# Glutamine is required for M1-like polarization in response to *Mycobacterium tuberculosis* infection

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**Abstract**: In response to *Mycobacterium tuberculosis* infection, macrophages mount proinflammatory and antimicrobial responses similar to those observed in M1 macrophages activated by LPS and IFN-γ . A metabolic reprogramming to HIF-1-mediated uptake of glucose and its metabolism by glycolysis is required for M1-like polarization, but little is known about other metabolic programs driving the M1-like polarization during infection. We report that glutamine serves as a carbon and nitrogen source for the metabolic reprogramming of M1-like macrophages. Widely targeted metabolite screening identified an association of glutamine and/or glutamate with highly impacted metabolic pathways of M1-like macrophages. Moreover, stable isotope-assisted metabolomics of $U^{13}C$ glutamine and $U^{13}C$ glucose revealed that glutamine, rather than glucose, is catabolized in both the oxidative and reductive TCA cycles of M1-like macrophages, thereby generating signaling molecules that include succinate, biosynthetic precursors such as aspartate, and the antimicrobial metabolite itaconate. $U^{15}N$ glutamine tracing metabolomics further revealed participation of glutamine nitrogen in synthesis of intermediates of purine and pyrimidine metabolism plus amino acids that include aspartate. The findings were corroborated by diminished M1 polarization from chemical inhibition of glutaminase (GLS), the key enzyme of the glutaminolysis pathway, and by genetic deletion of *GLS* in infected macrophages. Thus, the catabolism of glutamine through the TCA cycle as an integral component of metabolic reprogramming in activating macrophages, coordinates with elevated cytosolic glycolysis to satisfy the cellular demand for bioenergetic and biosynthetic precursors of M1-like macrophages. Knowledge of these new immunometabolic features of M1-like macrophages should advance the development of host-directed therapies for tuberculosis

## Characterization of infected bone marrow-derived macrophages by sm-RNA-FISH

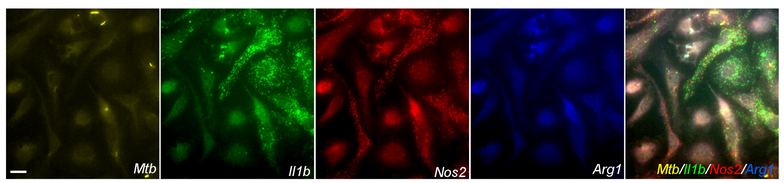


**Fig. 1. Single-cell mRNA analysis of immunometabolic markers in *Mtb*-infected BMDMs.** BMDMs seeded on coverslips were probed in 3-plex hybridization reactions using sm-RNA-FISH probes labeled with transcript-specific probe sets (about 50 oligonucleotides for each mRNA) that were coupled to tetramethylrhodamine (TMR), Texas Red, or Cy5 fluorophores. Images were acquired in Z-stacks of different fields of cells in different channels. Fluorescence spots corresponding to single mRNA molecules in individual cells were counted in the merged Z-stacks using a custom image-processing algorithm implemented in MATLAB. Representative images of GFP-labeled Mtb (yellow) and mRNA molecule spots for *Il1b* (Green), *Nos2* (Red), and *Arg1* (Blue) at 8 hrs p.i in Z-stacks of the same field of cells in different channels and merged together.

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## Metabolite screening identifies highly impacted metabolic pathways associated with glutamine

