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Glutamine Metabolism Is Required for Alveolar Macrophage Proliferation

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ABSTRACT: Alveolar macrophages (AMs) are critical for normal lung homeostasis, surfactant metabolism, and host defense against various respiratory pathogens. Despite being terminally differentiated cells, AMs are able to proliferate and self-renew to maintain their compartment without the input of the hematopoietic system in the adulthood during homeostasis. However, the molecular and metabolic mechanisms modulating AM proliferative responses are still incompletely understood. Here we have investigated the metabolic regulation of AM proliferation and self-renewal. Inhibition of glucose uptake or fatty acid oxidation did not significantly impact AM proliferation. Rather, inhibition of the glutamine uptake and/or glutaminase activity impaired AM mitochondrial respiration and cellular proliferation *in vitro* and *in vivo* in response to growth factor stimulation. Furthermore, mice with a genetic deletion of glutaminase in macrophages showed decreased proliferation. Our data indicate that glutamine is a critical substrate for fueling mitochondrial metabolism that is required for AM proliferation. Overall, our study is expected to shed light on the AM maintenance and repopulation by glutamine during homeostasis and following acute respiratory viral infection.

Keywords: Alveolar macrophage; Proliferation; Glutamine; Self-renewal

1. Introduction

Pulmonary homeostasis is important to maintain the status quo in the face of attacks from various pathogens, allergens, mineral particles or gases [1,2]. When this homeostasis is disrupted, the threshold for activation of the immune system is dysregulated, potentially leading to the development of diseases such as asthma, pneumonia and other acute or chronic lung conditions [3–6]. Alveolar macrophages (AMs) are the major immune cell types in the lung at the steady state [7]. They are critical in maintaining lung homeostasis under the physiological condition through their phagocytosis of surfactant proteins, invading pathogens or noxious particles. AMs also express high levels of immuno-suppressants including IL-10 and TGF-β, which are vital in actively inhibiting pulmonary inflammation during homeostasis [8]. Upon infection, however, AMs can sense invading pathogens to produce a plethora of inflammatory mediators to control pathogens and/or initiate inflammatory responses [9]. For instance, upon microbial invasion, AMs can produce a variety

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of inflammatory cytokines and chemo-attractive mediators to recruit other immune cells (such as neutrophils and monocytes), contributing to the development of pulmonary inflammatory responses [10]. AMs can also act as accessory cells by presenting antigens to T lymphocytes to facilitate adaptive immune responses [11,12]. Finally, upon pathogen clearance, AMs play a critical role in the repair and remodeling of connective tissue in the lung parenchyma [13]. Therefore, AMs are the main gatekeepers orchestrating pulmonary immune balance and tissue repair processes during homeostasis and inflammation.

Like many other tissue-resident macrophages, AMs are mainly developed from the fetal origin, yolk sac-derived macrophage progenitor cells and fetal monocytes [14]. Fetal monocytes begin to accumulate in the developing lung prenatally and differentiate into mature AMs via an immature macrophage stage during the first week after birth [15]. After development, AMs have the potential to proliferate and self-renew, allowing them to maintain themselves independent of the infiltration of bone marrow-derived monocytes in adulthood during homeostasis [16–21]. Emerging studies have shown the involvement of various growth cytokines and transcription factors in regulating AM self-renewal including GM-CSF, TGF-β, Bhlhe40, Bhlhe41 and mTOC1 etc. Additionally, mitochondrial oxidative metabolism was shown to be required for AM proliferation in vitro and for their proliferation and repopulation after partial depletion following influenza virus infection [15]. However, the molecular and metabolic mechanisms regulating AM proliferation and self-renewal remain to be incompletely understood.

