Glucocorticoid-induced immune-metabolic reprogramming in macrophages

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1. Abstract

Glucocorticoids (GC) are amongst the most frequently prescribed substances in clinical medicine. Their potent anti-inflammatory properties render them essential for treating disorders characterized by uncontrolled inflammation such as rheumatoid arthritis, asthma or inflammatory bowel disease. However, high serum levels with a concomitant excess in GC activity lead to side effects related to a subsequent dysregulated systemic energy metabolism.

Macrophages represent cells of the innate immune system that are present throughout the organism and involved in the initiation and resolution of the inflammatory response.

These cells represent a major target of glucocorticoids in an inflammatory setting. GC were shown to tailor metabolic pathways by directly affecting mitochondrial biology in inflammatory macrophages. However, past research did not link such GC-mediated metabolic adaptations of macrophages with the anti-inflammatory effects of these compounds.

In this study, we analyzed the effects of GC on metabolic reprogramming and mitochondrial dynamics in LPS-activated macrophages. We performed metabolic flux analysis as well as profiling of TCA metabolites and intermediates of glycolysis and studied the impact of a GC-mediated metabolic reprogramming of macrophages on their inflammatory response.

We show that GC rescue TCA cycle activity in LPS-activated macrophages and identify the TCA cycle-derived metabolite itaconate as a major mediator of the anti-inflammatory effects of GC. Our data thus identify a missing link between GC driven rewiring of the TCA cycle and their well-known impact on cytokine signaling.

Taken together, this thesis emphasizes the importance of GC during the immune-metabolic programming of innate immune cells. These insights could contribute to the discovery of novel targets for anti-inflammatory interventions during chronic inflammatory diseases.

2. Zusammenfassung

Glukokortikoid-induzierte immun-metabolische Neuprogrammierung von Makrophagen

Hintergrund und Ziele: Glukokortikoide zählen zu den am häufigsten verschriebenen Medikamenten im klinischen Alltag. Ihre potenten anti-inflammatorischen Eigenschaften machen sie auch heute noch zu einem unverzichtbaren Werkzeug in der Therapie chronisch-entzündlicher Erkrankungen wie der rheumatoiden Arthritis, dem Asthma bronchiale oder chronisch-entzündlicher Darmerkrankungen. Ohne streng geführte Therapiekontrolle können hohe Glukokortikoid-Serumspiegel jedoch zu schwerwiegenden Nebenwirkungen führen, welche in vielen Fällen mit einer Dysregulation des systemischen Energiestoffwechsels assoziiert sind.
Makrophagen gehören zu den Zellen des angeborenen Immunsystems und kommen ubiquitär im Organismus vor, wo sie Entzündungsantworten sowohl initiieren als auch auflösen können. Diese Zellen stellen ein zentrales pharmakologisches Ziel für Glukokortikoide im inflammatorischen Kontext dar. Neueste Arbeiten konnten zeigen, dass Glukokortikoide den mitochondrialen Stoffwechsel direkt beeinflussen. Ob diese Glukokortikoid-vermittelten Veränderungen im zellularen Metabolismus zu deren anti-inflammatorischen Effekten beitragen war bisher jedoch unklar.

Die vorliegende Arbeit hatte zum Ziel, die metabolischen Effekte von Glukokortikoiden in Makrophagen zu untersuchen und deren Bedeutung für die bekannten anti-inflammatorischen Eigenschaften dieser Substanzklasse zu verstehen.

Methoden: Makrophagen wurden aus dem Knochenmark von Wildtyp sowie LysM-Cre Twinkle-Knockout und IRG1-Knockout (Immune-responsive gene 1) Mäusen isoliert und in Kultur gehalten. Die in vitro Applikation von Lipopolysacchariden (LPS) über unterschiedliche Zeiträume diente hierbei als inflammatorisches Modell. Zur Bestimmung Glukokortikoid-induzierter Effekte wurden die Derivate Kortikosteron und Dexamethason in den Versuchen verwendet. Mittels *Enzyme-linked Immunosorbent Assays (ELISA)* konnte die Sekretion verschiedener Zytokine quantifiziert werden. *Realtime PCR (RT-PCR)* wurde zur Bestimmung der Transkription unterschiedlicher Proteine und Enzyme des Zellstoffwechsels und der mitochondrialen Dynamik verwendet. Darüber hinaus wurden bioenergetische Prozesse mittels *Seahorse Flux Analyzer* und der Quantifizierung bestimmter Stoffwechsel-Metabolite durch *massenspektrometrische Verfahren (LC/MS)* untersucht.

Ergebnisse und Beobachtungen: Wir konnten zeigen, dass Dexamethason nicht nur inflammatorische Effekte von LPS antagonisiert, sondern auch die mitochondriale Funktion, einschließlich sauerstoffabhängiger Energieproduktion, signifikant beeinflusst. Zusätzlich führte die Dexamethason Stimulation von LPS-aktivierten Makrophagen zur vermehrten Produktion des immunmodulatorischen Metaboliten Itaconat, welcher eine entscheidende Verbindung zwischen metabolischen und antiinflammatorischen Effekten von Glukokortikoiden in Makrophagen repräsentiert. Mittels Verwendung von Makrophagen aus IRG1-defizienten Mäusen, denen die Fähigkeit fehlt, selbstständig Itaconat zu produzieren, sowie durch externe Applikation eines Itaconat-Derivats, konnten wir eine zentrale Bedeutung von Itaconat bei der Sekretionshemmung pro-inflammatorischer Zytokine durch Dexamethason nachweisen.

Schlussfolgerungen: Immunmetabolische Effekte von Glukokortikoiden vermitteln deren anti-entzündliche Eigenschaften in Makrophagen, wobei die Glukokortikoid-induzierte Akkumulation des Metabolits Itaconat von zentraler Bedeutung ist. Weiterführende Experimente müssen helfen, die molekularen Mechanismen, welche dieser vermehrten Itaconat-Produktion durch Glukokortikoide zugrunde liegen, zu identifizieren.
Die Ergebnisse dieser Arbeit könnten zur Entdeckung neuartiger Signalwege beitragen, die als Zielstrukturen effektiver anti-inflammatorischer Medikamente dienen könnten.

3. Introduction

3.1. Glucocorticoids and glucocorticoid-receptor-signaling

3.1.1. Metabolic homeostasis and anti-inflammation

Glucocorticoids are steroid-based hormones, mostly synthesized in the *Zona fasciculata* of the adrenal cortex. Their release is controlled by stimulation of the adrenocorticotropic hormone (ACTH), a peptide produced by the anterior pituitary gland. ACTH-secretion itself is regulated by the corticotropin releasing hormone (CRH), which is generated by neurosecretory nerve terminals at the hypothalamus. This hypothalamic-pituitary-adrenal axis (HPA axis) works as a negative feedback loop, with the peripheral hormone controlling its own secretion [1] (Fig.1).

Cortisol represents the active version of glucocorticoids in the human endocrine system. To guarantee the adjustment of different activity levels throughout the day, cortisol production underlies a circadian rhythm: Serum concentrations are higher earlier in the morning and decline in the evening. In addition, the HPA axis is activated when the organism is exposed to different stimuli such as emotional stress, trauma or infection [2]. Once released into the bloodstream, GC influence numerous physiologically important processes, including electrolyte homeostasis, cardiovascular function, cognitive function, reproduction, and development [3].

Most importantly, their function is crucial for a stabilized systemic energy metabolism and a regulated immune response. They help to maintain stable blood glucose levels by promoting gluconeogenesis, glycogen metabolism, glucose uptake and utilization, and pancreatic endocrine secretion [4]. Moreover, they are crucial regulators of immune cell activation and exert potent anti-inflammatory effects [5]. This feature makes them effective pharmacological agents for the treatment of diverse inflammatory conditions such as rheumatoid arthritis, asthma and multiple sclerosis.

Nevertheless, the importance of their influence on homeostasis of both glucose metabolism and a well-balanced immune response is evidenced when the system of self-regulation is compromised or manipulated. High serum levels can result from an overproduction of cortisol, in most cases due to increased ACTH release by pituitary corticotroph tumors (Cushing´s disease), or be a consequence of prolonged administration of therapeutic GC with concentrations above the “cushing threshold” [6]. In either case, an excess of glucocorticoid activity can lead to disruption of homeostatic control and severe pathologies as described in 2.1.3 and illustrated in Fig.1.

3.1.2. GR signaling

Kendall´s discovery of “Compound E” guided decades of investigation followed by countless new insights into the biology of GC signaling [7]. Nevertheless, probably due to their pleiotropic effects, a clear concept of the key mechanisms behind their immune regulatory actions remains elusive. There is no doubt concerning their immunosuppressive properties, however, the detailed principles of how GC bring these into action has been challenged in the recent years [8].

There is increasing evidence for a cell-type specific and context-dependent immune-regulation rather than just “one fits and hits all” suppression [9, 10]. GC act dependently from the affected cell-type, duration of the stimulus and the state of inflammation [11]. In the setting of acute inflammation, GC inhibit extravasation of leukocytes, reduce blood flow to inflammatory sites, attenuate production of chemokines and cytokines, limit vascular dilatation and influence resolution by promoting phagocytosis of cellular debris by macrophages. Wound healing, followed by resolution, is impaired, in part through reduction of collagen synthesis and blocking of angiogenesis [12].

Their interaction with multiple inflammatory processes gives an idea of the complexity of their action on a molecular level. The current paradigm indicates a glucocorticoid receptor (GR)-dependent positive and negative regulation of gene expression, in total targeting over 9,000 genes of the human transcriptome [5]. Due to their steroid molecular structure, GC cross the cell’s double lipid layer without interacting with transmembrane proteins. After passing this barrier, GC bind to an intracellular receptor of the nuclear receptor family [13]. Disassociation from its chaperone-complex occurs, after which the activated glucocorticoid-receptor transfers to the cell´s nucleus [14]. Here, it interacts with the DNA either directly or by “tethering” other transcription factors (TF) and then controlling their action on gene-expression. The GR can take its effect as a monomer or after formation of a dimer, a conjunction of two GR [15]. As a monomer, it manipulates other TF by tethering or by independently interacting with their response elements [16]. Meanwhile, the dimerized GR binds to specific palindromic regions called Glucocorticoid-responsive-elements (GRE), which occur in regulatory sections of target genes, leading to either activation (transactivation) [17] or in some cases, repression of the transcriptional machinery [18]. Most of the anti-inflammatory effects have been proposed to be a consequence of “trans-repression”, which is defined as repressed transcription of pro-inflammatory mediators by tethering TFs like NF-kB (nuclear factor kappa-light-chain enhancer of activated B-cells), AP-1 (activator protein 1) or STAT-3 (signal transducer and activator of transcription 3)
[19-21].

After GR/TF-interaction, proinflammatory pathways are attenuated on multiple levels. For instance, a reduced production of pro-inflammatory cytokines such as interleukin-6 (IL-6) or

tumor necrosis factor alpha (TNF-α) results in down-regulated Toll-like-receptor signaling (TLR, see 2.2.1).[22].

In contrast to trans-repression, transactivation is suggested to be responsible for unwanted side effects like alterations of glucose metabolism or adipogenesis [23]. Nevertheless, several studies have demonstrated that GR dimerization and transactivation is also required for certain anti-inflammatory actions.[24-26]

For the attachment to GRE, the GR needs co-activators such as SRC-protein family members (steroid receptor coactivator), CBP (CREB-binding protein) or p300 to guarantee chromatin accessibility [27]. On the other hand, co-repressors like NCOR-1 and 2 (nuclear receptor co-repressor) lead to chromatin condensation and prevent gene expression.[28] The regulatory function of the chromatin landscape illustrates the fact that epigenetic modifications also play a crucial role in controlling GC immunosuppressive actions.[29]

Additionally, next to modulating gene expression in the nucleus, mitochondrial transcription is also shaped by GR. Indeed, the GR can affect apoptosis-related processes and modify mitochondrial function by directly interacting with the mitochondrial genome and proteins involved in mitochondrial respiration and energy metabolism.[30, 31]

Rapid GC-induced effects may be explained by non-genomic alterations, including modulation of cell-signaling pathways via a membrane-bound GR, non-specific membrane-intercalation or the direct interaction with kinases through the cytosolic GR [32]. This comprehensive and multilayered system emphasizes that the transactivation vs. trans-repression model seems to describe a simplified idea of GC way of action. Unlike once hoped [33], it fails to fully distinguish desired immunosuppressive from side effect related mechanisms.

3.1.3. Glucocorticoids in disease and pharmacological treatment

After Addison´s disease was described in the 19th century [34], it took nearly a hundred years for glucocorticoids to be available for patients suffering from an underproduction of cortisol or autoinflammatory disorders [7] .

GC showed their potential as potent anti-inflammatory agents when Hench and colleagues introduced them as a novel treatment for rheumatoid arthritis in 1948 [35, 36]. Since then, GC are indispensable in every field of medicine. Even after the introduction of new anti-rheumatic drugs like biologics, they still play an important role in treating conditions characterized by uncontrolled inflammation [37].

Next to rheumatoid arthritis, GC are utilized for the therapy of other autoimmune or allergic diseases like asthma, multiple sclerosis, inflammatory bowel disease and even hematologic cancers like multiple myeloma [38]. Due to their broad spectrum of application it is estimated that 1-3% of the adult Western population is receiving therapeutic GC [39].

Over the years, more synthetic derivatives of endogenous cortisol were produced, with different potency and reduced side effects [7, 40]. Optimizing GC therapy by looking for agents that mimic their immune-suppressive properties but simultaneously limit side effects is still an ongoing process in pharmacological development. Selective glucocorticoid receptor modulators (SEGRM) or targeted drug delivery systems could be potential solutions to this issue [41, 42]. However, today there are in general two major problems of GC therapy, with prolonged administration leading to GC resistance and limiting clinical efficacy on one hand, while an excess in GC activity results in numerous unwanted clinical symptoms on the other (Fig.1).

Considering the mechanism behind a regulated GC-secretion, high serum levels and long-term exposure can cause adrenal insufficiency following the loss of ability to regulate the body's answer to stress [43]. Furthermore, a pro-diabetic metabolism, partly due to promoted gluconeogenesis and lowered insulin sensitivity, can be a consequence. Impaired collagen synthesis, osteoporosis, muscle atrophy, impaired wound healing, depression, mood changes, skin thinning, and hypertension are pitfalls of GC therapy as well [12].

Overall, the poor therapeutic index of GC makes the adjustment of therapeutic strategies inevitable, especially concerning dose and duration of administration. Nevertheless, further investigation of key signaling pathways affected by GC could improve immunosuppressive therapies in the future.

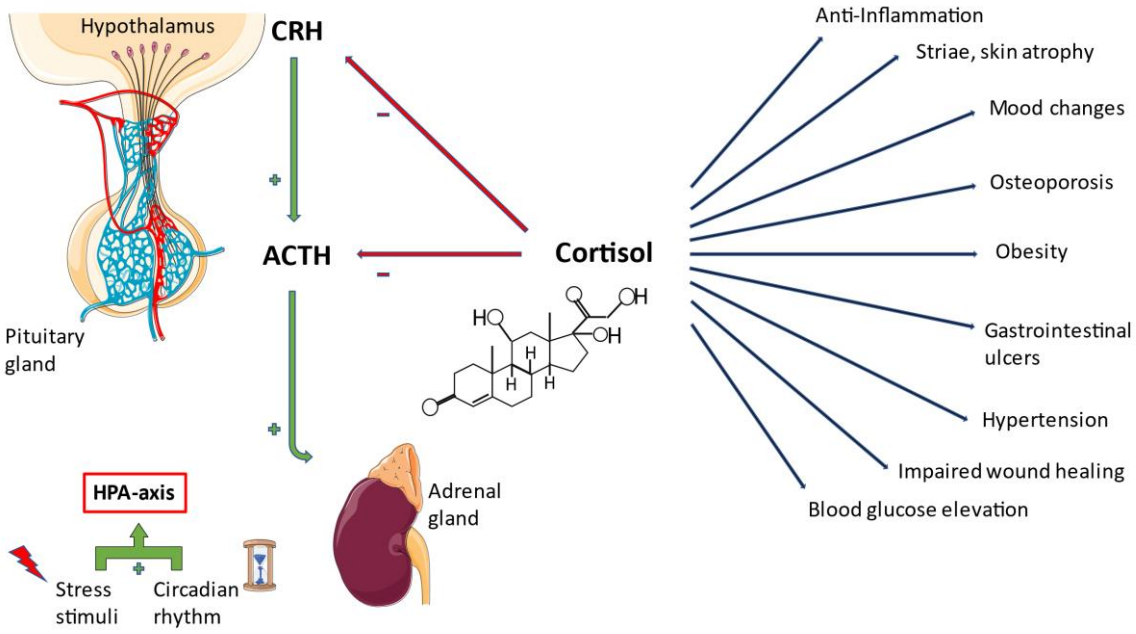

Figure 1: The HPA axis is a self-regulated system. Cortisol has pleiotropic effects on the organism. Prolonged high serum levels, often due to external administration, can result in broad clinical symptoms.

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3.2. Macrophages: In between inflammation, resolution and tissue repair

3.2.1. Origin and function

Macrophages are present in every compartment of the organism and are either tissue-resident or recruited to an inflammation site [44]. A major part of tissue resident macrophages develop from precursors of the yolk sac and fetal liver, others differentiate from blood monocytes that migrate to the tissue. Blood monocytes themselves emerge from common myeloid progenitor (CMP) cells in the bone marrow or myeloid stem cells (MSC), respectively [45]. Microglial cells in the brain, Kupffer cells in the liver, osteoclasts in the bone and bronchoalveolar macrophages in the lungs serve distinctly tissue-specific purposes. However, maintaining tissue homeostasis is a feature they all have in common. Additionally, as cells of the innate immune system, macrophages initiate and orchestrate the defense against an invading pathogen [46].

