)\(^7\), this is the first evidence of a role for ACSS2 in macrophage biology. Finally, we propose that in addition to a role of Akt in the induction and suppression of inflammatory genes through control of oxidative metabolism and thus citrate-derived Ac-CoA levels, Akt may also regulate P genes through control of ACSS2 by phosphorylation at conserved serine residues. Thus, Akt and ACSS2 act as additional regulatory modules integrating TLR signaling with shifts in macrophage metabolism to induce gene-specific responses to microbial encounter.
RESULTS

Magnitude and rate of metabolic shifts reflect strength of macrophage LPS stimulation

In our previous study, we demonstrated that the ETC acts as a rheostat to couple duration of LPS exposure to ETC directionality to modulate a balance between the induction and suppression of inflammatory responses. During this investigation, we also observed that such metabolic shifts are coupled not only to the duration but also the strength of LPS exposure. Indeed, activation of bone marrow derived macrophages (BMDMs) with low or high dose LPS resulted in distinct changes in the rates of extracellular acidification (ECAR) and oxygen consumption (OCR), reflective of the strength of stimulation (Fig. 3.1a,b). Further titration of LPS dose used for BMDM stimulation followed by analysis of acute inflammatory gene induction revealed similar dose-dependent effects on induction of *Il6* (Fig. 3.1c) and many other inflammatory genes (data not shown). We also observed that respiration shutdown was positively correlated with the strength of LPS activation (Fig. 3.1d) and the strength of tolerance to LPS restimulation (Fig. 3.1e), which was assessed using the strategy described in Fig. 2.8a. Thus, consistent with our previous study and building on the link between mitochondrial metabolism and responsiveness of inflammatory genes, ETC activity couples dose and time of LPS exposure to magnitude and duration of inflammatory gene induction.
Figure 3.1 Dose-dependent metabolic shifts couple strength of LPS stimulation to amplitude and duration of inflammatory gene induction in macrophages. (a,b) Seahorse extracellular flux analysis of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in real-time by BMDMs unstimulated (Naive) or LPS-activated with a low (LPS 10 ng/mL) or high dose (LPS 100 ng/mL). (c) BMDMs were unstimulated (Naive) or stimulated with the indicated doses of LPS for 3 hours followed by qPCR analysis of Il6 and Il1b gene expression. (d) Maximal mitochondrial respiration in BMDMs unstimulated (Naive) or stimulated with indicated doses of LPS for 12h. Maximal respiration was calculated by subtraction of non-mitochondrial respiration (OCR post-Rot/AA) from mean uncoupled respiration (OCR post-FCCP). (e) Representative induction of Il6 and Il1b genes in BMDMs tolerized with different doses of LPS. BMDMs were treated according to the schematic in Fig. 2.8a. Concentrations indicate dose of LPS used during 24h tolerization. All BMDMs were rechallenged with the same dose, as indicated in Fig. 2.X. *p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (c,e) or octuplets (a,b,d)).

Akt signaling integrates strength of LPS stimulation with glucose oxidation to control macrophage inflammatory responses

Our previous observations demonstrate that deployment of the glycerol phosphate shuttle (GPS) is an early event during LPS stimulation that critically drives ETC activity to support enhanced production of acetyl-CoA (Ac-CoA) for histone acetylation and inflammatory gene induction in macrophages. Is has also been reported that activation of Akt downstream of TBK1-IKKε signaling is necessary for rapid induction of glucose utilization through glycolysis in LPS-
stimulated dendritic cells (DCs). Given that low and high doses of LPS induce different rates of glycolysis and magnitudes of respiration in BMDCs during activation, we postulated that differences in Akt signaling may underpin this response. Analysis of Akt activation over a timecourse by immunoblot assay for Akt phosphorylation at S473 and T308, modifications both necessary for full activity, revealed differences in kinetics supportive of a role for Akt in integrating strength of LPS stimulation with rate and extent of glucose utilization (Fig. 3.2a). Consistent with this, pharmacological inhibition of Akt (AKTi) completely blunted the early increase in GPD2-dependent glucose oxidation (Fig. 3.2b), and thus also attenuated LPS-induced promoter region histone acetylation and expression of Il6 and Il1b (Fig. 3.2c,d).

**Figure 3.2** Akt signaling integrates LPS dose with glucose oxidation to regulate LPS activation in BMDCs. (a) Immunoblot analysis of activating phosphorylation of Akt at S473 and T308 during BMDCM activation induced by the indicated doses of LPS. (b) Maximal mitochondrial respiration in BMDCMs unstimulated (Naïve) or stimulated with LPS for 1h +/− pretreatment with the Akt inhibitor MK-2206 (AKTi). BMDCMs were stimulated with LPS for 0-3h +/− AKTi followed by analysis of Il6 and Il1b promoter region histone acetylation (c) and gene expression (d). *p ≤0.05, **p ≤0.01, ***p ≤0.001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (d), triplicates (c), or octuplets (b)).
The early increase in glucose oxidation necessary to support inflammatory gene induction during LPS activation ultimately leads to respiration shutdown, driving inflammatory gene suppression and blunted responsiveness, or tolerance, to subsequent LPS encounters. Inhibition of Akt protected against shutdown of respiration and suppression of Il6 and Il1b expression during prolonged LPS exposure (Fig. 3.3a,b) and was comparable to that observed by impairing glucose oxidation by 2DG cotreatment or GPD2 deletion (Fig. 2.9, 2.12, and Supplementary Fig. 3.1).

**Figure 3.3** Akt signaling leads to impaired glucose oxidation and inflammatory gene suppression during prolonged LPS exposure. BMDMs were unstimulated (Naïve) or stimulated with LPS for 12h +/- the Akt inhibitor MK-2206 (AKTi) followed by analysis of the following parameters. (a) Mitochondrial respiration, determined by oxygen consumption rate (OCR). Basal and maximal respiration were calculated by subtraction of non-mitochondrial respiration (OCR post-Rot/AA) from mean OCR pre-oligomycin and post-FCCP, respectively. (b) Il6 and Il1b gene expression. *p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (b) or octuplets (a)).

Such protection was attributed to effects of AKTi on glucose oxidation in part because inhibition of Akt resulted in significant attenuation of LPS-induced Gpd2 expression (Supplementary Fig. 3.2 and see Discussion). As expected, protection of respiration by AKTi cotreatment also rescued histone acetylation and inflammatory gene induction in tolerant macrophages (Fig 3.4a,b). Thus,
glucose oxidation via GPD2 and the GPS may be a novel target of Akt in LPS-activated macrophages, serving as a critical node at the nexus of TLR signaling and metabolism to regulate the magnitude and duration of inflammatory responses.

**Figure 3.4** Inhibition of Akt protects against induction of LPS tolerance in macrophages. (a) Promoter region histone acetylation and (b) expression of *Il6* and *Il1b* in N, N+L, T, and T+L+AKTi conditions. T+L+AKTi indicates cotreatment with Akt inhibitor (MK-2206) during tolerization followed by washout and LPS rechallenge without inhibitor (compare to Fig. 2.X). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (b) or triplicates (a)).

**Tolerant macrophages have a large repertoire of responsive genes that are regulated by Akt**

Although pro-inflammatory macrophage effector molecules, such as IL-6 and IL-1b, that are acutely induced and later suppressed following TLR stimulation by various PAMPs are the most commonly studied components of the innate response to microbial encounter, the LPS-inducible transcriptome of macrophages encodes a vast array of molecules important for myriad biological functions, including phagocytosis, ion transport, response to unfolded proteins, and more\(^4,18,19\). We performed RNA-seq analysis on naïve and tolerant BMDMs unstimulated or stimulated with LPS
as in Fig. 2.8a and grouped all LPS-inducible genes into three classes based on expression patterns across conditions. Proportions of each class within the total LPS-inducible gene set, as well as representative expression of genes from each class are presented in Fig. 3.5. Tolerized “T” genes (i.e. Il6 and Il1b) were defined as those that showed impaired induction in the tolerant state compared to the responsive state. Non-Tolerized “NT” genes (i.e. Lcn2 and Rantes) did not appear tolerant but were also not further induced in tolerant BMDMs after LPS restimulation. Finally, Primed “P” genes (i.e. Fpr1 and Irg1) were defined as those that, like NT genes, did not exhibit tolerance but also displayed strong induction in tolerant, LPS-restimulated BMDMs.