Glutamine or L-glutamine is the most abundant amino acid in the blood. Since glutamine can be synthesized by muscle and other organs, it is considered as a non-essential amino acid in the body even though diet serves as an important source of glutamine [22]. Glutamine is needed for protein and nucleotide synthesis, biomass accumulation and energy source in rapidly dividing cells including active lymphocytes, enterocytes of the small intestine and cancer cells [23]. In macrophages, glutamine plays an important role in macrophage polarization. M2 macrophages (macrophages that are polarized by type 2 cytokines) were shown to have increased accumulation in glutamine and the inhibition of glutamine metabolism diminished M2 but not M1 gene expression [24]. Additionally, it has been shown that increased accumulation of glutamine in macrophages under pathological conditions [25]. Nevertheless, the role of glutamine in regulating tissue-resident macrophage proliferation and/or homeostatic function remains to be explored.

In this study, we have examined the roles of glutamine and glutaminase in AM proliferation and self-renewal *in vitro* and *in vivo*. We found that the inhibition of glutamine uptake and glutaminase activity impaired AM proliferative responses *in vitro* and *in vivo*. Further, macrophage-specific deficiency of glutaminase impaired the optimal proliferation of AM in responses to growth factors. Mechanistically, we found that glutamine metabolism is required for mitochondrial oxidative phosphorylation and the expression of genes involved in macrophage proliferation and self-renewal. Our findings thus establish an important role of glutamine uptake and its downstream metabolism in regulating AM proliferation in response to growth signals.

2. Materials and Methods

The details of the key resources are described in Table 1.

2.1. Mice and Treatment

This study obtained the approval of the Mayo Clinic Institutional Animal Care and Use Committees (IACUC). The care of the mice and the experimental protocols were achieved in strict agreement with the standards for the Use of Laboratory Animals. Age- and sex-matched mice (8–12 weeks old) were employed. WT C57BL/6 mice and Lyz2-cre mice were purchased from the Jackson Laboratory and bred in-house. $GLS^{fl/fl}$ Mice were obtained from Dr. Jeff Rathmell (Vanderbilt University). $CPT2^{fl/fl}$ mice were obtained from Toren Finkel (UPMC). $GLS^{\Delta Lyz2}$, or $CPT2^{\Delta Lyz2}$ mice were generated by crossing $GLS^{fl/fl}$ or $CPT2^{fl/fl}$ mice with Lyz2-cre mice respectively. All mice were raised in a specific pathogen-free (SPF) animal housing facility.

For IAV infection, influenza A/PR8 /34 strain (~45 PFU/mouse) was diluted in FBS-free DMEM medium (Corning) on ice in anesthetized mice through intranasal route as described before [7].

For recombinant murine GM-CSF (Biolegend, San Diego, CA, USA) stimulation, mice were treated with GM-CSF (1 μg/mouse) or PBS through the intranasal route at day 1 to day 3. And the BAL cells were collected for analysis at day 4.

Table 1. Key resources table.