In general, inflammation is a coordinated response to different perturbations of tissue integrity. Infection or injury can result in a sequence of reactions involving cells and molecular mediators taking action against the underlying cause. If successful, this sequence terminates in resolution and tissue-repair [47]. Macrophages play a significant role in generation as well as in terminating inflammation [44]. By secreting pro-inflammatory cytokines and growth factors, engulfing invading microorganisms and recruiting other leukocytes, they sustain the initial process of host defense. As sensory cells, they express recognition receptors which detect pathogens or mediators derived from cellular damage. These receptors are called pattern-recognition-receptors (PRR) and are activated by pathogen-associated-molecular-patterns (PAMPs). TLR are membrane bound PRR that recognize components of pathogens such as LPS, peptidoglycan or RNA. Ligands of PRRs in the cytoplasm (NOD-like receptors, NLR or RIG-I-like receptors, RLR) include bacterial RNA, uric acid crystals and bacterial toxins, which must pass the cell membrane to be detected [48]. Upon activation, these receptors stimulate transcription factors that initiate the expression of pro-inflammatory genes [48].

By using their defense mechanisms, macrophages can destroy certain pathogens without the help of adaptive immune cells in early stages of an immune response. Yet they are, next to dendritic cells, important antigen-presenting-cells (APC) that bridge the process of pathogen recognition with a specific answer by cells of the adaptive immune system [45].

After elimination of the pathogen, macrophages coordinate tissue repair by adopting a pro-resolving, anti-inflammatory phenotype. This phenotype is induced by responding to anti-inflammatory mediators and prolonged by their secretion. Expression of cell surface receptors such as programmed cell death ligands 1 and 2 (PD-L1 and PD-L1) assist this change of function [49]

Preserving the fine-balanced dichotomy of initiation and resolving an inflammatory response is associated with heterogeneity that relies on a high level of plasticity of these cells. Moreover, their ability to control tissue homeostasis, ubiquitous presence and contributing role to the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis makes them essential targets for GC at all directions of macrophage polarization [50, 51].

3.2.2. Plasticity as a characteristic feature of macrophage biology

Depending on the signal, macrophages can be activated and polarized from a resting state (M0) to different phenotypes (Fig.2). M1- or classically activated macrophages (CAM) gain antimicrobial and pro-inflammatory features whereas M2- or alternatively activated macrophages (AAM) are responsible for their anti-inflammatory and pro-resolving nature [52]. Signals that drive “classical activation” include TLR-ligands and components of pathogens such as LPS, LTA (lipoteichoic acid), viral RNA/DNA or the cytokines interferon-y (IFN-y) and TNF-α [53, 54]. CAM express surface markers such as the cluster of differentiation molecules CD80, CD86 and enzymes supporting the inflammatory response, namely inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2). Transcription factors such as NF-kB or hypoxia-inducible factor 1 alpha (HIF1-a) initiate synthesis of high levels of pro-inflammatory cytokines. TNF-α, IL-6, IL-1ß, IL-12 and IL-23 support T-cell responses and maintain promotion of inflammation [54].

Typical inducers of M2-polarization are the cytokines IL-4 and IL-13, produced by cells of the innate and adaptive immune system. However, there have been several others identified that induce alternative macrophage activation such as IL-10, IL-33 or GC [55]. This led to further subdivision of M2-macrophages (M2a, M2b, M2c or M2d), characterized by different activation signals and effector mechanisms of each subtype [55]. In general, AAM express markers and effectors like CD163, CD36, STAT6, GATA3 (GATA binding protein 3), PPAR-y (peroxisome proliferator-activated receptor gamma) or ARG1 (arginase 1) that support a pro-resolving profile [53]. For example, the enzyme ARG1 depletes arginine and catalyzes the production of polyamines that are associated with tissue remodeling functions and wound healing [56]. Even though this concept emphasizes their ability to adopt a task-specific profile, it is an oversimplification and too static of a model as macrophages can rapidly adjust their phenotype in consonance with a permanently changing environment [52]. This is supported by recent studies that challenge the interchangeability of CAM, mainly described *in vitro*, with the M1-phenotype and AAM with M2-polarized macrophages [57]. The characteristic of adopting a distinct phenotype in a changing environment is accompanied with alterations in cell metabolism that assure immune cell effector functions leading to supporting or restraining an immune response.

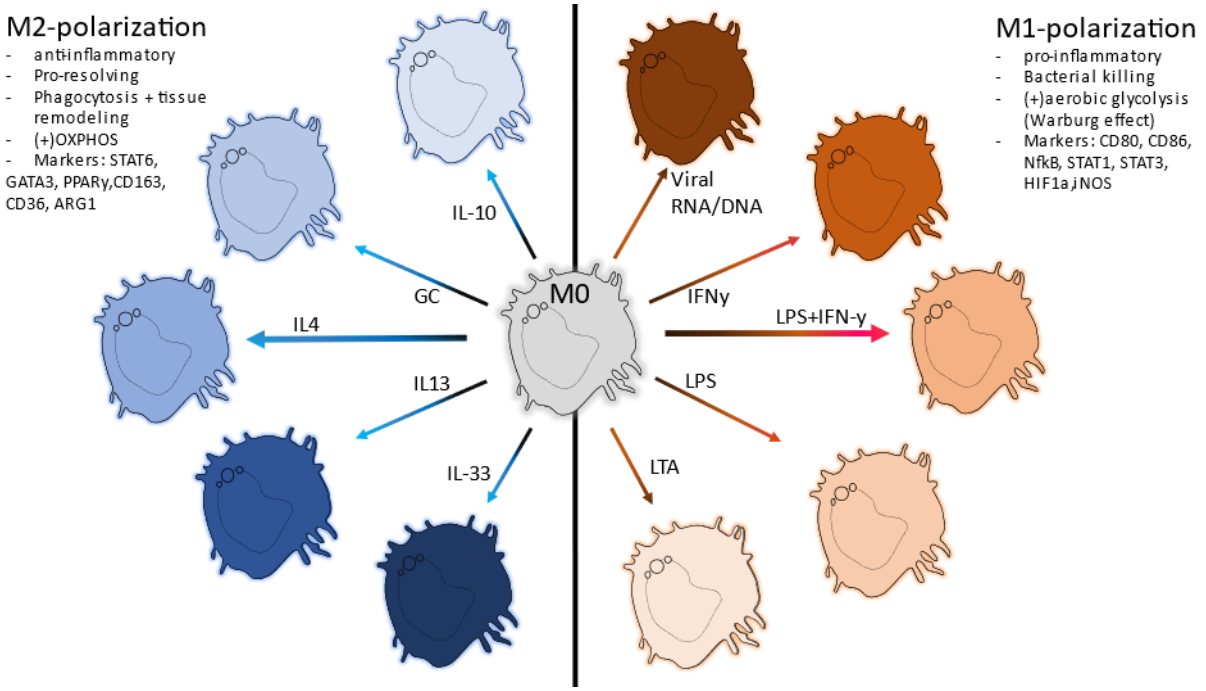


Figure 2: Macrophage polarization as a complex continuum rather than just two extremes. According to the dichotomous picture of macrophage phenotyping, M2-polarization is accompanied with anti-inflammatory and pro-resolving features and induced by signals like IL-4/IL-13, IL-10, IL-33 and GC. M1-macrophages are specialized in supporting inflammation to protect the host from a pathogen. Distinct markers characterize each phenotype. (This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license)

3.3. Immunometabolism: Linking key metabolic pathways with immune cell action

3.3.1. Metabolism is regulated by a cell´s environment

In general, cell metabolism can be divided into anabolism and catabolism. Anabolism unifies metabolic reactions that result in synthesis of molecules that are associated with cell growth and development. By contrast, catabolic metabolism is responsible for energy supply [58]. Cells generate energy via production of adenosine-triphosphate (ATP) by metabolizing carbon compounds. Glucose is the main source of carbon, yet fatty or amino acids can be channeled into metabolic pathways as well.

A sequence of enzyme-catalyzed reactions that includes glycolysis in the cytosol and citric acid cycle (TCA-cycle) in the mitochondria enable the cell to produce reduction agents that fuel the electron transport chain (ETC) at the inner membrane of mitochondria. Glucose is directed into the cytosol by glucose transporters (GLUT) and activated through phosphorylation by the enzyme hexokinase or glucokinase in the liver, respectively. Glycolysis results in production of pyruvate that is channeled into the mitochondria and converted into acetyl-CoA. Acetyl-CoA represents the central hub of cell metabolism as it is both the product of catabolic and educt of anabolic reactions. For example, it can be either the source for fatty acid synthesis in the cytosol or the product of fatty acid oxidation (FAO) in

the mitochondria. Glycolysis ensures the fast supply of a small amount of ATP and reduces the co-factors nicotinamide adenine dinucleotide (NAD) and flavine-adenine-dinucleotide (FAD). NADH and FADH are electron carriers necessary for a working ETC and consequently for mitochondrial respiration.

Inside the mitochondria, the TCA-cycle enzyme citrate-synthase catalyzes the synthesis of citrate from oxaloacetate and acetyl-CoA, followed by a sequence of reactions. An active TCA-cycle provides high quantities of NADH and FADH and ensures efficient energy production. Oxidation at the complexes of the ETC results in a proton gradient across the inner mitochondrial membrane. The gradient activates ATP synthases, which conserve a multiple of ATP compared to glycolysis alone. This oxygen- and mitochondria-dependent process is called “oxidative phosphorylation” (OXPHOS) and is typical for quiescent cells under normoxic conditions.[58]

Alterations of a cell´s environment can direct a change of utilized metabolic pathways. Under hypoxic conditions, the cell depends on glycolysis as the main source of ATP-production (anaerobic glycolysis). It generates lactate via oxidation of pyruvate to restore NAD levels. Other observations were made in a cancer microenvironment. The “Warburg effect” describes the phenomenon by which tumor cells tend to disrupt OXPHOS and support glycolysis-linked production of lactate, even if oxygen is present (aerobic glycolysis) [59]. Among a multitude of effects, the redirection of cancer cell metabolism results in accumulation of lactate and diversion of glycolytic intermediates that are used for nucleotide synthesis to support tumor growth.[60]

The ability to reprogram metabolic pathways to support distinct functions is seen in immune cells as well. Early studies suggested that activated macrophages and rapidly proliferating T-cells have a high demand of energy and depend on distinct metabolites that are required for their function and plasticity [61]. This interaction of immune cell function and metabolism has been referred to as “immunometabolism”. As mentioned above, macrophage activation is associated with signal specific alterations of key metabolic pathways and, simultaneously, a diversion of mitochondrial biology.[62] Representing potent mediators for anti-inflammatory macrophage polarization (see 1.2.2), GC have recently been linked to important alterations of macrophage “immunometabolic” reprogramming [63].

Overall, mitochondrial function and biogenesis are essential for immune cell polarization as they preserve the setting for oxygen dependent ATP-production and turnover of multifunctional metabolites (see 2.3.3).

3.3.2. Mitochondrial dynamics affect metabolic efficacy and immune cell signaling

Mitochondria are essential for energy supply of most organisms and often illustrated as the “powerhouse of the cell”. As described in chapter 2.3.1, they provide the location for the TCA cycle, fatty acid oxidation and oxygen-dependent ATP-production. Two membranes and the intermembrane space enable the organelle to create a proton gradient necessary for mitochondrial respiration.

A part of the mitochondrial proteins is encoded by its own genome, the mitochondrial DNA (mtDNA). However, the major part is transcribed in the nucleus and transferred into the mitochondria [64]. Their replication is independent from the nuclear genome. The replisome consists of the mtDNA helicase TWINKLE, the mtDNA polymerase (POLG) and the single stranded binding protein (mtSSB). The TWINKLE helicase is responsible for the unwinding of DNA that initiates the replication process [65]. Mitochondrial replication is crucial for their function and necessary for mitochondrial fission. Separation is needed for biogenesis on one hand and clearance of dysfunctional mitochondria on the other (mitophagy) [66]. Recent studies showed that “midzone fission” is associated with a promotion of mitochondrial proliferation. By contrast, “peripheral fission” leads to mitophagy.[67]

Moreover, the organelles are able to elongate, fuse and change their location within a cell. This mitochondrial dynamic of fusion and fission is a well-balanced process and depends on coordination of distinct proteins, acting at the outer and inner membrane of mitochondria. Optic-atrophy protein 1 (OPA-1) and mitofusin 1 and 2 (Mfn) orchestrate fusion whereas the dynamin-related protein 1 (DRP-1), in communication with the endoplasmatic reticulum and adaptor proteins like MFF, drives mitochondrial fission [68]. Dysfunction of balanced mitochondrial dynamics and biogenesis can have an influence on metabolism, apoptosis and immune cell function [64]. For example, different effector states of T-cells are suggested to be connected with alterations of mitochondrial dynamics. Memory T-cells have elongated mitochondria with more efficient OXPHOS. Increased fission and fragmentation of mitochondria in effector T-cells leads to oxidative stress and production of reactive oxygen species (ROS) [69].

Another link of mitochondrial integrity with inflammatory signaling was reported in human monocytes. Polarization towards a M1-phenotype upon LPS-stimulation was related to enhanced expression of miR-125b, a microRNA that shapes TLR-signaling. MiR-125b limits the effects of the fusion mediator MTP18 (mitochondrial protein 18kDa), resulting in reduced mitochondrial fusion and consequently dysregulated mitochondrial dynamics [70].

Additionally, in innate immune cells, mitochondrial dynamics are closely related to crucial signaling cascades after viral infection.[71] The mitochondrial adaptor protein MAVS (mitochondrial antiviral-signaling protein) is bound to the outer mitochondrial membrane and

part of pro-inflammatory RIG-I-receptor signaling [72]. In this context, mitochondrial fusion is suggested to positively regulate MAVS-mediated RIG-I-signaling via MFN-1 [73].
Furthermore, the latest investigations suggested an antagonizing role for GC on LPS-induced fragmentation of mitochondria and ROS production that could contribute to GC immunosuppressive effects [63].
Taken together, there are several networks that indicate a connection of inflammation with mitochondrial dynamics and integrity. This connection highlights the importance of mitochondrial function for immune cell regulation and as recently reported, potentially for anti-inflammatory actions of GC.

3.3.3. Immune-metabolic programming: A hallmark of macrophage plasticity

Classical activated macrophages (M1) promote glycolysis, the pentose phosphate pathway (PPP) for nucleic acid synthesis and redirect the TCA-cycle at different points. In alternatively activated macrophages (M2) on the other hand, glycolytic activity is limited in comparison to CAM and the TCA cycle remains intact with high rates of OXPHOS (Fig.3) [74]. In line with the observations of GC action on mitochondrial networks, Stifel *et al.* recently demonstrated that GC induce a M2-like phenotype by counteracting LPS-induced effects on glycolysis and TCA cycle function, however, not on oxidative phosphorylation [63].
Increased glycolysis is a hallmark metabolic change of rapidly activated immune cells. It provides a fast and sufficient way to generate ATP and metabolic intermediates that modulate effector functions, in macrophages mainly phagocytosis and cytokine production [61]. Promotion of glycolysis in CAM is organized on different levels. Distinct glycolytic enzymes have been linked to inflammatory signaling pathways. For example, increased hexokinase activity positively correlates with promotion of the NLRP3 inflammasome (NLR family pyrin domain containing 3) [75], the glycolysis regulating enzyme PFK2 (phosphofructokinase 2) was found to be activated upon LPS/IFN-y-challenge in macrophages [76] and PKM2 (pyruvatkinase M2) limits glycolytic flux and assures the redirection of glycolytic intermediates. Moreover, a dichotomous role of PKM2 links the glycolysis enzyme with activation of the inflammation-regulating transcription factor HIF1-a [77] that is suggested to be negatively modulated by GC in inflammatory macrophages [63].
Even glycolytic metabolites themselves show effects on immune function, supported by evidence that accumulation of lactate in CAM drives epigenetic modifications of inflammatory genes [78].
Finally, mTOR (mechanistic target of rapamycin), a central regulator of cell growth, development and inflammation has been associated with metabolic adjustments in differently activated immune cells [75, 79]. As mentioned above, AAM show decreased glycolytic activity in comparison to CAM. This observation could be linked to limiting expression of the

enzyme PFKB3 (6-phosphofructo-2-kinase isoenzyme 3) followed by a reduced glycolytic flux in the M2 phenotype [76].

As a consequence of M1 polarization, the TCA cycle is shifted towards the production of intermediates that either influence the immune response themselves or support pathways in charge. Key regulatory metabolites of the redirected TCA-cycle include citrate, succinate and itaconate. The levels of citrate, itaconate and succinate increase after downregulation of the TCA enzymes isocitrate dehydrogenase (IDH) and succinate dehydrogenase (SDH) [74]. Accumulation of citrate leads to production of itaconate that inhibits SDH in the mitochondria. [80]. The enzyme ACOD1 (aconitate decarboxylase) is encoded by the gene IRG1 (immune-response-gene 1) and catalyzes the synthesis of itaconate from citrate and cis-aconitate, respectively [81]. High succinate concentrations occur as a consequence of SDH inhibition and mediate stabilization of HIF1-a that induces the production of the proinflammatory cytokine IL-1β [82].