This third class, although the smallest of the three subsets, constituting only 8% of all LPS-inducible genes detected by our analysis, was of greatest interest, as induction of such P genes must be supported by an alternative source of Ac-CoA to that derived from citrate from glucose oxidation. Furthermore, because the expression pattern of P genes is antithetical to the concept of tolerance, P genes must belong to distinct and important functional clusters. Gene ontology
analysis was performed to further classify P genes based on the biological processes and cellular components to which they belong (Supplementary Figs. 3.3).

Although glucose oxidation is impaired in tolerant macrophages, we first turned to Akt in our investigation into the metabolic underpinnings of induction of P genes, as Akt activity is still induced by LPS stimulation of tolerant BMDCs (Fig. 3.6a), and relevant metabolic targets of Akt in control of macrophage activation remain incompletely characterized. Strikingly, treatment of tolerant BMDCs with AKTi before LPS restimulation (Fig. 3.6b) completely attenuated reinduction of some P genes (Fig. 3.6c). However, consistent with suppression of T genes due to impaired glucose oxidation and carbon flux into Ac-CoA for histone acetylation and gene induction (Fig. 2.9 and 2.10), inhibition of glucose utilization and ACLY activity by 2DG and ACLYi treatment between challenges did not lead to strong attenuation of P genes (Supplementary Fig. 3.4). It is worth noting that the additional rest between LPS washout and LPS restimulation was selected and incorporated to minimize the contribution of transcript stability to P gene expression levels measured in T and T+L conditions so that reinduction could be assessed with greater precision. This duration of rest between challenges was shown to be insufficient for natural reversal of tolerance, based on no recovery of T genes in the T+L condition (data not shown). Thus, Akt regulates induction of P genes upon restimulation of tolerant macrophages through a mechanism independent of enhanced ACLY-mediated conversion of glucose-derived citrate to Ac-CoA.
Figure 3.6 LPS-inducible Akt signaling regulates induction of P genes in tolerant BMDMs. (a) Immunoblot analysis of activating phosphorylation of Akt at S473 and T308 during LPS restimulation of tolerant BMDMs. (b) Schematic of workflow for analysis of P gene regulation. BMDMs were left unstimulated (naïve, N) or stimulated with LPS for 24h to induce LPS tolerance (tolerance, T). BMDMs were washed and left without LPS for 4h. LPS responsiveness was assessed by stimulating naïve (N+L) or re-stimulating tolerant (T+L) BMDMs with LPS. In the T+L+inhibitor condition, drug was added in the final hour of rest between tolerance induction and restimulation. Naïve and Tolerant cells were collected as baseline controls (N and T). (c) Representative induction of Fpr1 and Ptges genes in BMDMs treated according to (b) with the Akt inhibitor MK-2206 used in the T+L+inhibitor condition (T+L+AKTi). ***p ≤ 0.001 (Student’s t-test). Data are from one experiment representative of two (a) or three experiments (c) (mean and s.e.m. of duplicates (c)).

Induction of responsive genes in tolerant macrophages is supported by ACSS2 activity

Under conditions of impaired conversion of citrate to Ac-CoA such as in yeast lacking ACLY\textsuperscript{20}–\textsuperscript{22} and in cancer cells with truncated glucose oxidation due to preferential shunting of pyruvate towards lactate\textsuperscript{16}, acetyl-CoA synthetase enzymes (AceCS1/2) can provide Ac-CoA through an ATP-dependent reaction between acetate and CoA\textsuperscript{23}. Therefore, we measured protein levels of the
nucleocytosolic AceCS1 enzyme (ACSS2) in tolerant and tolerant restimulated BMDMs and found expression in both conditions (Fig. 3.7a). Pharmacologic inhibition of ACSS2 (ACSS2i) between challenges completely attenuated reinduction of P genes (Fig. 3.7b). To our knowledge, we are the first to report a role for ACSS2 in the regulation of LPS-inducible genes.

**Figure 3.7** ACSS2 is expressed in tolerant macrophages and regulates induction of P genes. (a) Immunoblot analysis of nucleocytosolic acetyl-CoA synthetase (ACSS2) in tolerant BMDMs restimulated with LPS for the indicated times. (b) Gene expression of *Fpr1* and *Bpifc* in BMDMs treated as in Fig. 3.6b, using an inhibitor of ACSS2 in the interval between tolerization and restimulation (T+L+ACSS2i). of workflow for analysis of P gene regulation. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment representative of three experiments (mean and s.e.m. of duplicates (b)).

The known major sources of acetate in mammalian cells are uptake from the extracellular environment\(^\text{24,25}\), conversion from pyruvate via activity of keto acid dehydrogenases\(^\text{12}\), and deacetylation of acetylated proteins such as histones\(^\text{12,13}\). Given the well-established role for enhanced histone deacetylase (HDAC) activity in LPS tolerant macrophages\(^\text{4}\) and the failure of 2DG treatment during the window between challenges to attenuate P genes, we focused on histone deacetylation as a source of acetate fueling ACSS2 activity in tolerant restimulated macrophages. Specifically, we postulated that suppression and induction of T and P genes in tolerant macrophages may be linked through a futile cycle of histone deacetylation and hyperacetylation, respectively. Such a mechanism would serve as a temporal regulatory circuit, allowing for hyper induction of P genes only after induction and suppression of T genes. This phasic response would also store carbons from glucose oxidation in acetylated chromatin at T genes during activation to
be used for acetylation of P genes in a setting of impaired glucose oxidation, thus turning the chromatin into a metabolic capacitor capable of storing acetyl groups and therefore acetylation potential. Consistent with this concept, treating BMDMs with the HDAC inhibitors sodium butyrate and trichostatin A (TSA) during the tolerizing LPS challenge rescued T genes and attenuated P genes (Fig. 3.8a).

**Figure 3.8** Histone deacetylation supports induction of P genes in tolerant BMDMs. Gene expression of *Il1b, Fpr1*, and *Ptges* in BMDMs treated as in Fig. 3.6b, with the HDAC inhibitors sodium butyrate (Butyrate) and trichostatin A (TSA) added during prior to tolerizing LPS stimulation (a) or prior to LPS restimulation (b), as indicated in by the corresponding schematics. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Student’s t-test). Data are from one experiment (mean and s.e.m. of duplicates (a,b)).

Unexpectedly, treatment with butyrate and TSA in the window between challenges also attenuated P genes while having only modest effects on T genes (Fig. 3.8b). Taken together, these data illuminate a role of deacetylation in supporting the induction of P genes in tolerant macrophages.
Furthermore, although inhibiting HDAC activity during tolerization suggests that P gene induction is linked in series to T gene suppression, attenuation of P gene induction by HDAC inhibition after acquisition of a tolerant state raises to question whether induction may be supported in parallel by deacetylation of histones at other gene sets.

Finally, we asked how ACSS2 activity is being regulated in tolerant macrophages. Canonically, ACSS2 is activated by deacetylation catalyzed by Sirt1\(^28\), possibly as a mechanism of sustaining histone acetylation under nutrient-limited conditions. Inhibition of Sirt1 in the window between challenges did not attenuate P gene induction in tolerant BMDMs, suggesting a non-canonical mode of ACSS2 regulation (Fig. 3.9). Given the enhanced activity of Akt in this setting and the effects of AKTi on P gene induction (Fig. 3.6), we investigated whether ACSS2 could be a target of Akt. A database search revealed several residues in ACSS2 that could be targeted by Akt-dependent phosphorylation, specifically S30, S263, and S267 (UniProtKB: Q9QXG4). Future studies will be focused on determining whether ACSS2 is activated by Akt in tolerant macrophages. This is covered in more detail in the Discussion section that follows.

![Graph](image)

**Figure 3.9** Sirt1 does not regulate the induction of P genes in tolerant macrophages. (a) Gene expression of *Fpr1* and *Ptges* in BMDMs treated as in Fig. 3.6b, using an inhibitor of Sirt1 in the interval between tolerization and restimulation (T+L+Sirt1i). *p ≤ 0.05 (Student’s t-test). Data are from two experiments (mean and s.e.m. of duplicates).
DISCUSSION

In this study, we make several findings regarding the metabolic regulation of macrophage effector functions during LPS activation and tolerance. First, we found that the strength of macrophage activation and tolerance, measured by induction of *Il6* and *Il1b*, reflect the dose of LPS exposure and correlate with early dose-dependent shifts in glycolysis, mitochondrial respiration, and Akt activation. These observations are consistent with previous reports that TLR stimulation results in dose-dependent metabolic adaptations in human monocytes and that Akt is activated downstream of TBK1/IKKe in LPS-activated dendritic cells to promote rapid induction of glycolysis.