Reagent or Resource	Source	Identifier
Antibodies		
Anti SiglecF-BV421 (clone E50-2440)	BD Biosciences	Cat# 562681
Anti SiglecF-PE (clone E50-2440)	BD Biosciences	Cat# 552126
Anti CD11b-PerCP-Cy5.5 (clone M1/70)	Biolegend	Cat# 101228, RRID: AB_893232
Anti CD11c-BV510 (clone N418)	Biolegend	Cat# 117338, RRID: AB_2562016
Anti CD64-BV711 (clone X54-5/7.1)	Biolegend	Cat# 139311, RRID: AB_2563846
Anti MerTK- APC (clone 2B10C42)	Biolegend	Cat# 151508, RRID: AB_2650739
Anti MerTK- FITC (clone 2B10C42)	Biolegend	Cat# 151504, RRID: AB_2617035
Anti F4/80-BV510 (clone BM8)	Biolegend	Cat# 123135, RRID: AB_2562622
Anti Ki67-FITC (clone SolA15)	eBioscience	Cat# 11-5698-82
Anti Ki67-APC (clone SolA15)	eBioscience	Cat# 17-5698-82
Bacterial and Virus Strains		
Influenza A/PR8/34	Laboratory of Thomas Braciale	[26]
Chemicals, Peptides, and Recombinant Proteins		
CB-839	TargetMol	Cat# T6797
BPTES	TargetMol	Cat# T6791
6-Diazo-5-oxo-L-norleucine	Sigma-Aldrich	Cat# D2141
5(6)-CFDA, SE	Invitrogen	Cat# 990192
Dimethyl 2-oxoglutarate	Sigma-Aldrich	Cat# 349631
2-Deoxy-D-glucose	Sigma-Aldrich	Cat# 154-17-6
7-AAD Viability Staining Solution	Biolegend	Cat# 420403
MethoCult™ Methylcellulose-based medium	STEMCELL Technologies	Cat# 03231
Seahorse XF Media & Calibrant	Agilent	Cat# 102353-100
Oligomycin	Sigma-Aldrich	Cat# 1404-19-9
FCCP	Sigma-Aldrich	Cat# 370-86-5
Rotenone	Sigma-Aldrich	Cat# 83-79-4
Antimycin A	Sigma-Aldrich	Cat# A8674
Bromodeoxyuridine (BrdU)	Biolegend	Cat# 423401
Zombie NIR™ Fixable Viability Dye	Biolegend	Cat# 423106
Recombinant mouse GM-CSF	Biolegend	Cat# 576308
Recombinant mouse M-CSF (carrier-free)	Biolegend	Cat# 576408
Recombinant mouse IL-4 (carrier-free)	Biolegend	Cat# 574306
Critical Commercial Assays		
GenElute™ Mammalian Total RNA Miniprep Kit	Sigma-Aldrich	Cat# RTN350
RNeasy Mini Kit	QIAGEN	Cat# 74104
Phase-Flow™ Alexa Fluor® 647 BrdU Kit	Biolegend	Cat# 370706
eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat# 00-5523-00
Seahorse XFp FluxPak	Agilent	Cat# 103022-100
Mouse Cytokine/Chemokine Magnetic Bead Panel	Luminex Cooperation	Cat# MCYTMAG-70K-PX32
Deposited Data		
Raw and analyzed data	This paper	GEO: GSE210076
Experimental Models: Organisms/Strains		
C57BL/6J	The Jackson Laboratory	Cat# 000664
Lyz2-cre	The Jackson Laboratory	Cat# 004781
CPT2$^{fl/fl}$	Laboratory of Toren Finkel	Mitsunori Nomura et al., 2016
GLS$^{fl/fl}$	Laboratory of Jeff Rathmell	Marc O Johnson et al., 2018
Software and Algorithms		
GraphPad Prism 7	GraphPad Software	http://www.graphpad.com
FlowJo (version 10.3)	LLC	http://www.flowjo.com
MeV software (version 4.9)	Dana-Farber Cancer Institute	http://mev.tm4.org
R language	The R Foundation	https://www.r-project.org/
GSEA	Broad Institute	http://software.broadinstitute.org/

2.2. Mouse AM Culture and Treatment In Vitro

Mouse AMs were obtained from bronchoalveolar lavage (BAL) as described previously [7]. Briefly, alveolar lavages were pooled from BAL washes (PBS with 2 mM EDTA). AMs were purified by adherence in complete medium

(RPMI-1640, 1% Pen/Strep, 10% FBS) for 2 h at 5% CO_2 and 37 °C. The non-adherent cells were discarded with warm PBS. The adherent AMs were cultured in complete medium with GM-CSF (10 ng/mL).

For AM treatment *in vitro*, AMs were subjected to the treatments with 2-DG (200 μM), DON (4 μM), BPTES (2 μM), CB-839 (1 μM), ETO (10 μM) or vehicle in complete medium overnight in the absence of GM-CSF. Then the cells were washed with warm PBS twice and treated with the above reagents in complete medium with GM-CSF overnight. Cells were then analyzed by Flow cytometry, and RNA-seq.