Moreover, citrate transfers to the cytosol and can be used for fatty acid synthesis for membrane biogenesis and generation of effector molecules of macrophages like prostaglandins and nitric oxide (NO). [83] In this context, NO is suggested to be involved in metabolic remodeling and alteration of mitochondrial respiration in macrophage polarization.[84]

Nevertheless, not every intermediate of a redirected TCA cycle exerts only pro-inflammatory actions. In fact, the metabolite itaconate facilitates microbial killing [85] while also contributing to anti-inflammatory functions of macrophages by affecting the production of IL-1β through the NLRP3 inflammasome and various other pro-inflammatory cytokines involved in TLR signaling. [80, 86]. Recent studies demonstrated that itaconate assists AAM polarization in IL-33-stimulated mononuclear cells and consequently mediates resolution of inflammation.[87]

However, the metabolic signature of macrophage polarization is not reduced to glycolytic catabolism and the redirection of the TCA cycle. FAO and glutamine metabolism are suggested to be crucial for M2 polarization as they fuel the active TCA cycle [74] IL-4-stimulated macrophages, an example of alternative activated macrophages, show enhanced OXPHOS concomitant with increased mitochondrial biogenesis and FAO that can, at least in part, be explained by upregulation of PGC1ß (PPAR-gamma coactivator 1 beta) [88]. PGC1ß is induced by STAT6 upon IL-4 stimulation (Fig.3). The M2 signature of these mediators was underlined by evidence that STAT6 and PGC1ß interact with M2 marker genes such as ARG1 [89]. An increase of arginase-1 activity in AAM, as mentioned in chapter 2.2.2, is in line with these findings [90].

In conclusion, immunometabolic reprogramming describes a central hallmark of macrophage polarization. The dogma of alternatively and classically activated macrophages seems to be

oversimplified, yet offers a plausible concept of how metabolic pathways could be (re)arranged to pave the way for pro- and in contrast, anti-inflammatory immune cell effector functions. In this context, endogenous and therapeutic GC represent substances that mediate anti-inflammation, as they organize macrophage polarization by evidently affecting key immunometabolic pathways and mitochondrial function.

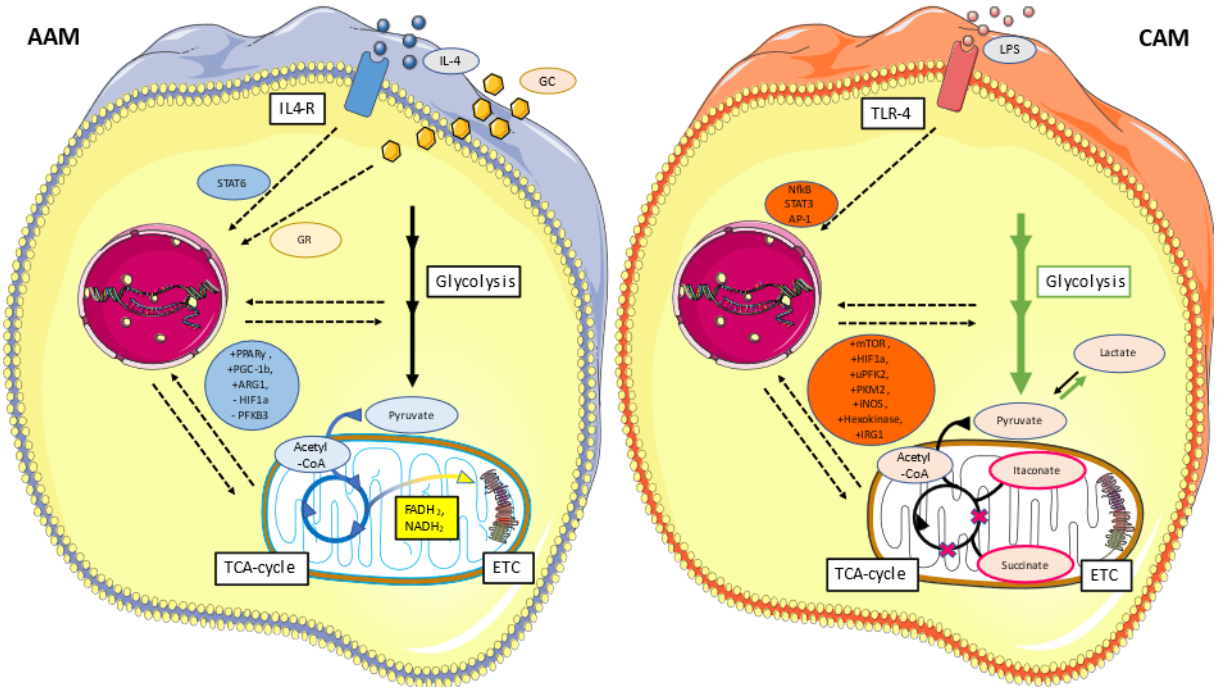


Figure 3: Metabolic signatures of macrophage polarization. AAM (left side) are characterized by low glycolytic activity but a functional TCA cycle and high rates of OXPHOS. CAM on the other hand, promote glycolysis and break the TCA cycle to redirect multifunctional metabolites. GC induce a M2-like phenotype by counteracting LPS effects on glycolysis and TCA cycle function (This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license)

4. Materials and Methods

4.1. Materials

Cell culture

DMEM (1x)	GIBCO® by Life Technologies (#41965-039)
RBC lysis buffer	8 g NH_4Cl 4.7 g HEPES 200 µl 0.5 M EDTA; pH 8.0 Adding 1000 ml VE-H_2O;
Penicillin/Streptomycin	PAN Biotech GmbH (#P06-07100)
Trypsin/EDTA 10x	PAN Biotech GmbH (#P10-024100)
DPBS 1x	GIBCO® by Life Technologies (#14190-094)

Cell culture plates

greiner bio-one	CELLSTAR®

Cytokines

LPS	Sigma-Aldrich (#L-2630)
Dexamethasone	Sigma-Aldrich (#D1756)
Dimethylitaconate	Sigma Aldrich (#592498)
DMSO	AppliChem (#A3608,001)

ELISA

Washing Buffer	0.05% Tween-20 (Sigma-Aldrich #P7949) in PBS
Blocking Buffer	1% BSA (Sigma-Aldrich #A7030) in PBS
ELISA-plate	Thermo Scientific (#442404)
ELISA-Kits	IL-10 (R&D Systems #DY417) IL-6 (R&D Systems #DY406) TNF-α (R&D Systems #DY410) IL-1β (R&D Systems #DY401)
Color-Reagent A+B	Thermo Scientific (#34021)

Stop-Solution	Invitrogen (#SS04)

Primer

Glut1	Forward: 5´-TCA ACA CGG CCT TCA CTG-3` Reverse: 5´-CAC GAT GCT CAG ATA GGA CAT C-3`
Drp1	Forward: 5´-CTG ACG CTT GTG GAT TTA CC-3` Reverse: 5´-CCC TTC CCA TCA ATA CAT CC-3`
Opa1	Forward: 5´-TCA GCA AAG CTT ACA TGC AGA-3` Reverse: 5´-TGC TTG GAC TGG CTA CAT TTT-3`
Mfn1	Forward: 5´-TGC CCT CTT GAG AGA TGA CC-3´ Reverse: 5´-AGA GCC GCT CAT TCA CCT TA-3´
Mfn2	Forward: 5´-GGG GCC TAC ATC CAA GAG AG-3´ Reverse: 5´-CCT TGG ACA GGT ACC CTT TG-3´
Nrf1	Forward: 5´-AGC ACG GAG TGA CCC AAA C-3´ Reverse: 5´-TGT ACG TGG CTA CAT GGA CCT-3´
Nrf2	Forward: 5´-TCT CCT CGC TGG AAA AAG AA-3´ Reverse: 5´-AAT GTG CTG GCT GTG CTT TA-3´
Il6	Forward: 5´-TCC TTC CTA CCC CAA TTT CC-3´ Reverse: 5´-GCC ACT CCT TCT GTG ACT CC -3´

RNA isolation

RNAlater® Solution	Ambion (#AM7021)
Precellys-Stahl-Kit 2,8 mm	PeqLab (#91-PCS-MK28)
peqGold Trifast	PeqLab (#30-2020)
TRIzol®	Invitrogen (#15596-026)

PCR

SYBR Select Master Mix	Applied Biosystems (#1601040)

Seahorse

<table>
<tr><td>Seahorse 96-well plate</td><td colspan="3">Agilent (#101085-009)</td></tr>
<tr><td>Cartridge</td><td colspan="3">Agilent (#102416-100)</td></tr>
<tr><td>XF Calibrant</td><td colspan="3">Agilent (#100840-000)</td></tr>
<tr><td>XF Base Medium</td><td colspan="3">Agilent (#103334-000)</td></tr>
<tr><td>Sodium Pyruvate 100 mM</td><td colspan="3">Sigma (#S8636)</td></tr>
<tr><td>Glucose</td><td colspan="3">Sigma (#G8769)</td></tr>
<tr><td>Glutamax</td><td colspan="3">Gibco (#35050-038)</td></tr>
<tr><td>MitoStress Test Kit</td><td colspan="3">Agilent (#103015-100)</td></tr>
<tr><td>GlycoStress Test Kit</td><td colspan="3">Agilent (#103020-100)</td></tr>
<tr><td>MitoStress Medium</td><td colspan="3">240 µl Glucose
600 µl Glutamax
600 µl Natriumpyruvate
- Added to 60 ml Medium
- Filtered (0.45µm filter) and adjusted to pH 7.4 using 0.2 M NaOH</td></tr>
<tr><td>GlycoStress Medium</td><td colspan="3">600 µl Glutamax
In 60 ml Medium
Filtered (0.45µm filter) and adjusted to pH 7.4 using 0.2 M NaOH</td></tr>
<tr><td>Reagents MitoStress Test Kit</td><td>Port</td><td>reagent</td><td>Concentration/ well</td></tr>
<tr><td></td><td>A</td><td>Oligomycin</td><td>1.0 µM</td></tr>
<tr><td></td><td>B</td><td>FCCP</td><td>1.5 µM</td></tr>
<tr><td></td><td>C</td><td>Rotenone/Antimycin A</td><td>0.5 µM</td></tr>
<tr><td>Reagents GlycoStress Test Kit</td><td>Port</td><td>reagent</td><td>Concentration/</td></tr>
</table>

		well
A	Glucose	10 mM
B	Oligomycin	1.0 µM
C	2-Desoxyglucose	50 mM

Perchloric acid extraction for Liquid chromatography-mass spectrometry (LC-MS)

$HClO_4$ (70%)	PanReac (#1321751611)
K_2CO_3	Roth (#P748.1)

4.2. Methods

Animals

Mice were maintained at the specific pathogen-free animal care facility (FPZ) of the university of Erlangen-Nuremberg and housed in a room at 23 +- 2°C, with 50 +- 10% humidity and a 12-hour light/dark cycle (lights from 8:00 a.m. to 8:00 p.m.). All mice had free access to water and regular rodent chow. C57BL/6JRj wild-type mice were obtained from Janvier Labs. Mice with a floxed Twinkle allele (R26-K320E-Twinkle$^{loxP/+}$) were provided from Prof. Dr. Rudolf Wiesner, institute of vegetative physiology from the University of Cologne (UKK). IRG1$^{-/-}$ mice (Irg1tm1a(KOMP)Wtsi) were obtained from Jackson Laboratories. Mixed-sex cohorts at 8-12 weeks of age were used for all the experiments.

Cell culture

- **Generation of bone marrow-derived macrophages (BMDMs)**

C57BL/6JRj wildtype, LysM-Cre Twinkle$^{fl/fl}$ and IRG1$^{-/-}$ mice were sacrificed at 8-12 weeks of age. Lower extremities were cut above the hips. Skin, tendons and muscles were removed. Femur and tibiae were washed for 30 s in 70% EtOH and afterwards placed in PBS. The bones were cut and opened at each end. Bone marrow was flushed into a petri dish using a 10 ml syringe, a 26 G cannula and 10 ml of PBS. The bone marrow was dispersed to create a cell suspension and afterwards transferred into a 50 ml falcon. The cell suspension was centrifuged at 1200 rpm for 8 min at room temperature. The supernatant was discarded and the pellet was suspended in 3 ml RBC lysis buffer for 3 min per mice. The erylysis was stopped by adding 27 ml of PBS and the solution afterwards homogenized through a 70 µm cell strainer. The cell suspension was centrifuged under the same conditions as described before. After discarding the supernatant, the resulting cell pellet was suspended in 10 ml BMDM medium and plated in a 10 cm petri dish. After incubation at 37°C and 5% CO_2 overnight, the supernatant, containing BMDMs in the suspension, were transferred into a 50

ml falcon tube. The suspension was centrifuged at 1200 rpm for 8 min at room temperature. The supernatant was removed and the cell pellet suspended in 10 ml warm BMDM medium. The cells were counted using a Neubauer counting chamber and plated at 5-6x10^6 cells per 10 cm cell culture dish.

ELISA

BMDMs were isolated as described above. After 5 days of incubation, cells were treated as indicated. Next, the supernatant was collected and ELISA was performed to measure the quantity of secreted cytokines after different stimulation profiles. Using the ELISA Kits for the cytokines listed above (4.1), the assay was performed according to the manufacturer's instructions. Optical density was determined at 450 nm with a wavelength correction at 540 nm.

RNA isolation

After indicated stimulation time, the cell were lyzed by adding 1 ml of TRIZOL into each well. Cell lysates were harvested and transferred into a low binding Eppendorf tube followed by adding 200 µl of chloroform. The tubes were shaken for 30 s and incubated for 10 min at room temperature. After centrifugation at 10700 rpm for 5 min at 4°C, 600 µl of the liquid supernatant was transferred into a new Eppendorf tube and mixed with the same volume of isopropanol. The samples were shaken for 30 s and placed for 15 min on ice. Afterwards, the samples were centrifuged again at 12600 rpm for 10 min at 4°C. The supernatant was discarded and the tubes were stored upside town. After drying, 1 ml of 75% ethanol was added and the samples were stored at -20°C overnight. Next, the tubes were centrifuged at 12800 rpm for 10 min at 4°C, the ethanol was discarded and the tube dried as described before. The pellet containing RNA was resuspended in 15 µl RNAse free water. Finally, by using NanoDrop analysis, the RNA concentrations were determined.

RT-PCR

1 µg of isolated RNA was used for the first-strand complementary DNA synthesis (Amersham Biosciences), which was then used for SYBR Green–based quantitative RT-PCR. Quantification of target gene expression was performed using a mathematical model by Pfaffl *et al*. [91]. Normalized gene expression values for each sample were calculated as the ratio of expression of messenger RNA (mRNA) for the gene of interest to the expression of mRNA for β-actin.

Agilent XF Seahorse flux analyzer: MitoStress Test and GlycoStress Test

Cellular bioenergetics were determined by using Agilent XF Seahorse flux analyzer for measurement of the cell´s oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), correlating with mitochondrial and glycolytic function, respectively. BMDMs were isolated as previously described. 80,000 cells per 200 µl and well were seeded on seahorse plates and activated according to the experimental protocol. On the day before measurement, the sensor cartridges were treated with 200 µl of sterile ultra-pure water (ddH_2O) and placed in a CO_2-free Seahorse incubator at 37°C overnight, along with 20 ml of XF calibrant solution. On the day of measurement, Mitostress or Glycostress medium was prepared according to the conditions described in chapter 4.1. The ultra-pure water was discarded from the cartridge and 200 µl of warm XF calibrant solution was added to each well, followed by incubation for 60 min in the incubator mentioned above. Cells were washed for a series of three times and at the end, 180 µl of the desired Seahorse Medium was added. The cells were placed in the Seahorse incubator for 60 min without the lid. Afterwards, the loading ports A, B and C were prepared and filled with the reagents (A: 20 µl, B:22 µl, C: 25 µl) necessary for either MitoStress Test or GlycoStress Test. The concentrations of the reagents needed for each experiment were standardized for BMDMs in separate experiments. The sensor cartridge was calibrated for 20 min and the Seahorse plate placed into the machinery followed by a measurement time of 1.45 h.

Metabolomics

- **Perchloric acid extraction**

For extraction of phosphorylated intermediates and carboxylates, $4x10^6$ BMDMs were seeded on a 10 cm cell culture dish to obtain 20 mg samples of four biological replicates. After cell treatment, the supernatant was discarded and the cells were washed with PBS. 10 ml of PBS per plate was added and the attached cells were scratched from the surface of the dish and transferred to a 15 ml falcon tube placed on ice. After centrifugation at 1200 rpm at 4°C for 7 min, the supernatant was discarded and the pellet shock-frozen using liquid nitrogen. For extraction, 0.5 ml of 1 M perchloric acid was added, vortexed and again shock frozen in liquid nitrogen. Afterwards, 0.5 ml of 0.1 M perchloric acid was added and the pellet resuspended. The solution was transferred to cooled 2ml Eppendorf tubes and centrifuged at 20800 x *g* at 4°C for 2 min. After centrifugation, 0.9 ml of the supernatant was transferred to cooled Eppendorf tubes and 25 µl of 5M K_2CO_3 was added immediately for pH adjustment. When the target value (pH=6-7) was reached, the solution was again centrifuged at 20800 x *g* at 4°C for 2 min and the supernatant afterwards transferred into a cooled Eppendorf tube. Samples were stored at -80°C until preparation for MS-analysis.

- **MS-Analysis**

For quantification, ionchromatography was applied with an ICS3000 HPLC system (Dionex) and ESI/MS/MS detection using a QTrap3200 Triple-Quadruple mass spectrometer with turbo V ion source (Applied Biosystems), operating in multiple reaction monitoring mode. MS-Analysis was performed by the biochemistry at the FAU Erlangen-Nürnberg.

Statistical analyses

For calculations of statistical significance, GraphPad Prism 8.3 was used. Data are expressed as mean +- SEM and were analyzed using One-way ANOVA with a Tukey post-hoc test for multiple comparisons. P-values less than 0.05 were considered statistically significant. All results are representative for at least 3 individual experiments.