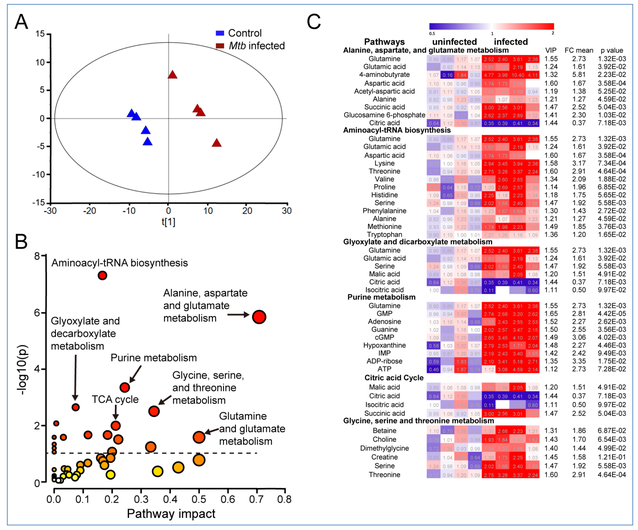


**Fig. 2 Identification of highly impacted metabolic pathways during *Mtb*-induced M1-like polarization by widely targeted small-metabolite screening.** Metabolites extracted from Mtb-infected BMDMs at 8 hrs p.i and uninfected controls were analyzed by the QTRAP 6500+ LC-MS/MS systems. Separation of M1-like macrophages from uninfected controls as scores plot from PLS-DA modeling (A). Highly impacted metabolic pathways in M1-like macrophages (B). The differential metabolites with variable importance in the projection (VIP) > 1 from the PLS-DA modeling of the two groups were subjected to pathway enrichment analysis by the Metaboanalyst (V5.0). Heatmaps of differential metabolites with VIP > 1 in highly impacted metabolic pathways (C). Data are shown as normalized values to the corresponding mean value of the uninfected group (4 biological repeats per group).

## Tracing metabolomics of $U^{13}C$ glutamine and glucose identifies anaplerosis of glutamine carbons through both the oxidative and reductive TCA cycle

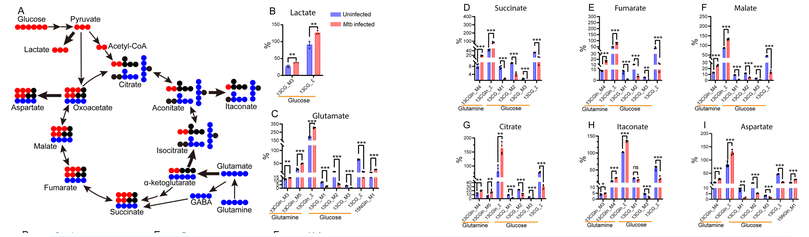


**Fig. 3. Isotope labeling distribution of $U^{13}C$ glucose and glutamine, and $U^{15}N$ glutamine during the M1-like polarization** Diagram of $^{13}C$ distribution from the catabolism of $U^{13}C$ glucose and $U^{13}C$ glutamine in glycolysis and/or the TCA cycle (A). Increased $^{13}C$ distribution from $U^{13}C$ glucose catabolism in glycolysis with the generation of M+3 lactate (B), and from the catabolism of $U^{13}C$ glutamine in the oxidative and reductive TCA cycle resulting in the generation of TCA cycle intermediates/derivatives (C - I). M1-like polarization was marked by diverting of the $^{13}C$ glucose carbon distribution from the TCA cycle M+2, M+1, and M+3 intermediates to the formation of M+3 lactate (A). In contrast, catabolism of $U^{13}C$ glutamine led to increased $^{13}C$ distribution in the form of M+4 TCA cycle intermediates/derivatives, including succinate (D), fumarate (E), malate (F), itaconate (H) and aspartate (I), as well as M+4 and M+5 citrate (G), indicating the simultaneous operation of both the oxidative and reductive TCA cycle. Increased $^{15}N$ distribution from $U^{15}N$ glutamine to M+1 aspartate (I) also indicates glutamine being a nitrogen source for the formation of nonessential amino acid aspartate. Solid red and blue circles represent $^{13}C$ from $U^{13}C$ glucose and $U^{13}C$ glutamine, respectively. Enrichment calculated from $U^{13}C$ glucose was marked as 13CG_M1, 13CG_M2, 13CG_M3, and 13CG_∑. ∑ was calculated with M1*1+M2*2…+Mn*n. Enrichment calculated from $U^{13}C$ glutamine was marked as 13CGln_M3, 13CGln_M4, 13CGln_M5, and 13CGln_∑. Enrichment calculated from $^{15}N$ glutamine was marked as 15NGln_M1.