2.3. Mouse PM Culture and Treatment In Vitro

Mouse peritoneal macrophages (PMs) were obtained from peritoneal lavage fluid. Briefly, peritoneal lavages were pooled from peritoneal washes (PBS with 2 mM EDTA). PMs were purified by adherence in complete medium (DMEM, 1% Pen/Strep, 10% FBS) for 2 h at 5% CO_2 and 37 °C. The non-adherent cells were discarded with warm PBS. The adherent PMs were cultured in complete medium with M-CSF (50 ng/mL) and IL-4 (500 ng/mL).

For PM treatment in vitro, PMs were subjected to the treatments with DON (4 μM) or vehicle in complete medium overnight in the absence of M-CSF and IL-4. Then the cells were washed with warm PBS twice and treated with the above regents in complete medium with M-CSF and IL-4 overnight. Cells were analyzed by Flow cytometry.

2.4. DON Treatment In Vivo

C57BL/6 WT or $GLS^{\Delta Lyz2}$ mice ($GLS^{fl/fl}$ as control) were injected with PBS or DON (4 mg/kg) from day 1 to day 3 in GM-CSF stimulation model, and BAL was collected at day 4 for analysis. C57BL/6 WT were injected with PBS or DON (1 mg/kg) at day 6 and day 7 in IAV infection model, and BAL was collected at day 8 for analysis.

2.5. Colony Formation Assays

Colony formation assays were performed as described [27]. Briefly, isolated AMs were cultivated in 35 mm culture dishes, pre-treated with or without DON (4 μM) and cultivated at 37 °C in 5% CO_2 conditions overnight. Next, the cells were rinsed using PBS before the adjunction of MethoCult medium (M3231, Stem Cell Technologies, Vancouver, Canada) added with 50 μg/mL penicillin/streptomycin, 2 mM glutamine and 10 ng/mL GM-CSF with or without DON (4 μM). The number of colonies were counted on day 18 after plating.

2.6. Flow Cytometry Analysis

For analysis of surface markers, cells were treated with specific antibodies for 15 min on ice in PBS added with 2% FBS. Fluorescence-conjugated FACS antibodies were purchased from BioLegend, BD Biosciences, or eBioscience. Cell populations were characterized as follows: ($CD11c^+$ Siglec F^+ $CD11b^{low}$), PMs ($CD11b^+$ $F4/80^+$). The 7-AAD (Invitrogen, Carlsbad, CA, USA) staining was used for excluding dead cells. To accurately confirm the phenotype of AMs, the fluorescence-minus-one (FMO) staining sets were adopted. The proliferation was evaluated by detecting the BrdU (Sigma-Aldrich, St Louis, MO, USA) and Ki67 incorporation cells *in vitro*. Frequencies of BrdU and Ki67 incorporations were determined by flow cytometry using corresponding cells stained by an isotype antibody as background controls. The analysis was done using the FACS Attune NXT flow cytometer (Life Technologies, Carlsbad, CA, USA) and FlowJo software (TreeStar, Ashland, KY, USA).

2.7. Bioenergetic Determination of Oxygen Consumption Rate (OCR)

To analyze the bioenergetic phenotypes of AMs, the Seahorse XF243 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) was used. Firstly, cells were cultured (40,000 cells/well) in the XF24 cell culture plate for 24 h in terms of which the culture medium was substituted by XF media (Seahorse Bioscience) deprived of FBS and sodium bicarbonate and subsequently was with XF media. Next, we the cells were cultured in non-CO_2 incubator for 1 h at 37 °C. OCR was detected for 3 min, accompanied by mixing for 3 min and medium re-oxygenation. Next, basal rate measurements were taken. Before a basal OCR reading, the cells were treated with 1 μM Oligomycin A (Sigma-Aldrich), followed by treatment with 750 1.5 μM FCCP (Sigma-Aldrich) to detect the maximal OCR. Next, a mixture of 0.5 μM rotenone and 0.5 μM Antimycin A (Sigma-Aldrich), 10 mM glucose (Sigma-Aldrich), and 50 mM 2-DG (Sigma-Aldrich) were added as indicated. OCR measurements were performed in triplicate and the OCR readings in each well were normalized in reference to total protein levels detected using the BCA protein assay kit (Pierce, Rockford, IL, USA). Data were analyzed with Wave Desktop software version 2.6 (Agilent Technologies, Santa Clara, CA, USA).