5. Results

5.1. GC decrease LPS-induced cytokine secretion in macrophages

Glucocorticoids are potent anti-inflammatory agents that exert this characteristic on transcriptional and posttranscriptional levels of cytokine receptor signaling [92, 93]. Suppression of pro-inflammatory mediators is suggested to represent a central mode of action. In addition, upregulation of anti-inflammatory cytokines is seen under certain conditions after GC treatment as well [10]. To analyze the anti-inflammatory potential of GC, we pre-stimulated bone marrow-derived macrophages (BMDMs) for 4 h with dexamethasone and corticosterone, followed by LPS treatment for different indicated timepoints. For IL-1β measurement, cells were additionally stimulated with ATP, 1 h prior to LPS stimulation. Extracellular ATP represents a potent activator of the NLRP3 inflammasome and consequently supports IL-1β release in macrophages. We observed that both GC-derivates reduced production of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β but, unlike described in previous studies [94], additionally reduced secretion of the anti-inflammatory cytokine IL-10 (Fig.4A-B). Downregulation of the cytokine IL-6 on transcriptional level is supported by suppressed mRNA expression levels after LPS and dexamethasone treatment (Fig.4C). Our data indicate that GC remarkebly reduce LPS induced secretion of pro- and anti-inflammatory cytokines.

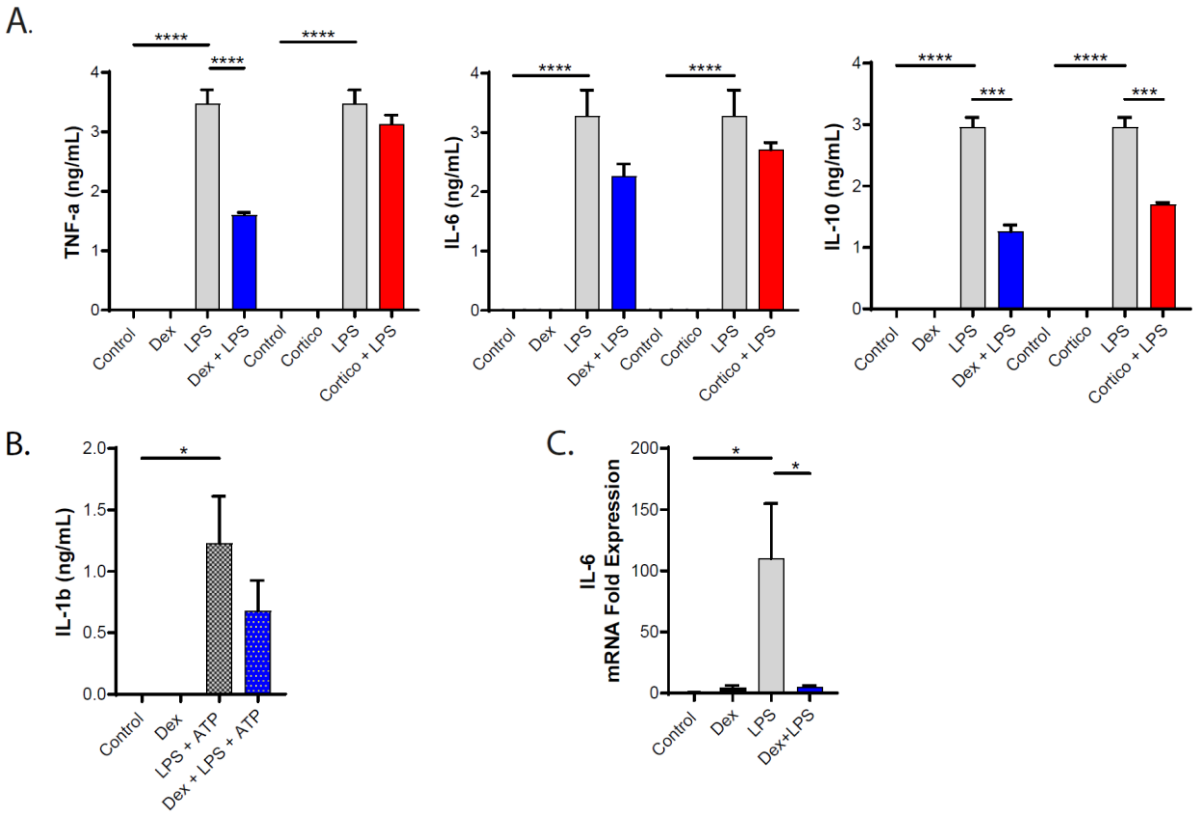
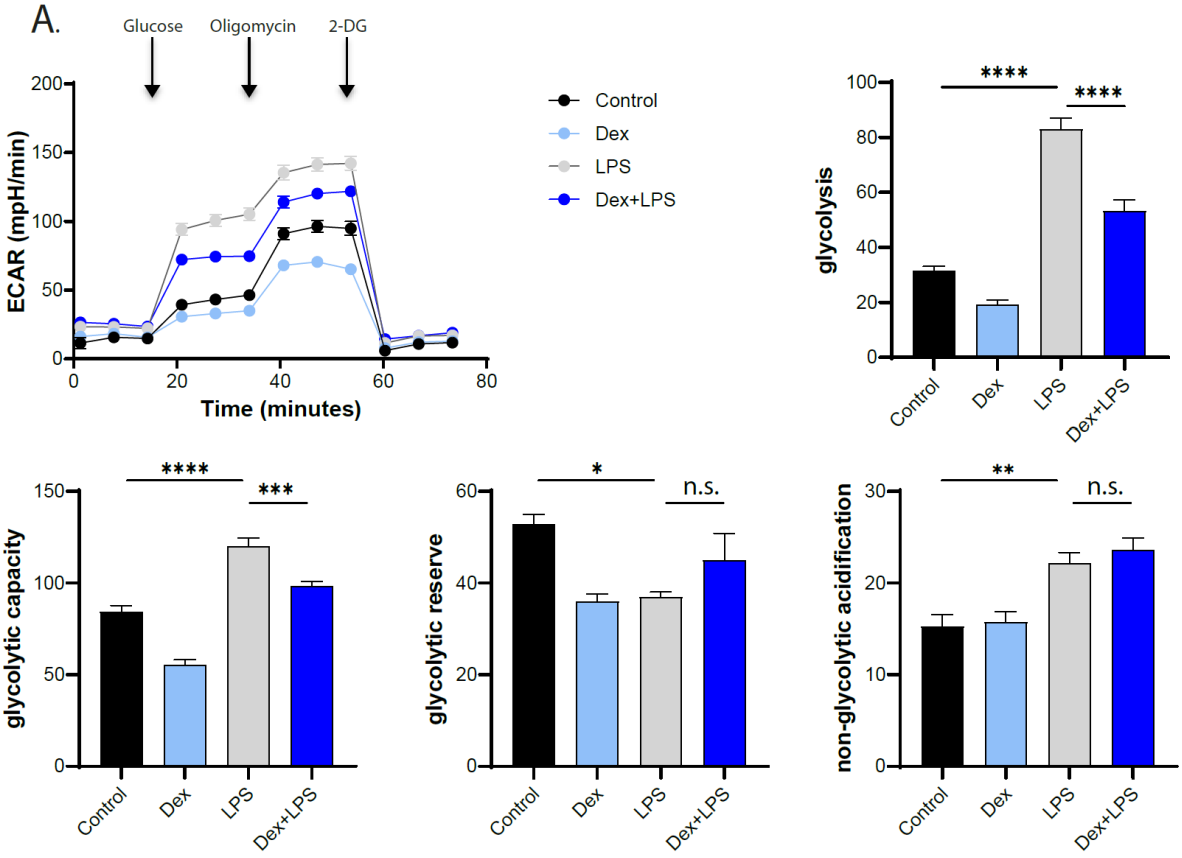


Fig.4: Glucocorticoids suppress production of pro- and anti-inflammatory cytokines in BMDMs. BMDMs were isolated 7 days prior to pre-treatment with 0.1 µM dexamethasone or corticosterone for 4 h followed by

stimulation with 100 ng/ml of LPS. (**A**) Secreted levels of pro- (TNF-a, IL-6) and anti-inflammatory (IL-10) cytokines after 6 h (TNF-a, IL-10) and 24 h (IL-6) of treatment with LPS, measured by ELISA. (**B**) Secreted levels of IL-1β after additional pre-treatment with 5 mM of ATP 1 h prior to stimulation with LPS. (**C**) Real-time PCR (RT-PCR) of mRNA expression levels of *Il6* after 24 h of LPS stimulation, normalized to *β-actin*. Statistical analysis was performed by one-way ANOVA using a Tukey post-hoc test. Data are expressed as mean ± SEM (n=4). *(p<0.05), ***(p<0.001), ****(p<0.0001) indicate significant differences between compared groups.

5.2. Dexamethasone limits glycolysis function by curtailing glucose flux and activation

Recent studies stated that the Warburg-like effect of increased glycolysis activity in inflammatory macrophages is suggested to be negatively modulated by glucocorticoids [63, 95]. However, there is a lack of evidence concerning the question if these GC-mediated alterations correlate with glucose intake and its hexokinase-mediated phosphorylation. Using Seahorse Extracellular Flux Analyzer, we observed increased extracellular acidification rates (ECAR) in the environment of BMDMs after 24 h of LPS treatment, indicating an increase of glycolytic functions in inflammatory macrophages. Moreover, LPS activated macrophages show ameliorated glycolytic capacity, after addition of oligomycin, in concordance with increased glycolysis function, yet dampened glycolytic reserve, suggesting a limited capability to quickly react to a high demand of energy during maximum glycolysis processing rates (Fig.5A).



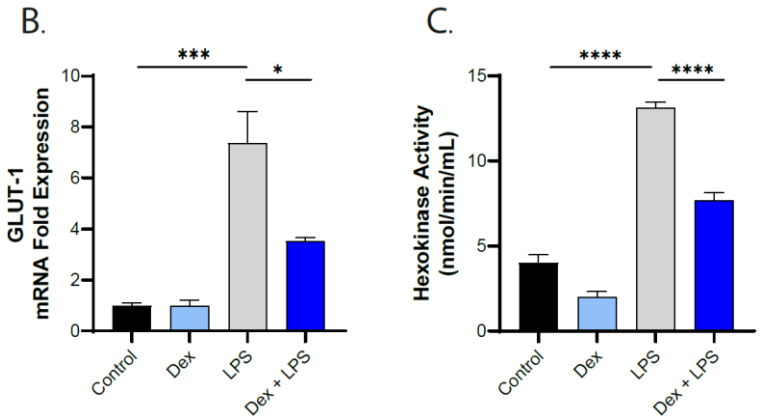
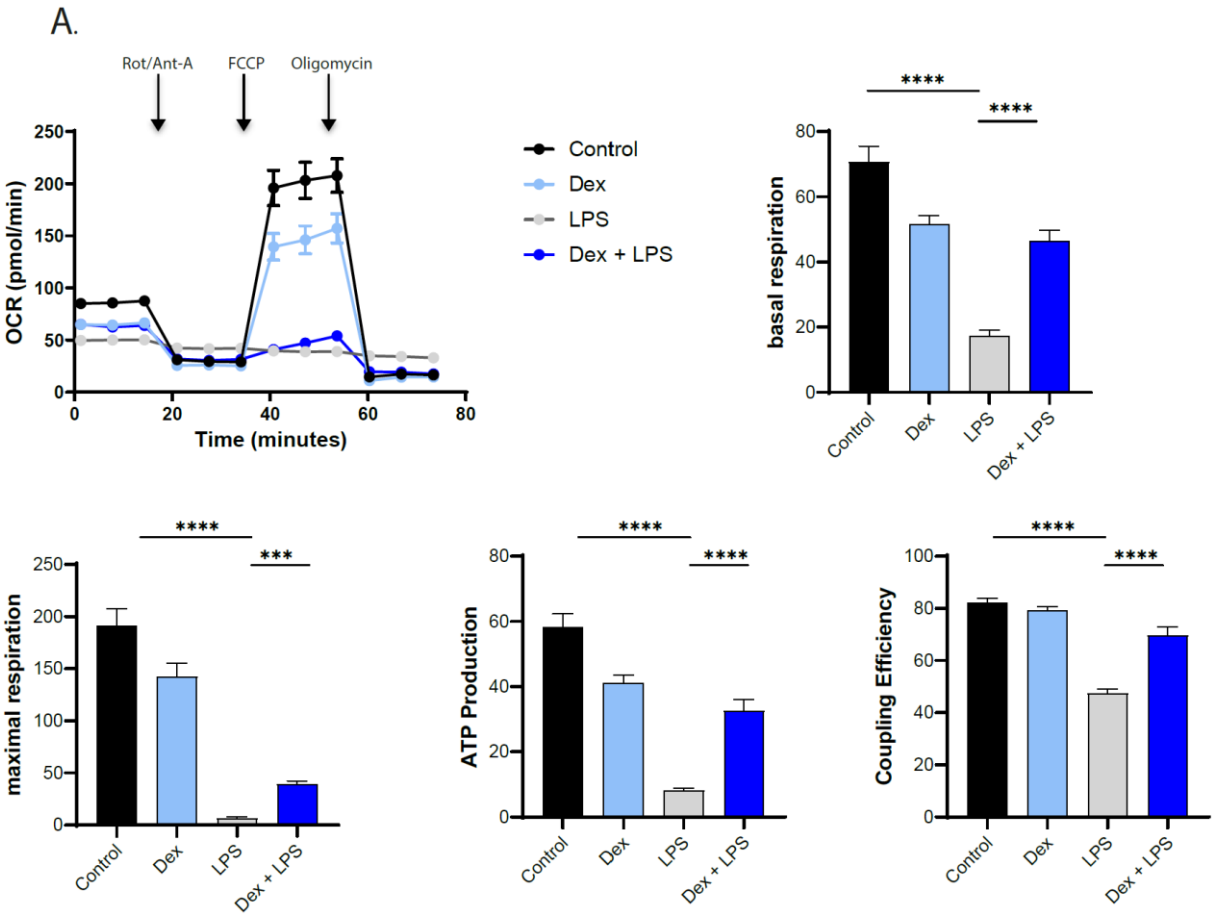


Fig.5: Dexamethasone reverses LPS-induced promotion of glycolysis in BMDMs. BMDMs were isolated 7 days prior to pre-treatment with 0.1 µM Dexamethasone for 4 h, followed by stimulation with 100 ng/ml of LPS. (**A**) The extracellular acidification rate (ECAR) was measured in real-time using Seahorse Extracellular Flux Analyzer after 24 h of LPS incubation. Basal glycolysis, glycolytic capacity, glycolytic reserve and non-glycolytic acidification were determined based on the measured values of ECAR. (**B**) RT-PCR was performed to measure relative mRNA expression of *Glut1* after 6 h of LPS incubation, normalized to *β-actin*. (**C**) Hexokinase activity was determined using ELISA after 24 h of LPS incubation. Statistical analysis was performed by one-way ANOVA using a Tukey post-hoc test. Data are expressed as mean ± SEM (n=4). *($p<0.05$), **($p<0.01$), ***($p<0.005$) and ****($p<0.0001$) indicate significant differences between compared groups; n.s.=not significant.

The change to a glycolytic state upon LPS activation is supported by an increased expression of the glucose transporter GLUT1 (Fig.5B) and hexokinase activity (Fig.5C), indicating a possible regulatory role of these proteins in the process of pro-inflammatory metabolic programming. We observed that treatment with dexamethasone, under the described conditions, resulted in downregulation of glycolytic function and negatively modulated the glycolysis promoting effects of LPS. This equally includes substantially reduced glycolytic capacity, reflecting lower glycolysis processing rates after dexamethasone treatment. Glycolytic reserve or non-glycolytic acidification, however, were not significantly increased. Interestingly, the glycolysis limiting effect of dexamethasone can likewise be seen besides an inflammatory setting (Fig.5A). Finally, down-regulation of mRNA expression of *Glut1* (Fig.5B) and reduction of hexokinase activity (Fig.5C) indicate that dexamethasone curtails glucose flux of LPS-activated macrophages. This could contribute to the observed LPS antagonizing effects of dexamethasone on metabolic reprogramming in macrophages.

5.3. Dexamethasone reinforces OXPHOS and mitochondrial fitness in inflammatory macrophages

Metabolic reprogramming in classically activated macrophages is not limited to increased glycolysis activity. In fact, decreased oxidative phosphorylation is often delineated as one of the key features of inflammatory macrophage polarization [74]. Measurement of mitochondrial respiration using Seahorse Extracellular Flux Analyzer demonstrated this effect after 24 h of LPS activation. As outlined in Fig.6, the oxygen consumption rate of inflammatory macrophages was strongly impaired, including basal and maximal respiration. Maximal respiration was determined after adding the uncoupling agent FCCP, which imitates a demand of energy by stimulating the respiratory chain to operate at maximum capacity. Moreover, the amount of oxygen used for ATP production in parallel with coupling efficiency was significantly decreased upon LPS stimulation. Additionally, we assumed impaired mitochondrial fitness of inflammatory macrophages, demonstrated by dampened spare respiratory capacity (SRC) in relation to quiescent cells, yet not significantly affected proton leak across the inner mitochondrial membrane. Recent studies claimed that GC could improve mitochondrial fitness by preventing the organelle from LPS-induced fragmentation and ROS production.[63] However, a direct effect of GC on mitochondrial respiration in a comparable context could not be well-delineated.