However, based on our previous study showing the importance of oxidative metabolism in supporting inflammatory macrophage activation and on our present findings illuminating parallels between LPS dose-dependent Akt signaling and changes in mitochondrial respiration, we chose to investigate the potential for Akt to regulate activation through control of glucose oxidation. We found that pharmacological inhibition of Akt during LPS stimulation blunted the early increase in mitochondrial respiration driven by enhanced GPD2-dependent glucose oxidation (see Chapter 2), thus also attenuating LPS-induced promoter region histone acetylation and expression of *Il6* and *Il1b*. Inhibition of Akt during prolonged LPS exposure also recapitulated the protection against respiration shutdown, inflammatory gene suppression, and LPS tolerance observed through impairing glucose oxidation by 2DG treatment or GPD2 deletion. Because these adaptations are a consequence of enhanced GPD2-driven glucose oxidation contributing to a switch from forward to reverse electron transport (FET to RET) during the transition from LPS activation to tolerance, we asked whether Akt could regulate GPD2 and found that AKTi treatment strongly attenuated LPS-inducible *Gpd2* expression. Taken together, these data support an updated version of our previous model (Supplementary Fig. 2.7), placing Akt as an additional regulatory node...
integrating TLR signaling with GPD2-dependent glucose oxidation to control inflammatory gene induction and suppression in macrophages (Supplementary Fig. 3.5). In addition to regulation of glucose oxidation via modulation of glycolytic flux and Gpd2 expression, Akt likely supports activation through direct positive regulation of ACLY by phosphorylation at S473 as has been reported in cancer\textsuperscript{30} and alternatively activated macrophages\textsuperscript{31}. This interaction is included in our updated working model (Supplementary Fig. 3.5a), but it will be important to validate the Akt-ACLY axis during LPS activation by immunoblot assay for ACLY S473 phosphorylation under conditions of inhibited Akt signaling. It is also noteworthy that the attenuation of LPS-inducible Gpd2 expression by AKTi treatment was observed at times later than the early increase in GPD2-dependent mitochondrial respiration. This is not inconsistent with a critical role for Akt in driving inflammatory gene suppression and tolerance through enhanced GPD2 activity, as the FET-to-RET transition occurs late during LPS stimulation and depends on continued glucose oxidation under conditions of impaired respiration (induced by many mechanisms including inhibition by nitric oxide\textsuperscript{32} and itaconate\textsuperscript{33–35}). Akt-dependence of the GPD2-driven respiration after 1h of LPS stimulation could be a result of increased flux through upper glycolysis and the glycerol phosphate shuttle due to enhanced hexokinase II (HK-II) activity permitted by its Akt-dependent translocation to the mitochondrial outer membrane\textsuperscript{15,36,37}. Given the delayed induction of glycolytic genes downstream of Akt-HIF-1α, enhanced glucose oxidation fueled by increased substrate flux through HK-II and the GPS is an attractive mechanism.

Second, we define a subset of LPS-inducible genes exhibiting enhanced responsiveness, or priming, following tolerization and identify a novel role for ACSS2 as a regulator of these genes in tolerant macrophages. These primed (P) genes were an interesting case study on the metabolic
regulation of macrophage effector functions because their pattern of expression was seemingly inconsistent with the concept of tolerance and our understanding of metabolic regulation of gene induction in LPS-stimulated macrophages. However, gene ontology analysis revealed that P genes belong to functional clusters distinct from pro-inflammatory cytokine and chemokine production, providing insight into which biological processes are prioritized in tolerant macrophages. As discussed in Chapter 1, the biochemical reactions in the metabolic networks of living organisms take on measurable changes in flux in response to the application of stress. “Steady-state” is the metabolic status as a function of the sum stress imposed by processes required for a cell’s existence (i.e. energy production, maintenance of ion gradients, and biosynthesis to balance molecular turnover), and any shifts in cellular metabolism in response to additional stress are calibrated to support specific biological functions to counter that stress and return to “steady-state.” Thus, given the existence of P genes in tolerant macrophages, we focused on uncovering the specific changes in metabolism calibrated to support induction and execution of P gene-related functions.

As oxidative metabolism is gradually shut down during tolerization, we postulated that P genes must be supported by an alternative pathway. Inhibitor experiments designed to block glucose utilization and citrate-derived Ac-CoA production between tolerization and LPS restimulation did not show strong attenuation of P genes, supporting our hypothesis. Strikingly, blocking acetate-derived Ac-CoA production by inhibiting ACSS2 completely attenuated P genes, suggesting a compensatory switch in response to impaired glucose oxidation. This switch from reliance on ACLY- to ACSS2-mediated production of Ac-CoA for gene induction is consistent with observations in other conditions of impaired conversion of citrate to Ac-CoA. Of course, future experiments measuring the size and substrate-dependency of the Ac-CoA pool in ACSS2-
deficient macrophages are needed, as well as analysis of promoter region histone acetylation at P genes, similar to what was performed in Chapter 2.

Although isotope tracing experiments have not yet been done in this setting, inhibition of HDACs during tolerization resulted in complete rescue of T genes and attenuation of P genes, supporting histone deacetylation as a source of acetate as has been previously reported\textsuperscript{16,17,27}. Moreover, our data showing failure of 2DG treatment between LPS challenges to attenuate P gene induction excludes dependency on acetate derived from glucose through the activity of alpha-keto acid dehydrogenases that has recently been proposed as an important source of acetate in other cell types\textsuperscript{26}. Given the known role of HDACs in the induction of LPS tolerance\textsuperscript{4}, we interpret this data as pointing towards a temporal regulatory circuit in which a futile cycle of histone deacetylation and hyperacetylation allows for hyper-induction of P genes after induction and suppression of T genes. Such a phasic response would store carbons from glucose oxidation in acetylated histones at T genes during activation to be used for acetylation of P genes in a setting of impaired glucose oxidation, thus turning chromatin into a metabolic capacitor capable of storing acetyl groups and therefore acetylation potential. Inhibition of HDACs after tolerization did not rescue \textit{Il1b} but completely attenuated the P genes \textit{Fpr1} and \textit{Ptges}, suggesting that there may be a larger pool of acetate from deacetylation of other genes that can also fuel P gene hyperacetylation and induction. Future experiments addressing this question will include ChIP-seq analysis of a larger set of T and P genes to determine whether they are regulated in a closed circuit or if other regions of chromatin can store acetylation potential. Isotope tracing experiments will also be performed to directly test whether histone acetylation at P genes is supported by acetate derived from deacetylation of histones that were previously acetylated downstream of glucose oxidation (see Chapter 4).
It is also noteworthy that the importance of P genes in assembly of CI and CV of the ETC (Supplementary Fig. 3.3) provides an additional layer of logic to the existence of a metabolic circuit linking the sequential induction of T and P genes. Indeed, as glucose oxidation critically supports T gene induction, we postulate that a rationale for the existence of P genes is to serve as a mechanism of reversing impaired mitochondrial oxidative capacity to restore responsiveness of inflammatory T genes after elimination of microbial infection. This conceptual framework is further supported by the natural recovery from tolerance that occurs after several days of LPS exposure\(^4\), coincident with a recovery of oxidative metabolism (data not shown). We predict that autocrine IL-10 may play a role in this time-dependent reversal\(^3\).

Third, we demonstrate that Akt regulates P genes and may do so through phosphorylation of ACSS2, constituting a novel mode of regulation of ACSS2. Our inhibitor experiments show that inhibition of Akt but not Sirt1 between LPS challenges results in attenuation of P gene induction comparable to blocking ACSS2 and HDACs. This observation was not recapitulated in BMDMs with constitutive Akt1/2-deficiency (data not shown), highlighting the importance of using methods conferring punctual inhibition in future studies investigating regulation of ACSS2 activity and induction of P genes. As additional evidence supporting Akt’s potential to directly control ACSS2, we found three serine residues in ACSS2 that are conserved phospho-targets of Akt (positions 30, 263, and 267). We are also aware of evidence for ACSS2 phosphorylation in situ, and future experiments will be focused on investigating such regulation in tolerant macrophages. Thus, in addition to a role of Akt in the induction and suppression of inflammatory genes through control of glucose oxidation, Akt may also regulate P genes through control of ACSS2 by direct phosphorylation (Supplementary Fig. 3.5b).
Taken together, the observations presented here extend our model for how GPD2-dependent glucose oxidation couples the duration of LPS exposure to induction and suppression of inflammatory genes, providing the first evidence of a role for ACSS2 in macrophage biology and highlighting Akt and ACSS2 as additional regulatory modules integrating TLR signaling with shifts in macrophage metabolism to induce gene-specific responses to microbial encounter.
MATERIALS AND METHODS