2.8. RNA-Seq

Total RNA was obtained from seeded FACS-purified AMs using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) based on the manufacturer-recommended protocol. Two pools per genotype were used for RNA-seq. The total RNA was purified (Agilent Bioanalyzer RIN > 7.0) and reverse-transcribed in cDNA. The RNA-Seq of the cDNA libraries was done using an Illumina HiSeq 4000 in conformity with the provider's protocol with the HiSeq 3000/4000 PE Cluster Kit and Illumina cBot. The RTA software (Illumina, version 2.5.2) was used for base-calling. Paired-end fastq files were mapped to the mouse mm10 reference genome using STAR version 2.6.0a [28]. Gene level counts were obtained using featureCounts from the Subread package version 1.4.6 [29]. Different expression analysis was done using DESeq2 version 1.28.1 [30]. Gene set enrichment analysis (GSEA) [31] was performed using gene lists ranked by log2 fold change using the mouse gene set database from Enrichment Map [32].

2.9. GC-MS Metabolite Analysis

First, cell activation was achieved *in vitro* for on a period of 4 days. Activated cells were incubated with glutamine free RPMI 1640 medium (Life Technologies: 11879020) supplemented with 10% dialyzed FBS (Life Technologies: 26400036), 1% P/S and 0.3 mg/mL [U-13C5] glutamine (Cambridge Isotope Laboratories: CLM-1822-H-0.1) and treated with CB-839 or vehicle. The cells were collected and washed once with PBS, then metabolites were extracted with 80% methanol and dried under a nitrogen stream in a sample concentrator. Dried metabolite samples were dissolved in 75 µL *N*,*N*-dimethylformamide (DMF), then derivatized with 75 µL *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-Butyldimethylchlorosilane (TBDMCS) (Thermo Scientific: TS48927). Samples were incubated at room temperature for 30 min and were analyzed using an Agilent 7890B GC coupled to a 5977A mass detector. 3 µL of derivatized sample were injected into an Agilent HP-5ms Ultra Inert column, and the gas chromatography (GC) oven temperature increased at 15 °C/min up to 215 °C, followed by 5 °C/min up to 260 °C, and finally at 25 °C/min up to 325 °C. The mass spectrometry (MS) was operated in split-less mode with electron impact mode at 70 eV. Mass range of 50–700 was analyzed, recorded at 1562 scans/second. Data was analyzed using Agilent Mass Hunter Workstation Analysis software. The following metabolites were detected as TBDMS derivatives: citrate (*m*/*z* 591), glutamate (*m*/*z* 432), succinate (*m*/*z* 289), and fumarate (*m*/*z* 287). IsoPat^2 software was used for isotopomer analysis to adjust for natural abundance as previously performed [33,34].

2.10. Statistical Analysis

The GraphPad Prism 7.0 (GraphPad Software) was executed for statistical analysis. The data is presented in the form of means ± SEM. The statistical methods for uncovering differences between groups were based on the paired or Unpaired t-tests, one-way ANOVA, or two-way ANOVA according to cases. A p-value < 0.05 was retained as a criterium for revealing statistical significance.