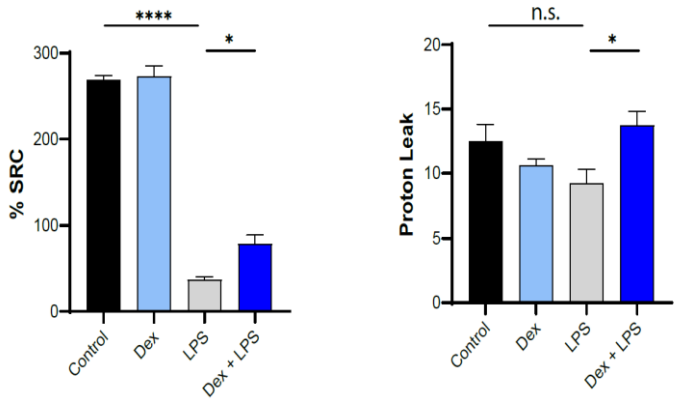


Fig.6: Dexamethasone restores LPS-induced suppressive effects on OXPHOS in BMDMs. BMDMs were isolated 7 days prior to pre-treatment with 0.1 µM Dexamethasone for 4 h, followed by stimulation with 100 ng/ml of LPS. (**A**) The oxygen consumption rate (OCR) was measured in real-time using Seahorse Extracellular Flux Analyzer after 24 h of LPS incubation. Maximal respiration, ATP-production linked respiration, coupling efficiency, proton leak and relative spare respiratory capacity were determined based on the measured values of OCR. Statistical analysis was performed by one-way ANOVA using a Tukey post-hoc test. Data are expressed as mean ± SEM (n=5). *(p<0.05), **(p<0.01), ***(p<0.005) and ****(p<0.0001) indicate significant differences between compared groups; n.s.=not significant.

Thus, we addressed the question whether treatment with dexamethasone could not only antagonize LPS-driven effects on glycolysis but on mitochondrial respiration as well. Indeed, in combination with LPS, dexamethasone restored mitochondrial function by reinforcing basal and maximal respiration, relative spare respiratory capacity, coupling efficiency and in parallel, oxygen-dependent ATP production. In concordance with the alterations on glycolysis, these data strongly suggest that dexamethasone promotes a metabolic phenotype that is characterized by reversing LPS-induced effects on metabolic programming and rescue of mitochondrial function in inflammatory macrophages.

5.4. Dexamethasone promotes mitochondrial dynamics in macrophages

Mitochondrial function is essential for immune cells during an inflammatory response [68]. Their dynamic fusion and fission enables the organelles to work efficiently as distinct mediators balance proliferation, mitophagy and growth. Additionally, recent studies demonstrated a link between GC treatment and changes in the mitochondrial network of inflammatory macrophages, as GC seem to prevent mitochondrial fragmentation and ROS production [63]. To address the question whether the fusion-fission machinery is affected by LPS and/or dexamethasone on a transcriptional level, we performed RT-qPCR to measure mRNA expression levels of crucial mediators involved in mitochondrial dynamics and biogenesis. Comparable to the experimental setting before, BMDMs were pre-stimulated for 4 h with dexamethasone prior to LPS activation. We analyzed the mRNA expression levels of the fusion mediators *Opa1*, *Mfn1* and *Mfn2*, the fission mediator *Drp1* and *Nrf1* and *Nrf2*, representing transcription factors essential for mitochondrial biogenesis and respiration. In

LPS-treated cells, expression levels of *Nrf1* and *Mfn1* were substantially reduced in comparison to the control, indicating possible regulatory effects of TLR signaling on mitochondrial dynamics and function (Fig 7). Expression levels of *Drp1*, *Opa1*, *Nrf2* and *Mfn2* appeared to be decreased, though not statistically different. Interestingly, dexamethasone positively modulated LPS-driven effects on production of these mediators. Furthermore, in a separate experiment we could observe that dexamethasone affected mitochondrial dynamics on the transcriptional level independently from LPS (Fig.7B). In conclusion, these results highlight a balancing role of dexamethasone on mitochondrial dynamics and biogenesis by antagonizing LPS induced downregulation of these proteins and, moreover, by affecting their expression in absence of an inflammatory setting.

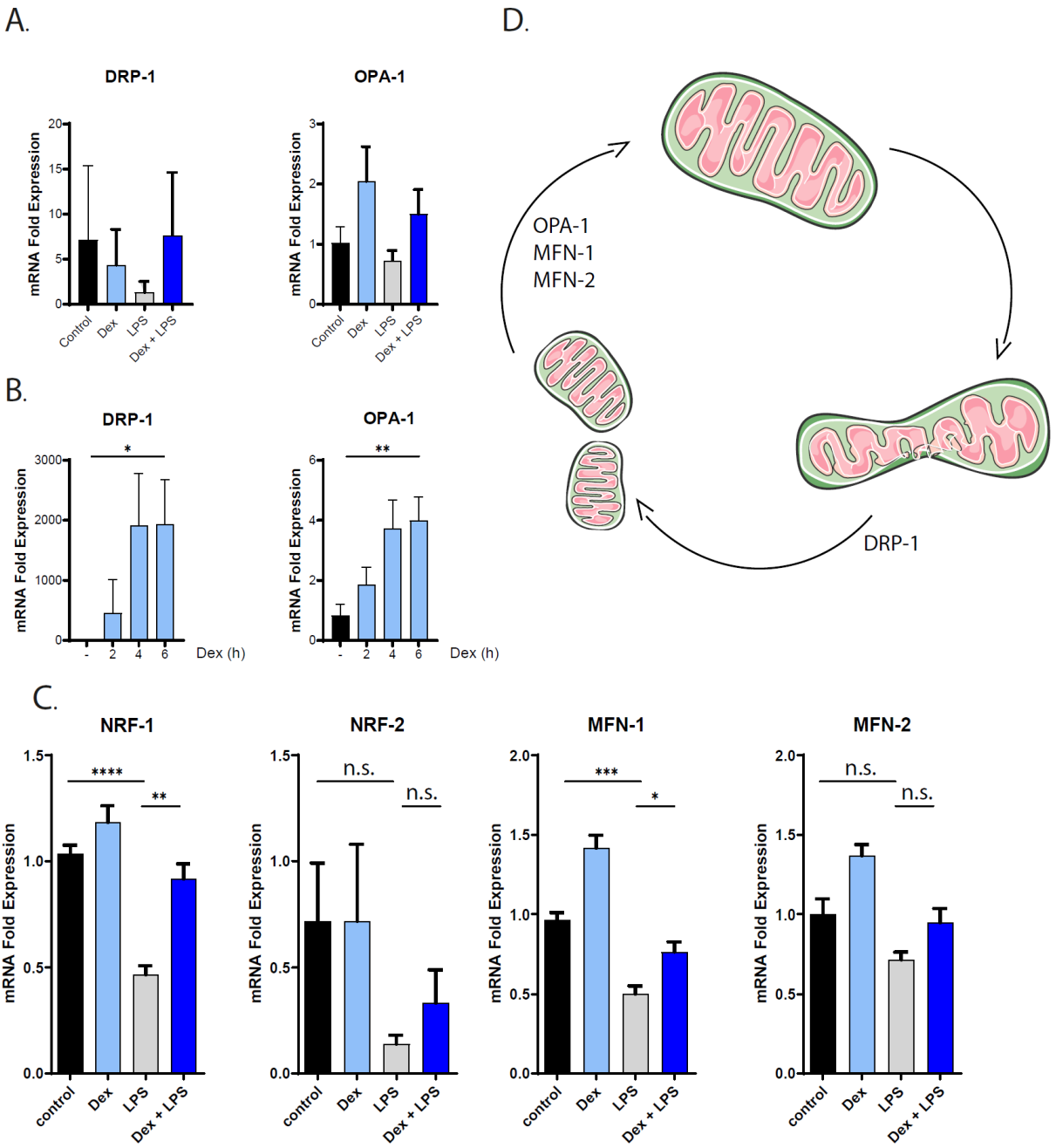


Fig.7: Dexamethasone promotes mitochondrial dynamics in BMDMs. (**A**)+(**C**) BMDMs were pre-treated with 0.1 µM dexamethasone 4 h prior to stimulation with 100 ng/ml of LPS for 6 h. RT-PCR was performed to measure relative mRNA expression of *Drp1*, *Opa1*, *Nrf1*, *Nrf2*, *Mfn1*, and *Mfn2*, normalized to *β-actin*. (**B**) BMDMs were activated with 0.1 µM of dexamethasone for 2, 4 and 6 h. RT-PCR was performed to measure relative mRNA expression of *Drp1* and *Opa1* (**D**) Illustration of the mitochondrial fusion-fission machinery with mediators involved. Statistical analysis was performed by one-way ANOVA using a Tukey post-hoc test. Data are expressed as mean ± SEM (n=3). *(p<0.05), ***(p<0.005) and ****(p<0.0001) indicate significant differences between compared groups; n.s.=not significant. (This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license)

5.5. Dysfunctional mitochondrial replication does not affect LPS or dexamethasone driven effects on metabolic reprogramming in macrophages

The mitochondrial dynamics induced by LPS and/or dexamethasone bolstered a rationale for the hypothesis that cytokine-induced immune-metabolic reprogramming is disturbed by mitochondria losing their ability to replicate efficiently. The helicase Twinkle is necessary for unwinding mitochondrial DNA during the process of mitochondrial replication [65]. BMDMs from wild-type (WT) and LysM-Cre Twinkle-knockout mice were treated for 4 h with dexamethasone prior to 24 hours of LPS stimulation. Cellular bioenergetics were measured via Seahorse Extracellular Flux Analyzer and cytokine levels were determined using ELISA after the indicated stimulation time. As suspected, dysfunctional mitochondrial replication resulted in impaired mitochondrial respiration, including relative SRC, proton leak and ATP-production linked oxygen consumption under every stimulatory condition (Fig.8A). LPS-induced effects or dexamethasone driven restoration of mitochondrial fitness, however, were not affected. In parallel, cytokine production and its inhibition by dexamethasone was not altered in Twinkle-deficient BMDMs either (Fig.8B). This data demonstrates that Twinkle-deficient macrophages lack mitochondrial fitness. However, this phenotype could not contribute to or disturb LPS and/or dexamethasone driven effects on mitochondrial respiratory activity or cytokine signaling.

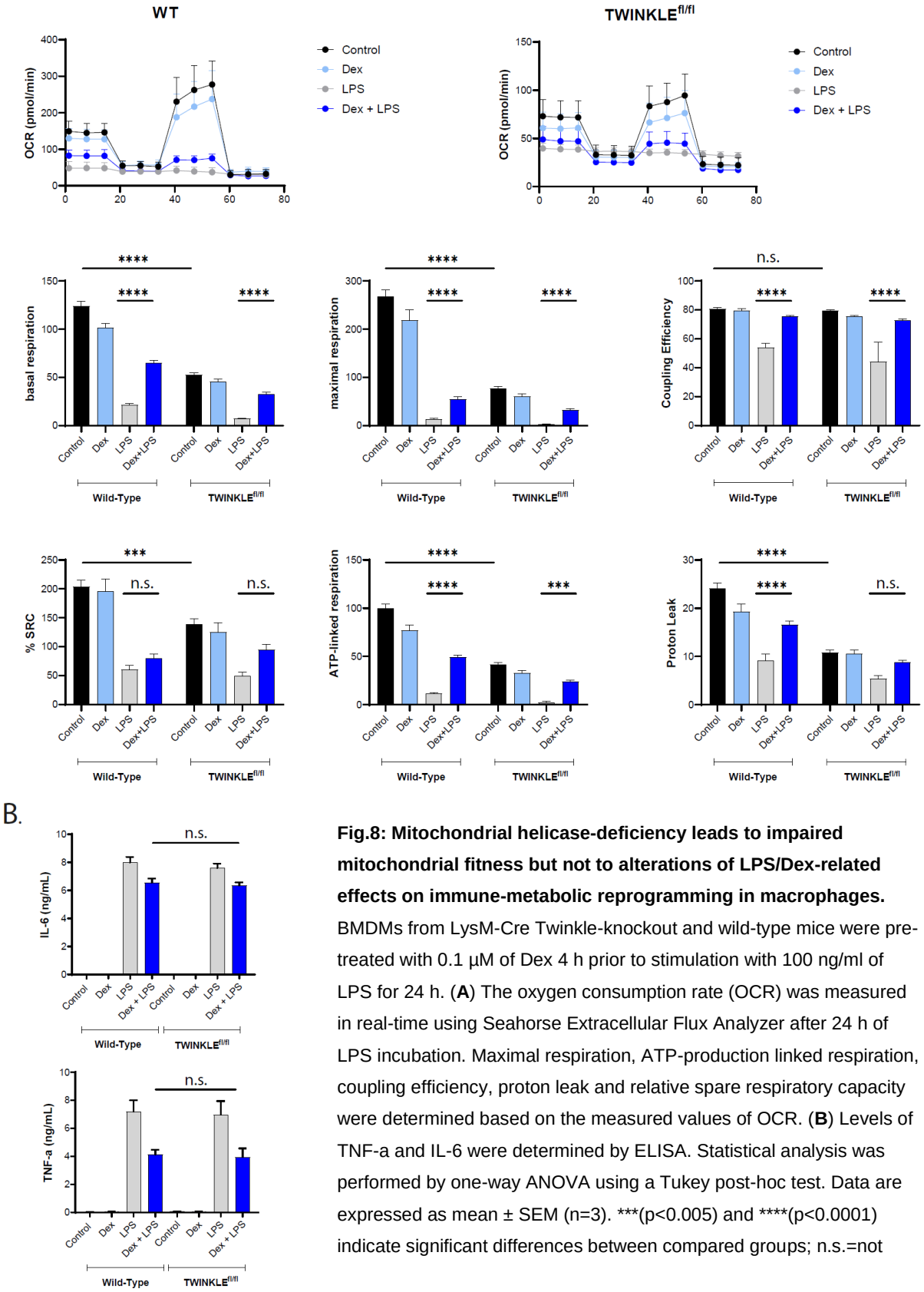


Fig.8: Mitochondrial helicase-deficiency leads to impaired mitochondrial fitness but not to alterations of LPS/Dex-related effects on immune-metabolic reprogramming in macrophages. BMDMs from LysM-Cre Twinkle-knockout and wild-type mice were pre-treated with 0.1 µM of Dex 4 h prior to stimulation with 100 ng/ml of LPS for 24 h. (**A**) The oxygen consumption rate (OCR) was measured in real-time using Seahorse Extracellular Flux Analyzer after 24 h of LPS incubation. Maximal respiration, ATP-production linked respiration, coupling efficiency, proton leak and relative spare respiratory capacity were determined based on the measured values of OCR. (**B**) Levels of TNF-a and IL-6 were determined by ELISA. Statistical analysis was performed by one-way ANOVA using a Tukey post-hoc test. Data are expressed as mean ± SEM (n=3). ***(p<0.005) and ****(p<0.0001) indicate significant differences between compared groups; n.s.=not

5.6. Dexamethasone modulates LPS-induced promotion of glycolysis and redirects TCA cycle metabolites and function

Increasing evidence revealed an influence of glucocorticoid treatment on glycolysis and TCA cycle function of LPS-activated macrophages [63]. By antagonizing LPS induced effects on these metabolic pathways, the authors suggested that glucocorticoids could mediate anti-inflammatory actions while restoring efficient energy supply in macrophages. Accumulation of multifunctional metabolic intermediates such as succinate and itaconate has been associated with pro- and anti-inflammatory features of macrophages. [96] However, the specific role of these metabolites in the context of anti-inflammatory signaling induced by glucocorticoids remains poorly understood. To gain a deeper insight into dexamethasone induced rewiring of glycolysis and mitochondrial respiration in inflammatory macrophages, we quantified essential metabolites being turned over during glycolysis and the TCA cycle after no treatment, a short (6 h) and a long (24 h) period of LPS- and/or dexamethasone stimulation. By performing mass spectrometry, we could measure the quantities of d-glucose, glucose-6-phosphate, fructose-6-phosphate, phosphoenolpyruvate, and lactate to illustrate glycolytic function under the indicated conditions. Since several studies reported a promotion of glycolysis function after LPS activation and our data could confirm enhanced glucose intake and activation in this context, we suspected high amounts of glycolysis intermediates after 24 h. Indeed, in comparison to quiescent and dexamethasone-treated cells, the quantities for glucose-6-phosphate and fructose-6-phosphate increased upon LPS treatment (Fig.9B). In parallel to our findings concerning the high extracellular acidification rate of LPS activated macrophages (Fig.5), we measured high levels of lactate after 24 h that could contribute to this observation. Since glycolytic activity and glucose flux is impaired in dexamethasone-treated cells during homeostatic and inflammatory conditions (Fig.5), our data confirm this effect by reduced levels of glycolysis intermediates, compared to LPS-treated cells at both timepoints.

Quiescent cells utilize a running TCA cycle for providing high amounts of NADH and FADH, acting as electron donors for the ETC. Classically activated macrophages are suggested to break and redirect the TCA cycle to alternate its function [74]. This redirection leads to an accumulation of multifunctional intermediates including citrate, itaconate and succinate. Indeed, we observed accumulation of citrate, isocitrate, α-ketoglutarate, fumarate, and malate after 24 h of LPS stimulation. Succinate and itaconate levels increased after 6 h, yet dropped after 24 h. Since dexamethasone enables LPS-treated macrophages to restore their ability to perform efficient oxidative phosphorylation, we hypothesized a recovery of TCA cycle function and its metabolites as well.