**BMDM culture.** BMDMs from male and female mice were differentiated and cultured as described previously\textsuperscript{39}. For LPS activation (0-3h post-LPS), BMDMs were stimulated with 1-100 ng/mL LPS (LPS Ultra-Pure, Sigma-Aldrich). All of the metabolic parameters associated with LPS activation, including glucose oxidation, Akt phosphorylation, and promoter histone acetylation, were assessed at early time points after LPS stimulation (0-3 h), while the downstream consequences of such metabolic events, i.e., inflammatory gene induction and cytokine production, were assessed at 3 h after LPS stimulation. For LPS tolerance (12-28h post-LPS), BMDMs were challenged with 100 ng/mL LPS for 24h to induce tolerance (T), washed, and then rechallenged with 10 ng/mL LPS (T+L) for the desired amount of time. In such experiments, naïve BMDMs were incubated in media for 24h (N), washed, and challenged with 10 ng/mL LPS (N+L) to provide responsive-cell controls. In Fig. 3.1, tolerance was induced using the concentrations indicated in the legend. When measuring induction of P genes, BMDMs were washed and media was replaced after 24h incubation in media or LPS, followed by a 4h rest before assay (N and T) or LPS rechallenge (N+L and T+L). Pharmacological inhibition of Akt (AKTi, 8 µM; MK-2206, Tocris, United Kingdom), ACLY (ACLYi, 80-160 µM; SB-204990, Tocris, United Kingdom), ACSS2 (ACSS2i, 25-50 µM; N-(2,3-di-2-thienyl-6-quinox- alinyl)-N0-(2-methoxyethyl)\textsuperscript{16}, ChemBridge), histone deacetylases (HDACi, sodium butyrate 3 mM and trichostatin A (TSA) 50-100 µM; Sigma-Aldrich), Sirt1 (Sirt1i, 0.5-1 mM; EX-527, Tocris, United Kingdom), and glucose utilization (2DG, 5-10 mM; 2-deoxy-glucose, Sigma-Aldrich), was achieved by pretreating BMDMs for 1h prior to LPS activation or in the final hour of 4h rest prior to LPS restimulation.
**Seahorse assays.** Basal and LPS-induced changes in oxygen consumption (OCR) and extracellular acidification (ECAR) rates were measured with a Seahorse XF96 Extracellular Flux Analyzer (Agilent). Assays were performed in Seahorse XF Assay Medium supplemented with glucose (11 mM) and adjusted to pH 7.4 with NaOH. Mix-wait-measure durations were set to 3, 2, and 3 minutes, respectively. For the MitoStress Test, oligomycin, FCCP, and rotenone/antimycin A were sequentially injected to achieve final concentrations of 1, 1.5, and 2 µM. Non-mitochondrial OCR was subtracted from the mean pre-oligomycin OCR and the mean post-FCCP OCR to calculate Basal and Maximal mitochondrial respiration, respectively. For measuring LPS-induced changes in OCR in real-time, media or LPS was injected after establishing basal OCR, followed by oligomycin (1 µM) and FCCP (1.5 µM). Real-time measurement of LPS-induced changes in ECAR was performed by injection of media or LPS followed by sequential injections of oligomycin (1 µM) and 2DG (500 mM) rather than oligomycin and FCCP. After each experiment, OCR and ECAR values were normalized to DNA content in each well by Hoechst 33342 staining (Life Technologies, Carlsbad, CA).

**Chromatin immunoprecipitation.** Acetylation at inflammatory gene promoters was measured by chromatin immunoprecipitation (ChIP) assay as described previously. Promoters are defined as the region between -500 bp and the transcription start site. Antibodies used were as follows: acetylated H3 (Millipore 06–599), acetylated H3K27 (Abcam ab4729), acetylated H4 (Millipore 06–866), and IgG (Santa Cruz, Dallas, TX, SC-2027). Fold enrichment was calculated as ChIP signals normalized to input.
**Gene expression.** RNA was isolated using RNA-Bee (Tel-Test) according to the manufacturer’s protocol. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCRs were run on a Bio-Rad C1000 Thermalcycler, and expression of target genes was calculated by the ddCT method using CFX Manager Software (Bio-Rad). Relative expression values for target genes were calculated by normalization to expression of hypoxanthine phosphoribosyltransferase (HPRT).

**RNA-seq.** Strand-specific libraries were generated using 500ng RNA input using TruSeq library preparation kit (Illumina, San Diego, CA). cDNA libraries were multiplexed using specific unique adaptors and sequenced using Illumina NextSeq 500 under single end 75bp read length parameters. Raw reads were trimmed off sequencing adaptors and low quality regions by btrim\(^23\). Trimmed reads were mapped to mouse genome (GRCm38) by tophat\(^21\). After the counts were collected, differential expression analysis was done by DEseq\(^2\), which calculated the fold changes and adjusted p-values. The fpkm values were calculated by DEseq2. The cutoff for defining the total LPS-inducible gene set was fold change >1.2 for N+L over N. Subsets of LPS-inducible genes were defined as follows: fold change >1.2 for N+L over T+L, labeled T genes; fold change between 0.8 and 1.2 for N+L over T+L, labeled NT genes; fold change >1.2 for T+L over N+L and fold change >1.2 for T+L over T, labeled P genes. Enrichment analysis of Gene Ontology (GO) terms in the P genes subset of LPS-inducible genes was performed using Enrichr\(^43,44\). Heatmaps were created using Prism software.

**Mice.** Wild-type C57BL/6J mice (The Jackson Laboratory) and whole-body Gpd2/-/- (KO) mice (generated as reported in Chapter 2) were maintained under specific pathogen-free conditions at
the Harvard T.H. Chan School of Public Health in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Male and female mice were used for endotoxic shock experiments and as a source of bone marrow for BMDM culture between 6 and 12 weeks of age. All protocols were approved by the IACUC of Harvard Medical School. In the endotoxic shock experiments, WT and GPD2 KO mice were injected with vehicle (saline) or 3 mg/kg LPS via intraperitoneal injection (IP), and serum IL-6 and IL-1β were measured 6 h later.

**Immunoblot and enzyme-linked immunosorbent assays.** For immunoblotting, BMDMs were lysed in 1% NP-40 buffer or RIPA buffer, and protein concentration was determined using the Bradford method or micro BCA assay (Pierce). Primary antibodies used were anti-phospho-AKT(S473), anti-phospho-AKT(T308), and anti-AKT (1:1000, Cell Signaling), anti-ACSS2 (1:1000, Abcam), and anti-α-Tubulin (1:5000, Sigma-Aldrich). For ELISA, cell culture supernatants and mouse whole blood were spun down, diluted in 5% BSA, and assayed for IL-6 and IL-1β using BioLegend Standard ELISA kits according to the manufacturer’s protocols.

**Statistical analysis.** Statistical significance was determined by a two-tailed Student’s *t*-test using Prism software (GraphPad). *, **, *** and **** in figures denote *p* value ≤0.05, 0.01, 0.001, and 0.0001, respectively.

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**Competing Interests**

The authors declare no competing interests.
Supplementary Figure 3.1 Attenuating glucose oxidation impairs suppression of inflammatory gene expression and cytokine production in macrophages and mice. Expression of $\text{Il6}$ and $\text{Il1b}$ and IL-6 cytokine production during prolonged LPS stimulation (12-24h) in WT BMDMs +/- 2DG (a,b) and in GPD2 KO BMDMs (c,d). Serum levels of IL-6 and IL-1β in 8-wk-old male WT and GPD2 KO mice injected IP with a high dose of LPS. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$ (Student’s $t$-test). Data are from one experiment representative of one (e) or three (a-d) experiments (mean and s.e.m. of duplicates (a-d) or n=4 mice per group (e)).

Supplementary Figure 3.2 LPS-induced expression of $Gpd2$ is regulated by Akt. (a) Gene expression of $Gpd2$ in BMDMs unstimulated (Naïve) or stimulated with LPS for 4h +/- Akt inhibitor (AKTi). $^*p \leq 0.05$ (Student’s $t$-test). Data are from one experiment representative of two experiments (mean and s.e.m. of duplicates).
Supplementary Figure 3.3 P genes belong to an array of gene ontologies. Gene set enrichment analysis performed on P genes using Enrichr GO Biological Process (a) and GO Cellular Component (b) reference lists. Pathways of interest are shown and ranked by p-value (Fisher exact test).