## Chemical inhibition and genetic manipulation of glutaminolysis pathway diminishes M1-like polarization

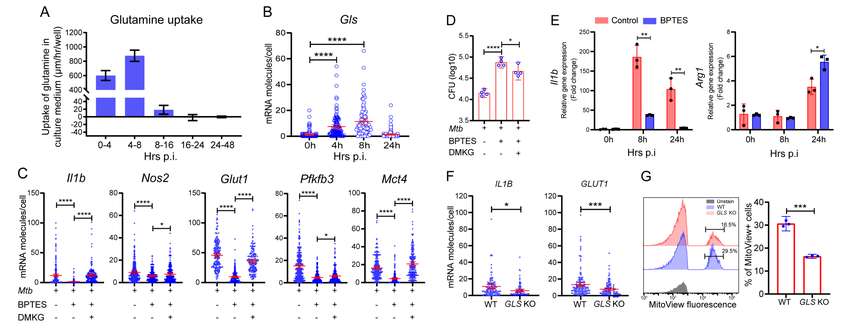


**Fig. 4. Requirement of glutamine for M1-like polarization.** High rate of glutamine uptake/utilization by *Mtb*-infected macrophages corresponding to the M1-like polarization (A). Cell culture supernatants collected at the indicated times were subjected to glutamine determination using the Glutamine/Glutamate-Glo (TM) Assay kit. Increased mRNA molecules for glutaminase gene *Gls* in M1-like macrophages (B). *Gls* mRNA molecules in infected BMDMs were detected and analyzed by sm-RNA-FISH. Diminished M1-like polarization by GLS inhibition with BPTES (10 μM), and alleviation of the inhibition by treatment with 1.5 mM DMKG (C). Enhanced Mtb growth by GLS inhibition with BPTES (D). CFU of *Mtb* was determined by plating assay of cell lysates of infected BMDMs with indicated treatments at day 3 p.i. Decreased expression of *Il1b* and increased *Arg1* in infected and BPTES-treated BMDMs (E). Expression of *Il1b* and *Arg1* were determined by RT-PCR and normalized to the expression level of *Actb*. Dampened M1 polarization in THP-1 *GLS* KO macrophages (F - G). Mitochondrial mass was evaluated using MitoView Fix 640 (Biotium) by flow cytometry and quantified (left: gating strategy; right: quantification) (G).

## Coordination of glucose and glutamine catabolism during M1-like polarization

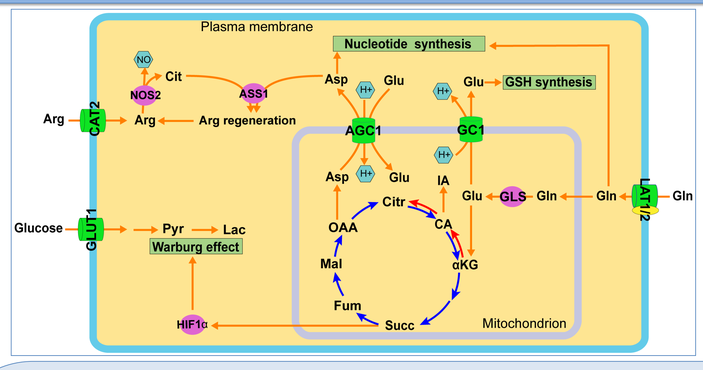


**Fig. 5. Coordination of glucose and glutamine catabolism during M1-like polarization**. M1-like polarization is marked with increased catabolism of glucose and glutamine (Gln) in distinctive subcellular compartments, but with coordinated functions. Increased glucose uptake, mediated by GLUT1, and its catabolism in the glycolysis pathway in cytosol result in the production of lactate (Lac). Increased Gln uptake, probably mediated by neutral amino acid antiporters, and its direct metabolite glutamate (Glu) participate in multiple pathways of the metabolic remodeling program of M1-like macrophages. Glu, the product of the glutaminolysis pathway by glutaminase (GLS) in mitochondria, enters the TCA cycle for anaplerosis reactions. Glu replenishes the TCA cycle leading to the generation of the antimicrobial metabolite itaconate (IA) and the signaling molecule succinate, which promotes stabilization of HIF1α and the subsequent Warburg Effect. Gln also contributes both carbon and nitrogen to the formation of the nonessential amino acid aspartate (Asp), which is then involved in biosynthetic pathways in the cytosol, including nucleotide synthesis and intracellular arginine regeneration by coupling with nitric oxide synthase 2 (NOS2)-derived citrulline (Cit) via argininosuccinate synthase 1 (ASS1) to sustain NO generation by NOS2. The export of mitochondrial Asp to the cytosol is mediated by the functional coupling between two mitochondrial carriers, wherein the glutamate carrier (GC1) exports the mitochondrial Glu deriving from GLS activity to cytosol. Cytosolic Glu then serves as a substrate of aspartate/glutamate carrier 1 (AGC1) for the net export of mitochondrial aspartate to the cytosol. Cytosolic Glu also participates in intracellular redox homeostasis involving the synthesis of glutathione (GSH).