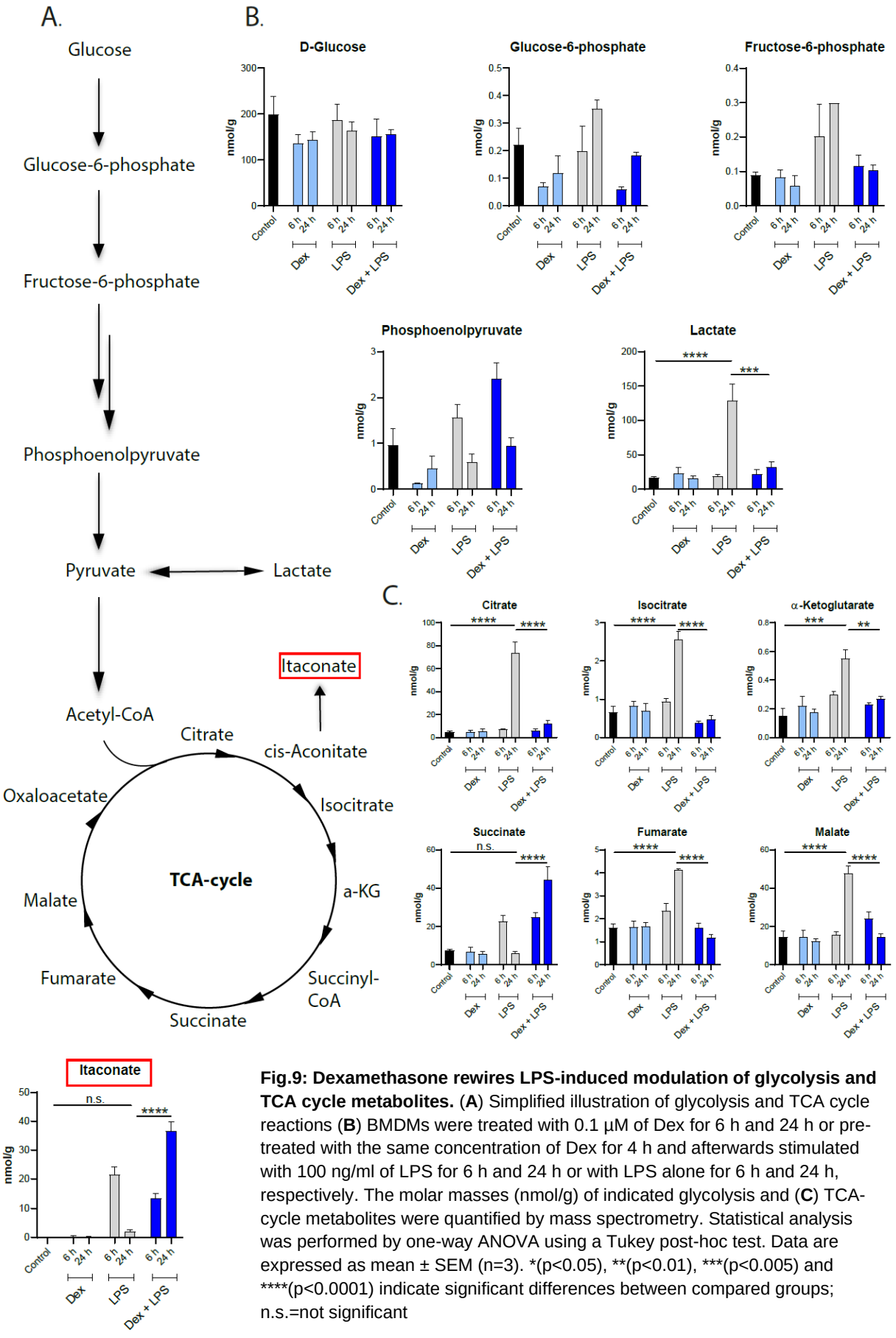


Fig.9: Dexamethasone rewires LPS-induced modulation of glycolysis and TCA cycle metabolites. (**A**) Simplified illustration of glycolysis and TCA cycle reactions (**B**) BMDMs were treated with 0.1 µM of Dex for 6 h and 24 h or pre-treated with the same concentration of Dex for 4 h and afterwards stimulated with 100 ng/ml of LPS for 6 h and 24 h or with LPS alone for 6 h and 24 h, respectively. The molar masses (nmol/g) of indicated glycolysis and (**C**) TCA-cycle metabolites were quantified by mass spectrometry. Statistical analysis was performed by one-way ANOVA using a Tukey post-hoc test. Data are expressed as mean ± SEM (n=3). *(p<0.05), **(p<0.01), ***(p<0.005) and ****(p<0.0001) indicate significant differences between compared groups; n.s.=not significant

As assumed, levels of citrate, isocitrate, α-ketoglutarate, fumarate and malate reach normal levels, comparable to untreated cells, upon dexamethasone-LPS co-treatment. However, dexamethasone treatment alone, at both time points, did not alter the quantity of TCA cycle metabolites. This contributes to the results of cellular bioenergetics and seems to necessitate an inflammatory setting for the dexamethasone-related effects on TCA cycle function and mitochondrial respiratory activity. As described above, itaconate and succinate accumulate after 6 h in LPS treated cells. In contrast to the observations recently made by Stifel *et al.*, our conditions of prolonged LPS challenge resulted in downregulation of succinate and itaconate, respectively [63]. However, in combination with dexamethasone, we observed significant accumulation of these metabolites. Taken together, our data indicate a dichotomous role of dexamethasone on glycolysis and TCA cycle function in this inflammatory setting. First, dexamethasone antagonizes LPS-induced effects on glycolysis promotion and accumulation of distinct intermediates of a broken TCA cycle. Second, glucocorticoids not only rewire the TCA cycle to possibly restore its energy generating features but also seems to induce a shift towards synthesis of the immune-modulating intermediate itaconate that could contribute to its anti-inflammatory characteristics.

5.7. Itaconate is required for anti-inflammatory effects of dexamethasone

Itaconate is an immune-modulating metabolite with antimicrobial [85] and anti-inflammatory features [86] that is produced by ACOD1, a decarboxylating enzyme encoded by immune response gene 1 (IRG1). In recent years, increasing evidence revealed that *Irg1* transcription and ACOD1 activity are increased upon different activation signals including LPS, IFN-y and IL-33 [87, 97]. Based on the observation of elevated levels of itaconate after 24 h of Dex-LPS co-treatment, we addressed the question of whether or not *Irg1* contributes to the observed anti-inflammatory effects of dexamethasone on cytokine signaling. Indeed, deletion of *Irg1* in BMDMs prevented dexamethasone-driven inhibition of TNF-a and IL-6 in LPS-treated cells (Fig.10A). To investigate whether exogenous itaconate could restore the anti-inflammatory effects of dexamethasone while endogenous itaconate is absent, we treated BMDMs from *Irg1* knockout mice with dimethyl itaconate (DMI), 1 h before activation with LPS. DMI-treated BMDMs presented a significant decrease of TNF-α and IL-6 levels upon dexamethasone and LPS co-treatment, in comparison to the untreated cells. This observation highlights a crucial role of itaconate on glucocorticoid-driven inhibitory effects on cytokine production and therefore presents evidence of a link between metabolic reprogramming and essential effector functions in macrophages.

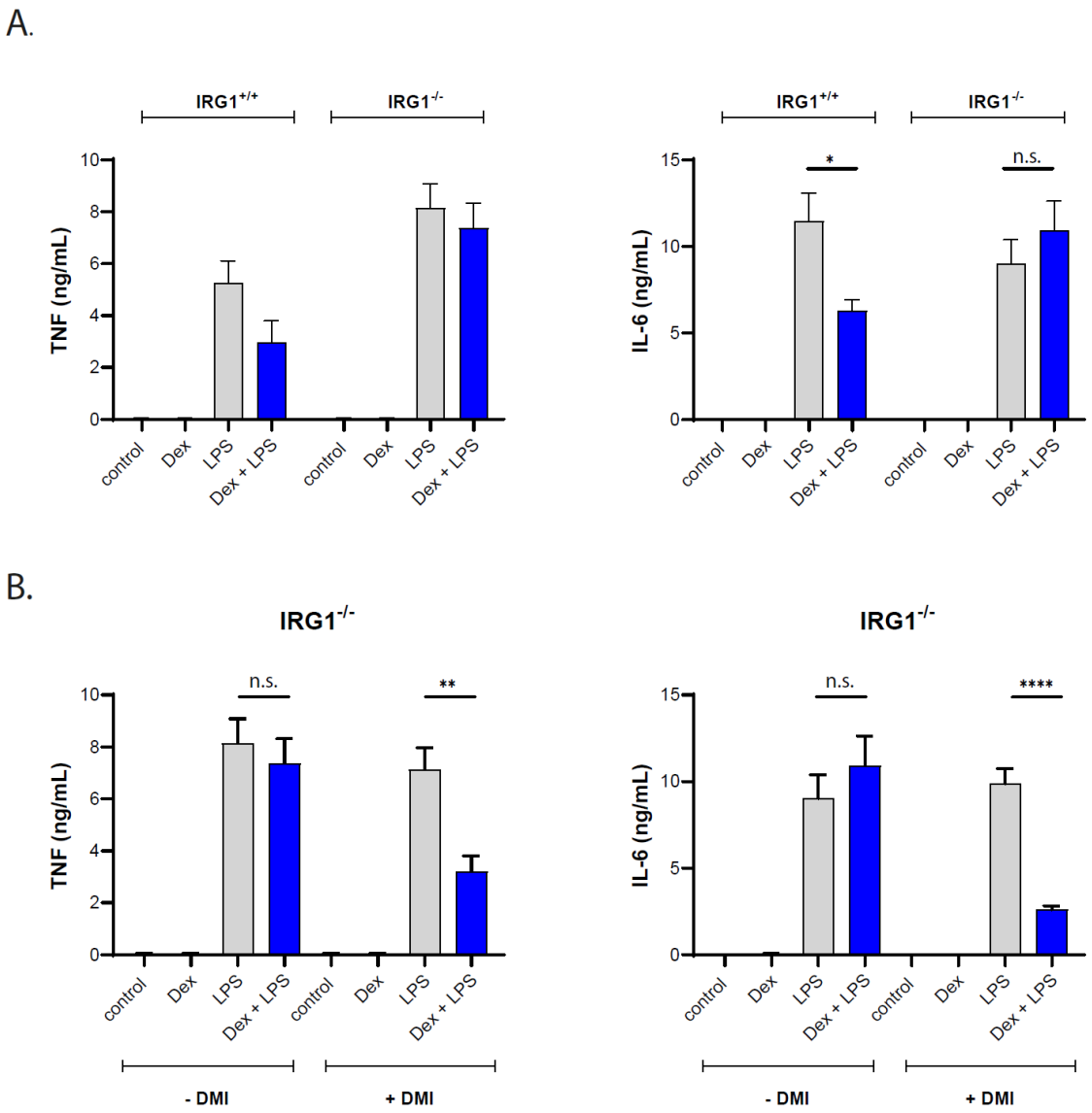


Fig.10: Itaconate is required for the anti-inflammatory effects of dexamethasone in BMDMs. (**A**) Quantification of TNF-α and IL-6 levels via ELISA of BMDMs from IRG1$^{+/+}$ and IRG1$^{-/-}$ mice after pre-treatment with 0.1 µM of Dex for 4 h, followed by stimulation with 100 ng/ml of LPS for 6 h (TNF-α) or 24 h (IL-6). (**B**) Secreted levels of TNF-α and IL-6 in BMDMs of IRG1$^{-/-}$ knockout mice treated (+) or not treated (-) with 62.5 µM of dimethylitaconate (DMI) 1 h before Dex-treatment. Statistical analysis was performed by one-way ANOVA using a Tukey post-hoc test. Data are expressed as mean ± SEM (n=3). *($p<0.05$), **($p<0.01$) and ****($p<0.0001$) indicate significant differences between compared groups; n.s.=not significant.

6. Discussion

6.1. GC affect pro- and anti-inflammatory cytokine secretion in macrophages

GC exert their anti-inflammatory effects in a cell type and context specific manner. These effects include affecting the communication between cells of the innate and adaptive immune system through production and secretion of cytokines. In the present work, we identified a GC mediated down-regulation of known pro-inflammatory cytokines that has been well described in BMDMs *in vitro* and *in vivo* [12, 98]. In most cases, this effect has been related to repression of transcription factors such as NF-kB, a major regulator of TLR signaling [21, 92]. Concerning inhibition of TNF-α production, post-transcriptional mechanisms of GC action have also been observed in other studies [93]. The inhibiting effect on a transcriptional level was confirmed by down-regulation of *Il6* mRNA levels following Dex-treatment. Surprisingly, our conditions of LPS- and Dex-treatment resulted in decreased production of the cytokine IL-10, similarly to the results seen with TNF-α, IL-6 and IL-1β. IL-10 is an anti-inflammatory cytokine that induces a pro-resolving phenotype in macrophages and is often up-regulated upon GC stimulation [10, 79]. As described in earlier *in vitro* studies, this effect is suggested to occur in a biphasic and context-dependent manner [99]. In these studies, higher doses of dexamethasone resulted in inhibition of IL-10 production *in vitro* whereas TNF-α production was downregulated at any concentration.

Similar concentrations of corticosterone, representing the main corticosteroid in rodents, could not suppress cytokine production in the same manner as dexamethasone. Corticosteroids used in clinical medicine such as prednisolone or dexamethasone are known to present different characteristics of pharmacodynamic and pharmacokinetic when administered systemically [100]. Early studies of binding capacity and pattern of corticosterone versus dexamethasone in different neural cell types could distinguish the mode of action of the two corticosteroids [101]. Moreover, analysis of corticosteroid interaction with the GR revealed higher binding affinity of dexamethasone in comparison to corticosterone in mice macrophages and human monocytes [102]. Consequently, the described observation can be explained by higher derivate potency or corticosteroid receptor binding affinity of dexamethasone.

6.2. GC reverse LPS-induced effects on glycolysis and recover mitochondrial respiratory activity in macrophages

LPS-activated macrophages typically upregulate glycolytic functions leading to an increased production of lactate, thereby shifting the cell towards an inflammatory phenotype, comparable to the Warburg effect of cancer cells [95]. Our data confirmed this characteristic by increased glycolytic function and elevated levels of glycolysis intermediates, including

lactate, in BMDMs after 24 h of LPS activation. Interestingly, in dexamethasone-treated cells, glycolytic function was impaired leading to the assumption that GC tailor this key immune-metabolic pathway to assist their anti-inflammatory actions. As recently reported, GC significantly regulate HIF-1α protein and mRNA expression and its target genes in context of their alterations on glycolytic metabolism in BMDMs and bone marrow-derived dendritic cells (BMDCs) [63] Notably, glycolytic reprogramming of activated immune cells and macrophage polarization in particular, has been associated with increased activity of mTOR and HIF-1α [103, 104]. mTOR is highly active and increases HIF-1α expression and activity in energy demanding situations, for example after TLR stimulation during an inflammatory response [105] Additionally, both transcription factors have been connected with pro-inflammatory actions of NF-kB upon bacterial stimulation [106, 107] The mTORC1/HIF-1α axis regulates glycolytic flux by controlling the expression of GLUT1 and rate limiting glycolytic enzymes such as hexokinase, which are both well-known target genes of HIF-1α [108] Thus, glucose uptake and utilization are both positively and negatively modulated by GLUT expression and hexokinase activity. In concordance with the reported link of mTOR/HIF-1α signaling and immune metabolic programming, we observed an impaired GLUT1 expression and hexokinase activity upon treatment with dexamethasone, in contrast to LPS, that could be a consequence of GC/GR mediated inhibiting effects on mTOR/HIF-1α- and NF-kB-related pathways. Indeed, deletion of HIF-1α leads to promoted GR actions in macrophages, indicating a possible link to GC glycolysis regulating features. [63] However, a clear picture of GC effects on mTOR or the mTOR/HIF-1α-axis remains elusive as studies report a necessary positive interference of mTOR on anti-inflammatory cytokine signaling of GC in human monocytes, suggesting that GC mediated inhibitory effects on HIF-1α and its target genes may be mTOR-independent. [109]

In addition to the observed effects on glycolysis function, analysis of cellular bioenergetics demonstrated a dexamethasone-mediated rescue of energy supply, mitochondrial fitness and respiratory activity in LPS-activated macrophages. Moreover, metabolomics revealed an abrogated block of the TCA cycle in this context, indicating a possible restoration of the metabolic pathway to produce reduction agents acting as electron donors at the electron transport chain (ETC). Decline of mitochondrial oxygen consumption in inflammatory macrophages critically relies on modulation of protein complexes at the ETC that includes induction of a reverse electron transport chain (RET) at complex I through oxidation of accumulated succinate and other electron donors such as glycerol-3-phosphate dehydrogenase (GAPDH) for generation of antimicrobial ROS [110, 111]. Interestingly, ROS, together with succinate, are able to inhibit prolyl hydroxylases (PHD) that represent crucial posttranslational negative regulators of HIF-1α stability, leading to increased production of

IL-1β upon LPS stimulation. [112] This reveals a connection between the block of OXPHOS or induction of RET, respectively, and HIF-1α mediated pro-inflammatory effector functions of macrophages. As recently reported, GC downregulate ROS production and HIF-1α expression in LPS-activated BMDMs, indicating a possible link to the effects observed herein on increased mitochondrial respiratory activity after dexamethasone treatment [63]. Moreover, nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is suggested to serve as a potent mediator of OXPHOS inhibition in LPS-treated cells. [113] Down-regulation of iNOS on a transcriptional level in a similar context has been reported in earlier studies, providing an additional explanation for the observed results. [114] Interestingly, increasing evidence uncovered a modulating role of a mitochondrial GR (mtGR) on cellular bioenergetics and mitochondrial integrity by affecting the mitochondrial transcriptome [30] and directly interacting with proteins and enzymes involved in balancing glycolytic and OXPHOS related energy production such as pyruvate dehydrogenase (PDH) [31] A possible mtGR/PDH axis and interaction with other mitochondrial enzymes and transcription factors could contribute to the observed metabolic shift seen after dexamethasone activation in inflammatory macrophages.