Supplementary Figure 3.4 Inhibiting the glucose-ACLY-Ac-CoA axis in tolerant macrophages does not attenuate induction of P genes. Expression of the P genes Ptges and Fpr1 in BMDMs treated as in Fig. 3.6b, using inhibitors of glucose utilization (2DG, a) and ATP citrate lyase (ACLYi, b) in the interval between tolerization and restimulation. *p ≤ 0.05 (Student’s t-test). Data are from one experiment representative of two experiments (mean and s.e.m. of duplicates).
Supplementary Figure 3.5 Integration of Akt and ACSS2 into previous model of metabolic regulation of gene induction in LPS stimulated macrophages. (a) During LPS activation, GPD2-dependent transport of electrons from the glycerol phosphate shuttle (GPS) through the ETC is upregulated to support an increase in glucose-derived carbon flux through glycolysis and the TCA cycle, providing increased availability of citrate and Ac-CoA. Increased glucose oxidation is also supported by increased activity and expression of glycolytic genes by Akt signaling. Coupled with an LPS-induced increase in ACLY activity downstream of Akt, enhanced production of Ac-CoA drives histone acetylation and induction of inflammatory genes like Il6 and Il1b. As oxidation of carbon substrates in the TCA cycle depends on the availability of electron acceptors such as NAD⁺, the cyclic reduction and oxidation of these molecules serves as an important link between oxidative metabolism in the TCA cycle and electron transport chain (ETC) activity. (b) During LPS re-stimulation of tolerant macrophages, glucose oxidation is impaired due to mitochondrial NAD⁺/NADH imbalance driven by GPD2-dependent reverse electron transport at complex I. Induction of primed genes (P) in this setting is fueled by Ac-CoA produced from acetate via the activity of ACSS2. Deacetylation of histones at other gene promoters, such as those of tolerized genes like Il6 and Il1b (T), contributes to the pool of acetate fueling histone acetylation and induction of P genes such as Fpr1 and Ptges.
REFERENCES


Chapter 4

Discussion and Future Directions
Summary and Conclusions

Systemic microbial infection in vertebrate animals elicits an acute systemic inflammatory response syndrome, characterized by activation of the innate and adaptive arms of the immune cells and elevated local and circulating pro-inflammatory cytokines (e.g. IL-6, IL-1β, and TNFα) and enhanced antimicrobial activity. This robust pro-inflammatory response is followed by prolonged immunoparalysis, characterized by general immunosuppression due to decreased pro-inflammatory cytokine production, impaired antigen presentation and costimulation, and marked T cell anergy. Immunosuppression in this context is believed to be a compensatory mechanism to limit damage to the host. However, protracted immunosuppression contributes a high percentage of sepsis-related hospital deaths. Central to coordinating the transition from activation to suppression are macrophages, which sense pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS). Macrophages mount an initial pro-inflammatory response followed by a persistent state of tolerance to subsequent bacterial challenges. Phase-two clinical trials aimed at suppressing inflammation during sepsis have been unsuccessful, turning attention toward preventing immunoparalysis. Thus, elucidating the mechanisms underpinning the critical inflection point in the biphasic response curve during sepsis could have significant therapeutic potential. Given the recent understanding that specific metabolic shifts critically support immunological recognition of infection and execution of effector functions, we pursued a metabolic underpinning to the biphasic response to microbial encounter.

Here, we uncovered and characterized several metabolic modules regulating the induction and suppression of gene-specific macrophage inflammatory responses to microbial encounter in vitro and in vivo. In Chapter 2, we carefully measured the time-dependent changes in metabolite
concentrations and mitochondrial respiration over the course of macrophage exposure to LPS and revised the current paradigm for how mitochondrial metabolism regulates inflammatory cytokine production, demonstrating that glucose oxidation initially supports but later suppresses induction of inflammatory genes through a metabolic pathway previously undescribed in macrophages. First, we showed that the link between glucose oxidation and transcription was by modulation of glucose-derived carbon flux through the TCA cycle and into the nucleocytosolic pool of acetyl-CoA (Ac-CoA), which serves as the substrate for transcription-activating histone acetylation at inflammatory gene promoters. We also show that the increase in glucose-derived Ac-CoA during activation depends on the enhanced activity of the enzyme ATP-citrate lyase (ACLY), which catalyzes the conversion of mitochondria-derived citrate to Ac-CoA and oxaloacetate. Next, we identified and characterized a novel role for the glycerol phosphate shuttle (GPS) and its limiting enzyme GPD2 (mitochondrial glycerol 3-phosphate dehydrogenase, mGPDH) in supporting the biphasic response to LPS by driving the acute LPS-inducible increase in glucose oxidation while also contributing to the shutdown of respiration characteristic of macrophages subjected to prolonged LPS exposure. Based on data obtained from experiments in bone-marrow-derived macrophages (BMDMs) from newly-generated Gpd2/- (KO) mice, we attributed the switch from increased to decreased glucose oxidation during the transition from LPS activation to tolerance to GPD2-dependent induction of reverse electron transport (RET). Indeed, based on specific changes in levels of complex I (CI)-associated superoxide and NADH, we proposed a model in which GPS activity, by delivering glucose-derived electrons to the electron transport chain (ETC), initially boosts oxidative metabolism to support increased acetyl-CoA (Ac-CoA) production and histone acetylation during LPS activation, while sustained GPS activity during prolonged LPS exposure overwhelms forward electron transport capacity leading to RET, thus reducing TCA cycling to
limit Ac-CoA production and inflammatory gene induction (Supplementary Fig. 2.12). Finally, the importance of GPD2-dependent regulation in the host response to microbial infection was demonstrated by using an in vivo model of LPS tolerance, in which a sublethal dose of LPS protects against septic shock triggered by a subsequent, lethal dose of LPS. In contrast to mice that did not receive the sub-lethal LPS pretreatment, mice that receive the pretreatment profoundly suppress IL-6 production during septic shock. Such suppression was attenuated in GPD2 KO mice, leading to enhanced IL-6 levels and exaggerated endotoxin-induced hypothermia. GPD2 KO mice that received sublethal LPS preconditioning also succumbed to subsequent lethal LPS challenge, while no lethality was observed in WT mice. Therefore, we illuminate the GPS is a hitherto unappreciated and unique regulator of LPS responses, using the ETC as a rheostat to couple duration of LPS exposure to ETC directionality to modulate a balance between the induction and suppression of inflammatory responses.

In Chapter 3, we extended our model for how GPD2-dependent glucose oxidation couples the duration of LPS exposure to induction and suppression of inflammatory genes by introducing an additional regulatory node at the nexus between TLR signaling and metabolism. We also identified a subset of LPS-inducible genes that displayed an expression pattern opposite to tolerance and were supported by a parallel metabolic module to glucose oxidation. Specifically, we showed that the serine/threonine kinase Akt (protein kinase B, PKB), a known target downstream of TLR signaling, integrated glucose oxidation with the strength and duration of LPS stimulation to control the amplitude and duration of inflammatory gene induction. We also identified a subset of genes, constituting 8% of the LPS-inducible transcriptome picked up by our analysis, that became hyper-responsive following prolonged LPS exposure, in contrast with the hypo-responsiveness of
inflammatory genes that is characteristic of the LPS tolerant state. These primed genes, or “P genes,” encode molecules important for biological processes distinct from tolerant genes. Moreover, consistent with the shift from increased to decreased oxidative metabolism over the course of LPS exposure as macrophages transition from activation to tolerance, P genes were not directly supported by glucose oxidation; rather, we showed that induction of P genes was supported by acetate-derived Ac-CoA production catalyzed by nucleocytosolic acetyl-CoA synthetase (AceCS1, or ACSS2). Moreover, we highlighted histone deacetylation as a source of acetate fueling this process. Finally, we proposed that in addition to a role of Akt in the induction and suppression of inflammatory genes through control of oxidative metabolism and thus citrate-derived Ac-CoA levels, Akt may also regulate P genes through control of ACSS2 by phosphorylation at conserved serine residues (Supplementary Fig. 3.5). Thus, we provide the first evidence of a role for ACSS2 in macrophage biology and highlight Akt and ACSS2 as additional regulatory modules integrating TLR signaling with shifts in macrophage metabolism to induce gene-specific responses to microbial encounter.