Collectively, our data revealed a GC-mediated restriction of glycolytic functions and restoration of mitochondrial respiratory activity in LPS-treated cells. Considering decreased glucose flux and activation, direct control of mTOR and/or HIF-1α expression or indirect destabilization by reducing ROS levels in dexamethasone-treated macrophages could provide an explanation for the observed effects on glycolysis and oxidative phosphorylation. Moreover, evidence for decreased iNOS expression through GC and direct interaction of the mtGR with key metabolic enzymes and proteins emphasize the significance of GC action on metabolic reprogramming in innate immune cells.

6.3. GC balance mitochondrial biogenesis while deficiency of mitochondrial replication does not contribute to or prevent LPS or GC-induced immune-metabolic programming in macrophages

Mitochondria play a key role in innate immune signaling. [115] The organelle provides the setting for metabolic pathways involved in balancing inflammatory processes and resolution of inflammation. This characteristic is brought to light when dysfunctional mitochondria prevent repolarization of pro-inflammatory into pro-resolving macrophages. [116]. We identified GC mediated up-regulation of mediators involved in promoting mitochondrial dynamics including DRP-1, OPA-1, MFN-1 and MFN-2, in comparison to LPS-treated cells after 6 h. DRP-1 and OPA-1 expression levels were additionally enhanced independently from LPS activation. Recent studies provided evidence for GC-linked prevention of LPS-induced mitochondrial fragmentation that could be in accordance with increased levels of the

fusion mediators OPA-1, MFN-1 and MFN-2 after dexamethasone treatment. [63] Mitochondrial fragmentation relies on the fission mediator DRP-1 and occurs as a stress response with concurrent increase of mtROS production, for renewal of dysfunctional mitochondria or, notably, as an indicator for promoted mitochondrial biogenesis. [67] In addition, balancing this process of mitochondrial dynamics is crucial for normal cell development and function as disruption can lead to degenerative disorders such as Parkinson´s disease. [117] Our findings indicate a non-specific control of these proteins on a transcriptional level through GC, suggesting that they do not shift mitochondrial dynamics into a certain direction but rather promote the process as a whole, possibly in order to affect multiple processes involved in mitochondrial biogenesis. This hypothesis is supported by the observation of the up-regulated transcription factors NRF-1 and NRF-2 upon dexamethasone treatment. NRF-1 and NRF-2 are both regulators of mitochondrial metabolism and proteins taking part in the respiratory chain such as cytochrome c oxidase. [118] Moreover, as nuclear transcription factors, they initiate expression of enzymes involved in mitochondrial replication and consequently bridge the communication between nucleus and mitochondria. [118] Additionally, NRF-2 evidently blocks pro-inflammatory cytokine transcription in macrophages. [119] Interestingly, identification of the promotor sequence of Von-Hippel-Lindau (VHL), revealed a recognition site for NRF-1, indicating that the expression of VHL might be under control of NRF-1. [120] By inducing degradation of HIF-1α, VHL negatively controls HIF-1α, subsequent glycolytic functions and possibly mitochondrial biogenesis. An upregulation of NRF-1 and control of VHL through GC on a transcriptional or protein level could therefore contribute to the effects seen on mitochondrial respiratory activity. Notably, a regulatory effect of GC on VHL function, directly or indirectly via NRF-1, in macrophages remains speculative as there are merely reports about GC promoting stabilization of HIF-1α by inducing degradation of VHL in zebrafish and human hepatocytes. [121] This observation, however, could indicate an antagonistic role of GC action on macrophages and possibly other immune cells in comparison to hepatocytes as GC evidently destabilize HIF-1α in macrophages. [63]

Due to the identified effects of GC and LPS on mitochondrial biogenesis, we hypothesized that mitochondrial replication, being crucial for biogenesis, might be part of the mechanism behind GC mediated rescue of mitochondrial function. We observed a lack of mitochondrial fitness in Twinkle-deficient macrophages, meaning decreased oxygen consumption rate at all conditions. However, Twinkle deficiency did not alter LPS and/or GC mediated effects on mitochondrial respiratory activity or cytokine production, suggesting that this phenotype does not contribute to or prevent GC induced immune-metabolic reprogramming in macrophages. Twinkle helicase is responsible for both mtDNA replication and mtDNA maintenance [122]. With respect to the fact that mtDNA is encoding key components of the respiratory chain and

OXPHOS, a defect of mtDNA maintenance would lead to impaired respiratory activity or oxygen consumption, respectively, and increased production of mtROS [123]. This is supported by the observation of increased mitochondrial biogenesis and decreased oxidative stress in cardiomyocytes upon Twinkle-overexpression in a model of myocardial infarction. [124] Interestingly, GC and LPS induced effects on mitochondrial respiratory activity were not compromised in Twinkle-deficient macrophages, suggesting that GC and LPS possibly do not affect regulation of OXPHOS-related proteins and enzymes encoded by mtDNA or that the pathways responsible for the observed effects do not fully rely on a balanced mitochondrial fusion-fission machinery. Notably, we identified upregulation of mediators involved in balancing mitochondrial biogenesis after a stimulation time of 6 h, indicating enhanced activity of these proteins possibly earlier than after 24 h of LPS and GC stimulation. Thus, protein analysis and different activation timepoints would be required to increase sensitivity and exclude kinetic related effects in this context.

In conclusion, we determined a positive correlation between GC action and mRNA expression levels of proteins crucial for mitochondrial biogenesis. Increasing activity of transcription factors such as NRF-1 and NRF-2 could be linked to the reprogramming of metabolic pathways by GC. Nevertheless, diminished mitochondrial biogenesis due to impaired mitochondrial replication does not influence the pathways responsible for GC mediated restoration of mitochondrial function and cytokine signaling.

6.4. GC remodel TCA cycle function for energy supply and production of itaconate as a mediator for anti-inflammatory actions on cytokine signaling in macrophages

As mentioned in chapter 4.2, we identified an abrogated block of the TCA cycle upon dexamethasone treatment in LPS-activated macrophages by mass spectrometry. In detail, prolonged (24 h) LPS stimulation resulted in relative abundance of TCA cycle intermediates, including citrate, isocitrate, α-ketoglutarate, fumarate and malate. Dexamethasone reversed these accumulations after 24 h, suggesting enhanced metabolization of the intermediates and possibly increased TCA cycle processing rates with concomitant electron transfer to the ETC, comparable to untreated cells. Surprisingly, succinate and itaconate did not accumulate after 24 h of LPS stimulation, but significantly after treatment with dexamethasone at this time point. This finding challenges the typical picture of a broken TCA cycle in inflammatory macrophages as classically activated macrophages after 24 h of stimulation are characterized by increased levels of isocitrate, succinate and itaconate, respectively [74]. In fact, the broken TCA cycle of CAM is associated with decreased activity of the TCA cycle enzymes isocitrate dehydrogenase (IDH) and succinate dehydrogenase (SDH), explaining the abundance of educts including citrate next to succinate and isocitrate. Increased

transcription of IRG1 in CAM leads to ACOD1 abundance and itaconate production by metabolization of citrate that itself, when accumulated, mediates distinct pro-inflammatory pathways [83]. However, succinate in particular is suggested to contribute to the pro-inflammatory phenotype of CAM as it supports downstream inflammatory processes such as activation of the NLRP3 inflammasome and stabilization of HIF-1α [82, 86]. Moreover, multiple TCA cycle components have the capacity to directly affect gene expression by modulation of the epigenetic landscape [125]. As mentioned, our observed decline of succinate and itaconate after 24 h of LPS challenge contrasts the paradigm of inflammatory metabolic remodeling in CAM. It is worth mentioning that classical activation of macrophages is generally defined by co-stimulation of LPS and additional IFN-y, which represents a strong mediator of pro-inflammatory phenotyping in macrophages [126]. Moreover, even though IRG1 is induced upon LPS activation in macrophages, IFN-y is suggested to significantly promote IRG1 transcription even if LPS is absent. [85] Interestingly, prolonged or repeated LPS challenge result in tolerization of macrophages to TLR stimuli that is linked to altered metabolic function and cytokine signaling [127]. Macrophage tolerization could provide an explanation for our observed effects on altered TCA cycle functionality after LPS treatment. Notably, considering a possible leading role of IFN-y in metabolic remodeling of CAM, studies could show that IFN-y abrogates LPS-induced tolerance in macrophages, indicating that IFN-y might be necessary for the “classic” TCA cycle break as it restores TLR induction and possibly inflammatory metabolic programming by LPS.[128]

Dexamethasone treatment in combination with LPS had no effect on succinate and itaconate abundance after 6 h but significantly increased concentration levels of succinate and itaconate after prolonged LPS activation time, indicating that GC mediated production of the anti-inflammatory intermediate might link immunosuppressive effects with metabolic reprogramming. To investigate the role of itaconate on the inhibitory effects of GC on pro-inflammatory cytokine signaling, we identified disturbed GC action in macrophages unable to produce itaconate, indicating a necessary role of itaconate for GC inhibitory effects on pro-inflammatory cytokine signaling. Supporting this hypothesis, the application of exogenous itaconate induced a rescue phenomenon of GC effects on secretion of TNF-α and IL-6 in IRG1 knockout macrophages. Itaconate is suggested to promote antimicrobial features of CAM, however, recent studies reported anti-inflammatory activities of the intermediate, including effects on pro-inflammatory cytokine signaling via modification of cysteine residues of signal transduction proteins such as Janus kinase 1 (JAK1) [129] The JAK1-STAT3 pathway is essential for cytokine receptor signaling of many cytokines, including IL-6 and IL-10. [130] In fact, posttranslational modification of STAT proteins is a well-known regulative mechanism of cytokine signaling. [131] Further, chemoproteomic profiling revealed interaction of the metabolite with glycolysis enzymes that mechanistically leads to limitation

of glycolytic flux and contributes to its anti-inflammatory properties, possibly in a synergistic manner to the effects of GC on glycolytic metabolism [132] Itaconate evidently inhibits SDH activity and consequently increases succinate levels, a mechanism that is mirrored by our observation that increased levels of itaconate after 6 h of LPS and 24 h of LPS and dexamethasone challenge positively correlate with measured succinate levels. As described above, succinate mediates pro-inflammatory signals in CAM. If the abundance of the intermediate is just a side-effect of itaconate-induced block of SDH or if it is involved in other signaling pathways needs to be evaluated in further studies.

These results strongly suggest that itaconate is required for anti-inflammatory effects of GC on cytokine signaling. A synergistic effect of transcriptional modulation of pro-inflammatory mediators via GC and posttranslational interactions of itaconate with proteins involved in cytokine signaling and cell metabolism, respectively, could provide an explanation for the observed results.

6.5. Concluding remarks

In conclusion, our data demonstrated that GC induce an anti-inflammatory metabolic phenotype in LPS-activated macrophages, which is defined by reorganization of glycolysis, promotion of mitochondrial dynamics, rewiring of the TCA cycle, and reinforcement of oxidative phosphorylation.

In this context, we identified rewiring of the TCA cycle and abundance of the immune-modulatory intermediate itaconate as the central hub that connects GC inhibitory actions on cytokine signaling with their impact on metabolic reprogramming in macrophages.

In concert with further research, this study could contribute to identification of downstream signaling pathways involved in this process and pave the way for the development of effective immune-suppressive agents and treatment strategies.

7. Abbreviations

AAM	Alternatively activated macrophages
ACOD	Aconitate decarboxylase 1
ACTH	Adrenocorticotropic hormone
AP-1	Activating protein-1
APC	Antigen presenting cell
ARG1	Arginase 1
ATP	Adenosinetriphosphate
BMDCs	Bone marrow-derived dendritic cells
BMDMs	Bone-marrow-derived macrophages
CAM	Classically activated macrophages
CBP	CREB binding protein
CD	Cluster of differentiation
CMP	Common myeloid progenitor
CoA	Coenzyme A
COX-2	Cyclooxygenase 2
CRH	Corticotropin-releasing hormone
DMI	Dimethylitaconate
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
DRP-1	Dynamin-related protein 1
ECAR	Extracellular acidification rate
ETC	Electron transport chain
FAD	Flavin adenin dinucleotide
FAO	Fatty acid oxidation
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
GATA 3	GATA binding protein 3
GC	Glucocorticoid
GLUT	Glucose transporter
GPDH	Glycerol-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HIF1a	Hypoxia-inducible factor 1-alpha
HPA axis	Hypothalamic pituitary-adrenal axis
IDH	Isocitrate dehydrogenase
IFN-y	Interferon-y

IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRG1	Immune response gene 1
JAK	Janus kinase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAVS	Mitochondrial antiviral-signaling protein
MFF	Mitochondrial fission factor
MFN	Mitofusin
miR-125b	Micro RNA 125b
MSC	Myeloid stem cell
mTOR	Mammalian target of rapamycin
MTP18	Mitochondrial 18kDa protein
mtSSB	Mitochondrial single-stranded DNA binding protein
NAD	Nicotinamide adenin dinucleotide
NCOR-1/2	Nuclear receptor corepressor 1/2
NfkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod like receptor
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
NRF-1	Nuclear respiratory factor 1
NRF-2	Nuclear respiratory factor 2
OCR	Oxygen consumption rate
OPA-1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen associated molecular pattern molecules
PDH	Pyruvate dehydrogenase
PD-L1/2	Programmed death ligand 1/2
PFK2	Phosphofructokinase 2
PFKB3	6-phosphofructo-2-kinase 3
PGC1b	Peroxisome proliferator-activated receptor-y coactivator 1b
PKM2	Pyruvatkinase M2
POLG	Polymerase gamma
PPARy	Peroxisome proliferator-activated receptor gamma
PRR	Pattern recognition receptor
RBC	Red blood cell
RET	Reverse electron transport chain

RLR	RIG-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SEGRM	Selective glucocorticoid receptor modulator
SEM	Standard error of the mean
SRC	Spare respiratory capacity
STAT	Signal transducers and activators of transcription
TCA-cycle	Tricarboxylic acid cycle
TF	Transcription factor
TLR	Toll-like receptor
TNF-a	Tumor necrosis factor alpha
VHL	Von-Hippel Lindau

8. References

1. Gjerstad, J.K., S.L. Lightman, and F. Spiga, *Role of glucocorticoid negative feedback in the regulation of HPA axis pulsatility.* Stress, 2018. **21**(5): p. 403-416.
2. Spiga, F., et al., *HPA axis-rhythms.* Compr Physiol, 2014. **4**(3): p. 1273-98.
3. Timmermans, S., J. Souffriau, and C. Libert, *A General Introduction to Glucocorticoid Biology.* Front Immunol, 2019. **10**: p. 1545.
4. Kuo, T., et al., *Regulation of Glucose Homeostasis by Glucocorticoids.* Adv Exp Med Biol, 2015. **872**: p. 99-126.
5. Franco, L.M., et al., *Immune regulation by glucocorticoids can be linked to cell type-dependent transcriptional responses.* J Exp Med, 2019. **216**(2): p. 384-406.
6. Orth, D.N., *Cushing's syndrome.* N Engl J Med, 1995. **332**(12): p. 791-803.
7. Benedek, T.G., *History of the development of corticosteroid therapy.* Clin Exp Rheumatol, 2011. **29**(5 Suppl 68): p. S-5-12.
8. Vettorazzi, S., et al., *A guide to changing paradigms of glucocorticoid receptor function-a model system for genome regulation and physiology.* FEBS J, 2021.
9. Ehrchen, J.M., J. Roth, and K. Barczyk-Kahlert, *More Than Suppression: Glucocorticoid Action on Monocytes and Macrophages.* Front Immunol, 2019. **10**: p. 2028.
10. Galon, J., et al., *Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells.* FASEB J, 2002. **16**(1): p. 61-71.
11. Cruz-Topete, D. and J.A. Cidlowski, *One hormone, two actions: anti- and pro-inflammatory effects of glucocorticoids.* Neuroimmunomodulation, 2015. **22**(1-2): p. 20-32.
12. Cain, D.W. and J.A. Cidlowski, *Immune regulation by glucocorticoids.* Nat Rev Immunol, 2017. **17**(4): p. 233-247.
13. Vandevyver, S., L. Dejager, and C. Libert, *On the trail of the glucocorticoid receptor: into the nucleus and back.* Traffic, 2012. **13**(3): p. 364-74.
14. Pratt, W.B. and D.O. Toft, *Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery.* Exp Biol Med (Maywood), 2003. **228**(2): p. 111-33.
15. Glass, C.K. and K. Saijo, *Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells.* Nat Rev Immunol, 2010. **10**(5): p. 365-76.
16. Hudson, W.H., et al., *Cryptic glucocorticoid receptor-binding sites pervade genomic NF-kappaB response elements.* Nat Commun, 2018. **9**(1): p. 1337.
17. Frijters, R., et al., *Prednisolone-induced differential gene expression in mouse liver carrying wild type or a dimerization-defective glucocorticoid receptor.* BMC Genomics, 2010. **11**: p. 359.
18. Zhang, G., L. Zhang, and G.W. Duff, *A negative regulatory region containing a glucocorticosteroid response element (nGRE) in the human interleukin-1beta gene.* DNA Cell Biol, 1997. **16**(2): p. 145-52.
19. Langlais, D., et al., *The Stat3/GR interaction code: predictive value of direct/indirect DNA recruitment for transcription outcome.* Mol Cell, 2012. **47**(1): p. 38-49.
20. Yang-Yen, H.F., et al., *Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction.* Cell, 1990. **62**(6): p. 1205-15.
21. Ray, A. and K.E. Prefontaine, *Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor.* Proc Natl Acad Sci U S A, 1994. **91**(2): p. 752-6.
22. De Bosscher, K., W. Vanden Berghe, and G. Haegeman, *The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression.* Endocr Rev, 2003. **24**(4): p. 488-522.
23. Vandevyver, S., et al., *New insights into the anti-inflammatory mechanisms of glucocorticoids: an emerging role for glucocorticoid-receptor-mediated transactivation.* Endocrinology, 2013. **154**(3): p. 993-1007.

24. Tuckermann, J.P., et al., *Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy.* J Clin Invest, 2007. **117**(5): p. 1381-90.
25. Clark, A.R. and M.G. Belvisi, *Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor.* Pharmacol Ther, 2012. **134**(1): p. 54-67.
26. Ronchetti, S., G. Migliorati, and C. Riccardi, *GILZ as a Mediator of the Anti-Inflammatory Effects of Glucocorticoids.* Front Endocrinol (Lausanne), 2015. **6**: p. 170.
27. Escoter-Torres, L., et al., *Fighting the Fire: Mechanisms of Inflammatory Gene Regulation by the Glucocorticoid Receptor.* Front Immunol, 2019. **10**: p. 1859.
28. Guenther, M.G., O. Barak, and M.A. Lazar, *The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3.* Mol Cell Biol, 2001. **21**(18): p. 6091-101.
29. Oh, K.S., et al., *Anti-Inflammatory Chromatinscape Suggests Alternative Mechanisms of Glucocorticoid Receptor Action.* Immunity, 2017. **47**(2): p. 298-309 e5.
30. Kokkinopoulou, I. and P. Moutsatsou, *Mitochondrial Glucocorticoid Receptors and Their Actions.* Int J Mol Sci, 2021. **22**(11).
31. Karra, A.G., et al., *Proteomic analysis of the mitochondrial glucocorticoid receptor interacting proteins reveals pyruvate dehydrogenase and mitochondrial 60 kDa heat shock protein as potent binding partners.* J Proteomics, 2022. **257**: p. 104509.
32. Buttgereit, F. and A. Scheffold, *Rapid glucocorticoid effects on immune cells.* Steroids, 2002. **67**(6): p. 529-34.
33. Sundahl, N., et al., *Selective glucocorticoid receptor modulation: New directions with non-steroidal scaffolds.* Pharmacol Ther, 2015. **152**: p. 28-41.
34. Ten, S., M. New, and N. Maclaren, *Clinical review 130: Addison's disease 2001.* J Clin Endocrinol Metab, 2001. **86**(7): p. 2909-22.
35. Hench, P.S., E.C. Kendall, and et al., *The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis.* Proc Staff Meet Mayo Clin, 1949. **24**(8): p. 181-97.
36. Manger, B. and G. Schett, *[80 milestones in rheumatology from 80 years- I. 1940-1960].* Z Rheumatol, 2021. **80**(6): p. 497-505.
37. Dorner, T. and G. Schett, *[80 milestones in rheumatology from 80 years-IV. 2000-2020].* Z Rheumatol, 2021. **80**(6): p. 528-538.
38. Caratti, G., et al., *Glucocorticoid receptor function in health and disease.* Clin Endocrinol (Oxf), 2015. **83**(4): p. 441-8.
39. McDonough, A.K., J.R. Curtis, and K.G. Saag, *The epidemiology of glucocorticoid-associated adverse events.* Curr Opin Rheumatol, 2008. **20**(2): p. 131-7.
40. Burns, C.M., *The History of Cortisone Discovery and Development.* Rheum Dis Clin North Am, 2016. **42**(1): p. 1-14, vii.
41. Meka, R.R., et al., *Peptide-targeted liposomal delivery of dexamethasone for arthritis therapy.* Nanomedicine (Lond), 2019. **14**(11): p. 1455-1469.
42. Van Moortel, L., K. Gevaert, and K. De Bosscher, *Improved Glucocorticoid Receptor Ligands: Fantastic Beasts, but How to Find Them?* Front Endocrinol (Lausanne), 2020. **11**: p. 559673.
43. Husebye, E.S., et al., *Adrenal insufficiency.* Lancet, 2021. **397**(10274): p. 613-629.
44. Gordon, S. and A. Pluddemann, *Tissue macrophages: heterogeneity and functions.* BMC Biol, 2017. **15**(1): p. 53.
45. Murphy, K. and C. Weaver, *Janeway's immunobiology*. 9th edition. ed. 2016, New York, NY: Garland Science/Taylor & Francis Group, LLC. xx, 904 pages.
46. Ginhoux, F. and M. Guilliams, *Tissue-Resident Macrophage Ontogeny and Homeostasis.* Immunity, 2016. **44**(3): p. 439-449.
47. Medzhitov, R., *The spectrum of inflammatory responses.* Science, 2021. **374**(6571): p. 1070-1075.
48. Barton, G.M., *A calculated response: control of inflammation by the innate immune system.* J Clin Invest, 2008. **118**(2): p. 413-20.

49. Wynn, T.A. and K.M. Vannella, *Macrophages in Tissue Repair, Regeneration, and Fibrosis.* Immunity, 2016. **44**(3): p. 450-462.
50. Diaz-Jimenez, D., J.P. Kolb, and J.A. Cidlowski, *Glucocorticoids as Regulators of Macrophage-Mediated Tissue Homeostasis.* Front Immunol, 2021. **12**: p. 669891.
51. Udalova, I.A., A. Mantovani, and M. Feldmann, *Macrophage heterogeneity in the context of rheumatoid arthritis.* Nat Rev Rheumatol, 2016. **12**(8): p. 472-85.
52. Smith, T.D., et al., *Regulation of macrophage polarization and plasticity by complex activation signals.* Integr Biol (Camb), 2016. **8**(9): p. 946-55.
53. Viola, A., et al., *The Metabolic Signature of Macrophage Responses.* Front Immunol, 2019. **10**: p. 1462.
54. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.
55. Rőszer, T., *Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms.* Mediators Inflamm, 2015. **2015**: p. 816460.
56. Pesce, J.T., et al., *Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis.* PLoS Pathog, 2009. **5**(4): p. e1000371.
57. Orecchioni, M., et al., *Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages.* Front Immunol, 2019. **10**: p. 1084.
58. Judge, A. and M.S. Dodd, *Metabolism.* Essays Biochem, 2020. **64**(4): p. 607-647.
59. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
60. Vaupel, P. and G. Multhoff, *Revisiting the Warburg effect: historical dogma versus current understanding.* J Physiol, 2021. **599**(6): p. 1745-1757.
61. O'Neill, L.A., R.J. Kishton, and J. Rathmell, *A guide to immunometabolism for immunologists.* Nat Rev Immunol, 2016. **16**(9): p. 553-65.
62. Russell, D.G., L. Huang, and B.C. VanderVen, *Immunometabolism at the interface between macrophages and pathogens.* Nat Rev Immunol, 2019. **19**(5): p. 291-304.
63. Stifel, U., et al., *Glucocorticoids coordinate macrophage metabolism through the regulation of the tricarboxylic acid cycle.* Mol Metab, 2021. **57**: p. 101424.
64. Annesley, S.J. and P.R. Fisher, *Mitochondria in Health and Disease.* Cells, 2019. **8**(7).
65. Milenkovic, D., et al., *TWINKLE is an essential mitochondrial helicase required for synthesis of nascent D-loop strands and complete mtDNA replication.* Hum Mol Genet, 2013. **22**(10): p. 1983-93.
66. Burman, J.L., et al., *Mitochondrial fission facilitates the selective mitophagy of protein aggregates.* J Cell Biol, 2017. **216**(10): p. 3231-3247.
67. Kleele, T., et al., *Distinct fission signatures predict mitochondrial degradation or biogenesis.* Nature, 2021. **593**(7859): p. 435-439.
68. Mills, E.L., B. Kelly, and L.A.J. O'Neill, *Mitochondria are the powerhouses of immunity.* Nat Immunol, 2017. **18**(5): p. 488-498.
69. Buck, M.D., et al., *Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming.* Cell, 2016. **166**(1): p. 63-76.
70. Duroux-Richard, I., et al., *miR-125b controls monocyte adaptation to inflammation through mitochondrial metabolism and dynamics.* Blood, 2016. **128**(26): p. 3125-3136.
71. Arnoult, D., et al., *Mitochondria in innate immunity.* EMBO Rep, 2011. **12**(9): p. 901-10.
72. Kawai, T., et al., *IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction.* Nat Immunol, 2005. **6**(10): p. 981-8.
73. Castanier, C., et al., *Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway.* EMBO Rep, 2010. **11**(2): p. 133-8.
74. Jha, A.K., et al., *Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization.* Immunity, 2015. **42**(3): p. 419-30.

75. Moon, J.S., et al., *mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation.* Cell Rep, 2015. **12**(1): p. 102-115.
76. Rodríguez-Prados, J.C., et al., *Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation.* J Immunol, 2010. **185**(1): p. 605-14.
77. Palsson-McDermott, E.M., et al., *Pyruvate Kinase M2 Regulates Hif-1α Activity and IL-1β Induction and Is a Critical Determinant of the Warburg Effect in LPS-Activated Macrophages.* Cell Metab, 2015. **21**(2): p. 347.
78. Zhang, D., et al., *Metabolic regulation of gene expression by histone lactylation.* Nature, 2019. **574**(7779): p. 575-580.
79. Ip, W.K.E., et al., *Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages.* Science, 2017. **356**(6337): p. 513-519.
80. Lampropoulou, V., et al., *Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation.* Cell Metab, 2016. **24**(1): p. 158-66.
81. Wu, R., et al., *ACOD1 in immunometabolism and disease.* Cell Mol Immunol, 2020. **17**(8): p. 822-833.
82. Tannahill, G.M., et al., *Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha.* Nature, 2013. **496**(7444): p. 238-42.
83. Infantino, V., et al., *The mitochondrial citrate carrier: a new player in inflammation.* Biochem J, 2011. **438**(3): p. 433-6.
84. Bailey, J.D., et al., *Nitric Oxide Modulates Metabolic Remodeling in Inflammatory Macrophages through TCA Cycle Regulation and Itaconate Accumulation.* Cell Rep, 2019. **28**(1): p. 218-230.e7.
85. Naujoks, J., et al., *IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid.* PLoS Pathog, 2016. **12**(2): p. e1005408.
86. Hooftman, A., et al., *The Immunomodulatory Metabolite Itaconate Modifies NLRP3 and Inhibits Inflammasome Activation.* Cell Metab, 2020. **32**(3): p. 468-478.e7.
87. Faas, M., et al., *IL-33-induced metabolic reprogramming controls the differentiation of alternatively activated macrophages and the resolution of inflammation.* Immunity, 2021. **54**(11): p. 2531-2546 e5.
88. Kelly, T.J., et al., *GCN5-mediated transcriptional control of the metabolic coactivator PGC-1beta through lysine acetylation.* J Biol Chem, 2009. **284**(30): p. 19945-52.
89. O'Neill, L.A. and D.G. Hardie, *Metabolism of inflammation limited by AMPK and pseudo-starvation.* Nature, 2013. **493**(7432): p. 346-55.
90. Mantovani, A., et al., *Macrophage plasticity and polarization in tissue repair and remodelling.* J Pathol, 2013. **229**(2): p. 176-85.
91. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
92. Göttlicher, M., S. Heck, and P. Herrlich, *Transcriptional cross-talk, the second mode of steroid hormone receptor action.* J Mol Med (Berl), 1998. **76**(7): p. 480-9.
93. Smoak, K. and J.A. Cidlowski, *Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling.* Mol Cell Biol, 2006. **26**(23): p. 9126-35.
94. Mozo, L., A. Suárez, and C. Gutiérrez, *Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes.* Clin Exp Allergy, 2004. **34**(3): p. 406-12.
95. Soto-Heredero, G., et al., *Glycolysis - a key player in the inflammatory response.* Febs j, 2020. **287**(16): p. 3350-3369.
96. O'Neill, L.A., *A broken krebs cycle in macrophages.* Immunity, 2015. **42**(3): p. 393-4.
97. Strelko, C.L., et al., *Itaconic acid is a mammalian metabolite induced during macrophage activation.* J Am Chem Soc, 2011. **133**(41): p. 16386-9.
98. Rogatsky, I. and L.B. Ivashkiv, *Glucocorticoid modulation of cytokine signaling.* Tissue Antigens, 2006. **68**(1): p. 1-12.

99. Franchimont, D., et al., *Tumor necrosis factor alpha decreases, and interleukin-10 increases, the sensitivity of human monocytes to dexamethasone: potential regulation of the glucocorticoid receptor.* J Clin Endocrinol Metab, 1999. **84**(8): p. 2834-9.
100. Czock, D., et al., *Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids.* Clin Pharmacokinet, 2005. **44**(1): p. 61-98.
101. De Kloet, R., G. Wallach, and B.S. McEwen, *Differences in corticosterone and dexamethasone binding to rat brain and pituitary.* Endocrinology, 1975. **96**(3): p. 598-609.
102. Werb, Z., R. Foley, and A. Munck, *Interaction of glucocorticoids with macrophages. Identification of glucocorticoid receptors in monocytes and macrophages.* J Exp Med, 1978. **147**(6): p. 1684-94.
103. Shi, L.Z., et al., *HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells.* J Exp Med, 2011. **208**(7): p. 1367-76.
104. Byles, V., et al., *The TSC-mTOR pathway regulates macrophage polarization.* Nat Commun, 2013. **4**: p. 2834.
105. Land, S.C. and A.R. Tee, *Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif.* J Biol Chem, 2007. **282**(28): p. 20534-43.
106. Rius, J., et al., *NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha.* Nature, 2008. **453**(7196): p. 807-11.
107. Weichhart, T., et al., *The TSC-mTOR signaling pathway regulates the innate inflammatory response.* Immunity, 2008. **29**(4): p. 565-77.
108. Kelly, B. and L.A. O'Neill, *Metabolic reprogramming in macrophages and dendritic cells in innate immunity.* Cell Res, 2015. **25**(7): p. 771-84.
109. Weichhart, T., et al., *Inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells.* Blood, 2011. **117**(16): p. 4273-83.
110. Mills, E.L., et al., *Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages.* Cell, 2016. **167**(2): p. 457-470.e13.
111. Scialò, F., D.J. Fernández-Ayala, and A. Sanz, *Role of Mitochondrial Reverse Electron Transport in ROS Signaling: Potential Roles in Health and Disease.* Front Physiol, 2017. **8**: p. 428.
112. Yin, M. and L.A.J. O'Neill, *The role of the electron transport chain in immunity.* Faseb j, 2021. **35**(12): p. e21974.
113. Everts, B., et al., *Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells.* Blood, 2012. **120**(7): p. 1422-31.
114. Di Rosa, M., et al., *Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages.* Biochem Biophys Res Commun, 1990. **172**(3): p. 1246-52.
115. Banoth, B. and S.L. Cassel, *Mitochondria in innate immune signaling.* Transl Res, 2018. **202**: p. 52-68.
116. Van den Bossche, J., et al., *Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages.* Cell Rep, 2016. **17**(3): p. 684-696.
117. Youle, R.J. and A.M. van der Bliek, *Mitochondrial fission, fusion, and stress.* Science, 2012. **337**(6098): p. 1062-5.
118. Scarpulla, R.C., *Nuclear control of respiratory gene expression in mammalian cells.* J Cell Biochem, 2006. **97**(4): p. 673-83.
119. Kobayashi, E.H., et al., *Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription.* Nat Commun, 2016. **7**: p. 11624.
120. Kuzmin, I., et al., *Identification of the promoter of the human von Hippel-Lindau disease tumor suppressor gene.* Oncogene, 1995. **10**(11): p. 2185-94.
121. Vettori, A., et al., *Glucocorticoids promote Von Hippel Lindau degradation and Hif-1α stabilization.* Proc Natl Acad Sci U S A, 2017. **114**(37): p. 9948-9953.
122. Peter, B. and M. Falkenberg, *TWINKLE and Other Human Mitochondrial DNA Helicases: Structure, Function and Disease.* Genes (Basel), 2020. **11**(4).
123. Nunnari, J. and A. Suomalainen, *Mitochondria: in sickness and in health.* Cell, 2012. **148**(6): p. 1145-59.

124. Inoue, T., et al., *Twinkle overexpression prevents cardiac rupture after myocardial infarction by alleviating impaired mitochondrial biogenesis.* Am J Physiol Heart Circ Physiol, 2016. **311**(3): p. H509-19.
125. Langston, P.K., et al., *Glycerol phosphate shuttle enzyme GPD2 regulates macrophage inflammatory responses.* Nat Immunol, 2019. **20**(9): p. 1186-1195.
126. Nathan, C.F., et al., *Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity.* J Exp Med, 1983. **158**(3): p. 670-89.
127. Seeley, J.J. and S. Ghosh, *Molecular mechanisms of innate memory and tolerance to LPS.* J Leukoc Biol, 2017. **101**(1): p. 107-119.
128. Chen, J. and L.B. Ivashkiv, *IFN-γ abrogates endotoxin tolerance by facilitating Toll-like receptor-induced chromatin remodeling.* Proc Natl Acad Sci U S A, 2010. **107**(45): p. 19438-43.
129. Runtsch, M.C., et al., *Itaconate and itaconate derivatives target JAK1 to suppress alternative activation of macrophages.* Cell Metab, 2022. **34**(3): p. 487-501.e8.
130. Murray, P.J., *The JAK-STAT signaling pathway: input and output integration.* J Immunol, 2007. **178**(5): p. 2623-9.
131. Stark, G.R. and J.E. Darnell, Jr., *The JAK-STAT pathway at twenty.* Immunity, 2012. **36**(4): p. 503-14.
132. Qin, W., et al., *S-glycosylation-based cysteine profiling reveals regulation of glycolysis by itaconate.* Nat Chem Biol, 2019. **15**(10): p. 983-991.