**Lessons from a worked example in immunometabolism**

Exploration into how shifts in intermediary metabolism support the four tasks of the immune system – recognition, effector functions, regulation, and memory – has widened the lens through which we view host defense and has led to the genesis of the relatively-new subfield of immunology called immunometabolism\(^9\). However, immunometabolism still largely sits at the fringes of its two parent disciplines, not biochemical enough for the metabolic biologists and not immunobiologically-important enough for classical immunologists. Of course there are papers that stand as exceptions. We like to think that our work is included with these exceptions.
Recall that in **Chapter 1**, the foundational relationship between metabolism and cell functions was described from a thermodynamic perspective. It was iterated that animate organisms are constrained by the same laws that govern the behavior of inanimate chemicals and that the reactions of such heterogeneous substances in an organism’s global metabolic network take on measurable changes in flux in response to the application of stress. Modern reluctance to accept the critical importance of metabolic shifts in the optimal execution of specific stress responses is partially a consequence of the focus of early work in biochemistry and partially a result of poor education in metabolism. The 19th and 20th centuries brought tremendous progress towards our understanding of biochemistry, as basic principles and tools of chemistry were applied to the study of heterogeneous substances within biological systems and how specific behaviors of such substances could support life. Such understanding has been crammed into a conceptual closet labeled “cell maintenance” by most until recent years (excluding perhaps the physiologists and some cancer biologists). Our worked example of how glucose oxidation regulates macrophage inflammatory responses is one with the potential to change this way of thinking, as it takes well-described macrophage effector functions during LPS activation and tolerance and systematically demonstrates regulation by modules in intermediary metabolism (Ac-CoA synthesis, TCA cycle, and GPS). A summary of this example and its broader application to other fields of study follows.

In our example, consider the macrophage as an open system. LPS is the imposed stress on the system, and the gene-specific inflammatory response is the system’s functional requirement of highest priority. TLR signaling, although of absolute necessity for transducing the stress into the functional response in this system (see **Chapter 1**), is excluded from this example because of our data demonstrating glucose oxidation as a layer of regulation downstream of TLR signaling acting
as a rheostat for the functional response rather than as a regulator of TLR signaling itself. At the foundation of this functional response to stress are the chromatin modifications that have been described as necessary events during gene-specific activation and tolerance\textsuperscript{10–12}. We demonstrated that such modifications depend on the metabolic status of the system, specifically on the flux of external glucose-derived carbons into the system, through glycolysis and the TCA cycle, into the nucleocytosolic pool of Ac-CoA, and into histones. Furthermore, we demonstrated that selectively blocking this flux path by pharmacologic or genetic manipulation results in attenuation of the gene-specific functional response. Similarly, tolerance was revealed to depend on this flux path such that rescue of responsiveness of the system to stress was achieved by protection of glucose oxidation and gene-specific histone acetylation. Such regulation of specific functional responses through the influence of metabolism on chromatin via changes in acetylation is a mode of metabolic control seen in other systems\textsuperscript{13–15} and in response to other stresses\textsuperscript{16–19}. Moreover, modification of chromatin by metabolites other than Ac-CoA has also been shown to integrate metabolism with functional shifts in the face of imposed stress, including histone demethylation by FAD-dependent LSDs and AKG-dependent JmjC demethylases and DNA demethylation by TET proteins\textsuperscript{20–22}. Although not investigated in the work in Chapters 2 and 3, post-translational modifications of non-histone proteins by spontaneous and enzyme-catalyzed reactions with metabolites is a general principle from biochemistry and is an important mode of regulation to consider when thinking about how metabolism might influence a system’s specific stress response\textsuperscript{23}. Indeed, examples in inflammatory macrophages have recently been reported\textsuperscript{24,25}.

The enzyme- and substrate-dependent synthesis of metabolites fueling modifications to histones and other proteins is another layer of metabolic regulation of specific functional responses
described by our work and others. In **Chapter 2**, we show that activation of the enzyme ACLY by phosphorylation at S455 is necessary for supporting the induction of inflammatory genes in response to LPS. Such regulation has previously been described in alternatively activated macrophages\(^{16}\) and is likely dependent on Akt, given our data in **Chapter 3** and work in cancer cells\(^{26}\). Moreover, we demonstrate that different substrate-enzyme-product axes may support distinct functional responses, supported by our evidence that acetate-ACSS2-Ac-CoA rather than citrate-ACLY-Ac-CoA regulates the induction of P genes. Such differences in substrate dependency may seem esoteric; however, they provide valuable insight into the system at the time of observation, highlighting prioritized functions (i.e. those of P genes versus T genes) and metabolic status (i.e. glucose oxidation off versus on). Illuminating these system properties informs the design of interventions to support or inhibit functions of the system in the corresponding context. For example, one might target acetate metabolism in cancer cells that have switched to acetate-dependent histone acetylation under nutrient-limited conditions resembling a tumor microenvironment\(^{27-30}\). In our macrophage example, limiting glucose oxidation strongly attenuated inflammatory gene induction during activation and enhanced responsiveness to restimulation in tolerant macrophages, underpinning a profound lack of protection against a lethal dose of LPS administered to GPD2 KO mice even after induction of tolerance using a sublethal dose of LPS. The observations from our mouse experiments are comparable to the protective effects of carbohydrate restriction and calorie restriction in mice given a systemic bacterial infection\(^{31}\).

Further upstream of Ac-CoA production, our work revealed that GPD2 and the GPS drive glucose oxidation to induce different functional states at different times following application of stress in
the form of LPS, initially supporting activation and later contributing to tolerance. This mechanism provides an example of a single metabolic module regulating a dynamic response over time. Such regulation is consistent the concept of metabolic shifts being calibrated to support a stress-specific functional demand placed on a system. GPD2-dependent glucose oxidation follows this thinking, as induction of pro-inflammatory genes is initially supported by increased GPS activity to enhance Ac-CoA production and histone acetylation, while suppression of inflammatory gene induction is achieved by respiration shutdown to limit the availability of citrate for ACLY-dependent Ac-CoA production and thus attenuate histone acetylation. Temporal regulation of gene-specific functions by metabolism is also found in the tolerant, restimulated system, as HDAC activity appears to fuel induction of P genes after suppression of T genes. Again, this metabolic shift reflects a change in priority of cellular functions. It is also possible that the increase in HDAC activity in the tolerant state serves the added role of countering the intracellular acidification that results from enhanced lactate production\textsuperscript{32}. Finally, we observed that many P genes encode subunits of CI and CV of the ETC (Supplementary Fig. 3.3). We postulate that this is a mechanism of reversing impaired mitochondrial oxidative capacity to restore responsiveness of inflammatory genes after complete elimination of microbial infection. Thus, our work provides an example of how a single metabolic module and clusters of metabolic modules support time-dependent changes in the functional requirements of a system in the face of a prolonged stress. If the goal of one’s work is to reorganize the hierarchy of functions in a system over time (i.e. restore responsiveness of inflammatory genes), then identification and targeting of such metabolic modules is an option. Another recent example of exploiting this modality was in the reversal of LPS tolerance by treating human monocytes with β-glucan \textit{ex vivo} after tolerization \textit{in vivo}. Rescue was achieved presumably by reversal of itaconate-mediated impairment of respiration, although additional evidence is needed\textsuperscript{33}.  

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Finally, the dynamic regulation of inflammatory responses by glucose metabolism in macrophages assigns novel biological importance to fundamental principles of biochemistry and bioenergetics that have not been implicated in many settings but constitute foundational mechanisms that may play a role in a wide array of biological systems. Specifically, we showed the critical importance of sufficient electron acceptor molecules in the oxidation of carbon substrates to support induction of histone acetylation and expression of inflammatory genes. Indeed, deployment of the GPS was necessary for maximal induction of glucose uptake and oxidation in LPS-activated BMDMs, consistent with its role as a redox shuttle in other tissues requiring high rates of glucose utilization\textsuperscript{34,35}. Moreover, histone acetylation and inflammatory gene induction were rescued in tolerant macrophages treated with the NAD\textsuperscript{+} precursor nicotinamide (NAM) (Supplementary Fig. 2.9), supporting the need for electron acceptors for TCA cycling proposed by Williamson and Krebs\textsuperscript{36} and demonstrated recently in cancer cells\textsuperscript{37}. Additionally, we demonstrated that GPD2-driven respiration can lead to RET in macrophages, underpinning its anti-inflammatory activity in the tolerant state. Indeed, GPD2-driven RET limits inflammatory gene induction by inducing accumulation of NADH at the expense of NAD\textsuperscript{+} return to the TCA cycle, thus impinging on substrate oxidation in the mitochondrial matrix. The switch from FET to RET is a consequence of thermodynamics and could actually be calculated if substrate oxidation rates and ETC component concentrations in the system were known. As mentioned in Chapter 1, the mitochondrial respiratory chain can be ordered based on the Gibbs energies of its components; however, more conventional is organization based on redox potentials ($E_h$). RET is favored under conditions of high proton motive force ($\Delta p$), due to an increase in one or both of its components. Shown in the mathematical representation of $\Delta p$ at 37°C in Eq. 4.1, these components are chemical ($\Delta pH$) and
electrical (ΔΨ) and result from differences in protons and differences in electrical potential across the mitochondrial inner membrane, respectively\textsuperscript{38}.

\[
\Delta p = \Delta \Psi - 61 \Delta \mathrm{pH}
\]

(Equation 4.1)

Prolonged exposure to LPS increases Δp in macrophages, as measured by increased ΔΨ (data not shown and \textsuperscript{39}); however, as GPD2 is poorly coupled to the proton circuit across the inner membrane because its redox activity is not coupled to translocation of protons, it acts as a continuous source of electron flow from oxidation of glycerol 3-phosphate (G3P) despite high Δp. This leads to over-reduction of the mobile electron carrier ubiquinone (Q), resulting in an \( E_h \) for Q/QH\textsubscript{2} that favors reverse electron flow through CI to reduce NAD\textsuperscript{+} to NADH. Although the enhanced expression and activity of GPD2 in LPS-stimulated macrophages favors RET through this mechanism, RET can be driven by other ETC components feeding into Q, as well (except for CI)\textsuperscript{40}. In addition to modulation of the NAD\textsuperscript{+}/NADH ratio, RET is also a source of superoxide through electron leak at the Q-facing site of CI, or site I\textsubscript{Q}\textsuperscript{41,42}. Thus, induction of RET by substrate feeding into the Q pool under conditions of high Δp represents a mechanism by which oxidative metabolism and bioenergetics may act on specific biological functions. Although isolated mitochondria from several cells and tissues have been shown to produce RET ROS\textsuperscript{43-47}, there are currently only a few settings in mammalian cells in which RET has been implicated, including LPS-stimulated macrophages (Fig. 2.13, 2.14, and \textsuperscript{39}), tissues subjected to ischemia-reperfusion injury\textsuperscript{40,48}, and in primary astrocytes\textsuperscript{49}. Therefore, RET remains an under-investigated mechanism for metabolic regulation of cellular and tissular stress responses.
Concluding Thoughts and Future Directions

Every scientist can appreciate the joy of being the first to observe a phenomenon of the natural world. To conceive, test, and realize an idea is a euphoric experience that justifies the negative emotions elicited by “failed” experiments, lost sleep, and monetary and technical hurdles. In the present case, the work shown and discussed in Chapters 2 and 3 started with the simple thesis, “There must be a metabolic basis of endotoxin tolerance.” This idea was born out of an obsession with immunological memory that likely stemmed from the first primary papers I read during my undergraduate training with Jason M. Grayson. To have realized this thesis and to have, in the process, uncovered the elegant mechanisms of metabolic regulation of inflammatory macrophage activation and tolerance discussed in previous Chapters, has been a remarkable experience. However, exploration into the unknown illuminates many more areas in need of investigation. The sections that follow will focus on describing these areas in addition to providing suggestions for future experiments to strengthen the conceptual framework built by the present work.

When discussing the observations from Chapter 2 in seminars or with colleagues, it is often asked “Is this true for stimuli besides LPS?” This is a fine question and one that, at present, can only be answered with a soft “yes.” In a collaboration with Linden Hu’s Laboratory at Tufts University School of Medicine, we have shown that glucose oxidation is initially enhanced and later repressed following exposure of BMDMs to the TLR2 ligand Pam3Cys-Ser-(Lys)4 (PamCSK) or to the live bacteria known for causing Lyme Disease, Borrelia burgdorferi (Bb). Changes in promoter region histone acetylation are important for inflammatory gene induction and suppression during activation and tolerance following PamCSK or Bb stimulation of BMDMs, and impairing glucose oxidation by inhibiting glucose utilization (2DG) or GPS activity (GPD2 KO) results in rescue of
histone acetylation and inflammatory gene induction in tolerant BMDMs comparable to that observed in our LPS tolerance experiments. Much of this work was performed by Urmila Powale at Tufts and is now a primary focus in the Hu Laboratory. Although these observations suggest that glucose oxidation is a conserved metabolic requirement for inflammatory gene induction downstream of TLR signaling, it will be necessary to test other TLR ligands. Additionally, whether glucose oxidation is a requirement for optimal induction of macrophage transcriptional responses downstream of sensing through other pathogen recognition receptors (PRRs) or cytokine receptors (i.e. IL-4R, IL-13R, IL-10R) is also of great interest. Indeed, the phenotypes of macrophages exposed to different signals fall along a spectrum as a function of the specific signal and the shifts in metabolism, but just as transcriptional profiling has revealed cores of genes conserved between subsets and phenotypes of immune cells, there must also be core metabolic pathways shared between macrophages exposed to different signals. Supportive of this concept, BMDMs alternatively activated by IL-4 stimulation have an acute increase in Akt-dependent glucose utilization similar to that observed in macrophages classically activated by LPS. In contrast, modulation of whole-body metabolism by dietary manipulation results in opposing effects of fasting and feeding on antibacterial and antiviral responses in mice. As GPS activity is primarily controlled by selective expression of Gpd2, discovery of additional settings in which enhanced glucose oxidation is enhanced could be initiated by screening a library of PRR ligands and cytokines for the ability to induce Gpd2 expression in BMDMs at different times after stimulation. Based on the examples provided above, a reductionist approach would need to be applied in each case of enhanced Gpd2 expression to verify that GPS activity is also increased and supports specific effector functions (i.e. glucose tracing into glycerol 3-phosphate (G3P), Seahorse analysis of glucose oxidation, and measurement of effector functions in GPD2-deficient BMDMs and mice).
In addition to exploring the role of GPD2-dependent glucose oxidation in macrophages polarized to other states, the importance of GPD2 in other cells is also an area of investigation illuminated by our work. As stated above, GPD2 and GPS activity are regulated by tissue-specific expression of Gpd2. There are currently only a few biological contexts in mammals and mammalian cells in which GPD2 has been implicated, including insulin secretion by pancreatic β-cells, liver gluconeogenesis, regulation of blood glucose and serum glycerol and triglyceride levels in female mice, and the growth of certain cancers. There is also evidence of time-dependent changes in Gpd2 expression in skeletal muscle following contraction and in T cells following activation. In the majority of these settings, enhanced GPD2 activity resulting from increased expression is functionally linked to specific biological functions through modulation of mitochondrial reactive oxygen species (ROS) levels.

The oxidation of G3P was first shown in tissue preparations by Meyerhof in 1919, while the glycerol phosphate shuttle was not proposed until 1958 after characterization of the cytosolic and mitochondrial glycerol 3-phosphate dehydrogenases. Tissue-restricted expression of GPD2 is thought to be an adaptation to minimize ROS production in tissues that do not require high rates of glucose oxidation. Although it is unclear how ROS produced at different sites within the ETC lead to distinct biological functions, it is worth noting that G3P oxidation by GPD2 can drive ROS production at multiple locations, including its own F and Q domains, as well as site I_Q from RET. One may postulate that production of superoxide towards the outer versus inner side of the mitochondrial inner membrane (i.e. by G_F versus G_Q, respectively) could have different biological effects based on the differential availability and chemical character of substrates and detoxifying enzymes. However, recall that the importance of GPD2 in the regulation of macrophage
inflammatory responses was not attributed to RET ROS but rather to induction of redox imbalance due to reduction of NAD\(^+\) at CI, a lesser-appreciated yet fundamental consequence of RET\(^70\). This mechanism of regulation was further supported by the observation that treating BMDMs with site I\(_O\) electron leak inhibitors (S1QELs)\(^{45,49}\) during tolerization to LPS did not rescue inflammatory gene induction upon restimulation (data not shown). Preventing RET in LPS-stimulated BMDMs by ectopic expression of the alternative oxidase (AOX) as was done previously\(^39\), but effects on tolerance were not measured. As AOX activity would prevent both RET ROS and NAD\(^+\) reduction at CI, it is likely that AOX-expressing macrophages would be resistant to LPS-induced tolerance. It would be exciting to induce endotoxin tolerance in WT and AOX-expressing mice and determine their level of protection against a subsequent lethal dose of LPS, similar to what was done in GPD2 KO mice in \textit{Chapter 2}.

Of course, changes in carbon flux and in ROS levels as a consequence of enhanced GPD2 activity could play concomitant roles in coupling changes in oxidative metabolism with the execution of specific effector functions. Taking again the example of time-dependent changes in GPD2 activity in the skeletal muscle following contraction, the acute increase\(^{63,64}\) and subsequent decrease\(^65\) in activity could be to support enhanced mitochondrial ATP production during the period in which contractions are being elicited, and such oxidative metabolism could enhance ROS from multiple sites in the ETC, inducing a hormetic response through redox-sensitive signaling networks. Thermogenesis by BAT is another context in which GPD2 activity is presumed to be important – cold exposure increases GPD2 expression\(^34\), and mice lacking GPD2 have 15% lower resting energy expenditure at thermoneutrality\(^61\) – however, a mechanism for how GPD2 is supporting the
thermogenic activity of brown adipose tissue (BAT) is lacking\textsuperscript{71}. Thus, elucidating the importance and regulatory interaction of GPD2 within other biological contexts is an open area of investigation.

Current knowledge on the transcriptional regulation of GPD2 is limited. GPD2 is encoded by a single gene on chromosome 2 of humans and 3 of rodents, containing approximately 2432 base pairs and 17 exons\textsuperscript{34,35}. There are three different first exons of \textit{Gpd2}: 1a, found in brain; 1b, found in all tissues; and 1c, found predominantly in the testis\textsuperscript{35}. The 1b exon contains a thyroid hormone receptor responsive element, which may explain the modulation of GPD2 activity in BAT and skeletal muscle in rodents given thyroid hormones and during hibernation\textsuperscript{72}. GPD2 protein contains 727 amino acid residues, which constitute FAD-, G3P-, and calcium-binding domains\textsuperscript{34}.

In \textbf{Chapter 3}, we proposed that LPS-inducible GPD2 and GPS activity may be Akt-dependent, as pretreatment with AKTi attenuated \textit{Gpd2} expression during LPS activation. This result should be validated in genetic models of macrophage Akt-deficiency, as have been used in the study of polarization previously\textsuperscript{73,74}. As a more comprehensive investigation into the path connecting TLR signaling with induction of \textit{Gpd2} expression, it would be useful to systematically test the importance of known modules coupling TLR signaling with transcription events\textsuperscript{75} through mutagenesis or knock-out studies. Such studies could even be performed by mining existing publically-available sequencing data. It is worth noting that uncovering common signaling modules integrating TLR engagement with enhanced \textit{Gpd2} expression and GPS activity would allow generalization across ligands and between immune cell subsets.

As final commentary on the biology surrounding the GPS, our discovery of the importance of GPD2 in control of macrophage inflammatory responses presents a novel regulatory module that
can be targeted by an array of interventions to modulate the host’s response to microbial encounter. Most appealing is the off-label use of the diabetes drug metformin as an inhibitor of GPD2. Indeed, although metformin’s glucose-lowering effects have been attributed to several mechanisms, including inhibition of CI\textsuperscript{76}, some reports suggest that GPD2 may also be inhibited by metformin\textsuperscript{59,60}. Therefore, the well-known anti-inflammatory effects of metformin\textsuperscript{77–80} may be underpinned by antagonism against GPD2. It would be useful to test this in a cell autonomous system by performing Seahorse extracellular flux analysis of G3P-driven respiration in permeabilized BMDMs untreated or treated with metformin and stimulated with LPS for different times. As oral administration of metformin leads to GPD2 inhibition in the liver, where it is first metabolized, alternative modes of delivery would better suit its anti-inflammatory application. For example, a slow-release injectable formulation was recently developed and tested for antitumor effects\textsuperscript{81}. Injection of metformin into the joints of an animal or human with Lyme arthritis due to late-stage Bb infection may have significant anti-inflammatory effects on resident immune cells by inhibiting GPD2 activity and glucose oxidation.

As an alternative approach to inhibiting GPD2 activity directly, proximal processes affected by increased GPD2 activity may also be therapeutically targeted to modulate inflammatory responses. In the case of GPD2-dependent inflammatory gene suppression, we identified GPD2-driven RET as a mechanism underpinning shutdown of respiration and restriction of TCA cycling due to NAD\textsuperscript{+}/NADH imbalance at CI. Recall that RET involves electron flow from a source of oxidized substrate (i.e. G3P) to the Q/QH\textsubscript{2} pool, which at a sufficient $E_h$ under high $\Delta\rho$ is able to donate electrons thermodynamically “uphill” to CI, driving reduction of NAD\textsuperscript{+} and electron leak onto O\textsubscript{2} at I\textsubscript{Q} to produce superoxide. Although not measured, it is possible that the concentration of
available Q molecules in the inner membrane space is limiting during prolonged LPS exposure and that modulation of the Q pool may protect against induction of RET by sustained GPD2 activity. Indeed, it has been proposed that Q deficiency plays a role in a number of human diseases and that supplementation with coenzyme Q$_{10}$ (CoQ) can correct such deficiency and ameliorate disease symptoms$^{82}$. Delivery of CoQ is difficult due to its exceptionally high hydrophobicity. Preliminary experiments in our cell autonomous system for studying macrophage LPS tolerance did not show protection of inflammatory gene induction after prolonged LPS exposure when high-dose CoQ was supplied (data not shown), even when handled as described previously$^{83}$. It will be important to quantify the pool size and redox status of the Q/QH$_2$ pair in macrophages over an LPS timecourse to determine whether there is a deficiency that could be corrected by CoQ supplementation, as such knowledge would inform the design of future animal experiments and would also strengthen the model of LPS-induced RET in macrophages. Moreover, if CoQ supplementation is shown to prevent RET and thus protect against LPS tolerance in vitro and/or in vivo, it would suggest that RET may play a role in pathologies resulting from CoQ deficiency in other settings such as diet-induced obesity$^{84}$.

Finally, regarding the availability of Ac-CoA for the induction of transcriptional responses following LPS stimulation, there are many additional experiments required for improving clarity, especially in the case of ACSS2-dependent P genes. A common question in response to the work described in Chapter 2 is, “What determines the gene specificity?” Although we demonstrated that the histone acetyltransferase (HAT) p300 was involved in coupling the increase in glucose-derived Ac-CoA with enhanced histone acetylation in the promoter region of pro-inflammatory genes, we were surprised to find that add-back of Ac-CoA by supplying a high concentration of
exogenous acetate did not rescue induction of pro-inflammatory genes in tolerant macrophages (data not shown). It is noteworthy that induction of P genes was not measured in these experiments but presents an important avenue for further exploration, as this subset of genes was shown to be profoundly dependent on ACSS2 in Chapter 3, while pro-inflammatory T genes may be specifically regulated by citrate-derived Ac-CoA. On the ACLY-dependency of T gene induction and lack of rescue by acetate, it is possible that spatial regulation of ACLY activity supports compartment-specific control of Ac-CoA.\textsuperscript{85} Production of a localized pool of Ac-CoA at T genes by spatially-associated ACLY activity is an interesting area for further investigation.\textsuperscript{86} Similarly, substrate source fueling ACSS2-dependent activity also requires many additional experiments. Most critical is the measurement of Ac-CoA levels and P gene expression levels in macrophages with silenced ACSS2 activity. Substrate dependency should then be elucidated by tracing of isotopically-labeled acetate into Ac-CoA and histones as was done for glucose in Chapter 2. Pulse-capture experiments in which isotopically-labeled glucose is transiently supplied during activation and then removed followed by measure of HDAC-inhibitor-sensitive labeled acetate will also provide insight into the proportion of acetate that is available for induction of P genes in tolerant macrophages. Of course, as mentioned previously, identification of the upstream signaling proteins responsible for enhancing ACSS2 activity in the tolerant state is also an area in need of further exploration. Although we have preliminary data showing that AKTi recapitulates the effects of ACSS2i in P gene induction, direct evidence for Akt-dependent phosphorylation of ACSS2 in this setting is needed.

Investigation into the areas discussed above will undoubtedly extend our appreciation for the many important roles of metabolism in supporting the context-specific functional requirements of
eukaryotic organisms. The phenotypes one can observe through experiments in immunobiology and physiology are often striking and very enjoyable to study. Metabolism provides a foundation of regulation and support of these phenotypes that tethers the complex biological phenomena to the organized set of first principles explaining the behavior of chemical species. Such an understanding of biological responses from a chemical perspective is not only clarifying but also illuminates many powerful points of intervention to exert control over the behavior of organisms. It is exciting to think on what wonderful phenomena lie ahead as continued exploration pushes back the barrier of the unknown to uncover new knowledge.
REFERENCES