3. Results

3.1. Glutamine Regulates the Proliferation of AMs In Vitro

We have previously shown that mitochondrial metabolism is essential in AM proliferation and self-renewal [35]. To investigate the effects of different metabolic pathways that fuel the mitochondria, we analyzed AM Ki67 expression in the presence of 2-Deoxy-D-glucose (2-DG, inhibitor of glycolysis), etomoxir (ETO, inhibitor of fatty acid mitochondria oxidation through the suppression of Carnitine palmitoyl transferase (CPT) I activity) and 6-diazo-5-oxo-L-norleucine (DON, glutamine antagonist) following GM-CSF culture *in vitro*. The presence of DON but not 2-DG nor ETO greatly impaired AM Ki67 expression (Figure 1A,B), indicating that glutamine metabolism but not glycolysis nor fatty acid oxidation is required for AM proliferation. We further confirmed that fatty acid oxidation is not required for AM proliferation *in vitro* by using the CPT2-deficient macrophages isolated from Lyz2 $CPT2^{fl/fl}$ ($CPT2^{\Delta Lyz2}$) mice (Figure 1C,D). Consistent with the important roles of glutamine metabolism in regulating AM proliferation *in vitro*, the absence of glutamine in the culture medium led to significantly decreased cell proliferation while the glutamine treatment dose-dependently increased cell proliferation as indicated by the Ki67-positive AMs (Figure S1A,B). To further explore the effect of glutamine metabolism on the proliferation of AMs, we treated the cells with glutaminase inhibitors bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide (BPTES) and CB-839. Similar to DON treatment, the presence of glutaminase inhibitors such as BPTES and CB-839 also led to decreased percentage of Ki67-positive AMs (Figure 1E,F). We next used a reported CFSE (Carboxy fluorescein succinimidyl ester) dilution [36]

assay to assess AM proliferation following treatment of various inhibitors (Figure 1G). In this assay, the dilution of CFSE dye due to cell proliferation can be measured by flow cytometry. We found that DON, CB-839 or BPTES, but not 2-DG nor ETO, treatment diminished CFSE dilution), further confirming that glutamine metabolism plays a significant role in the proliferation and renewal of AMs, possibly through the glutaminase activity. In contrast, DON, BPTES, CB-839 only slightly induced the death of macrophages as revealed by 7-AAD staining assay (Figure 1H,I). Upon cellular entry, glutamine is metabolized by glutaminase and glutamate dehydrogenase into α-ketoglutarate. We found that the addition of α-ketoglutarate into the glutamine-free medium partially rescued the proliferation deficiency of AMs (Figure 1J,K), suggesting that glutamine conversion into α-ketoglutarate is at least partially required for AM proliferation. To further confirm the results obtained with DON treatment, we crossed glutaminase-1 gene floxed ($GLS^{fl/fl}$) mice with Lyz2-Cre mice and generated $GLS^{\Delta Lyz2}$ (Lyz2-Cre $GLS^{fl/fl}$) mice, which lack glutaminase 1 in myeloid cells. We isolated AMs from WT control ($GLS^{fl/fl}$) or $GLS^{\Delta Lyz2}$ mice and then treated AMs *in vitro* with GM-CSF to examine the roles of GLS deficiency in AM proliferation. The deletion of GLS in AM caused decreased Ki67 expression in AMs (Figure 1L,M). Together, these results demonstrated that glutamine metabolism via glutaminase-dependent pathways is required for optimal AM proliferation *in vitro*. To determine whether glutamine metabolism is also required for the proliferation of other macrophages, we stimulated peritoneal macrophages (PMs) with IL-4 and M-CSF and then treated the PMs with or without DON. As shown in Figure S2, the inhibition of glutamine metabolism by DON significantly dampened PM Ki67 expression, indicating that glutamine metabolism also fuels PM proliferation *in vitro.*

3.2. Glutaminolysis Regulates AM Mitochondrial Oxidative Phosphorylation

Previously, we have shown that mitochondrial metabolism is critical for AM proliferation [37]. We therefore investigated whether glutamine metabolism affects mitochondrial respiration using the Seahorse extracellular flux analyzer. We determined the oxygen-consumption rates (OCR) of AMs treated with different molecules. Vehicle- and ETO-treated AMs showed higher basal oxygen consumption rate (basal OCR) and maximal mitochondrial respiratory capacity (max. respiration) than glutamine-deprived and DON-treated AMs (Figure 2A). Furthermore, basal OCR was higher in Vehicle- and ETO-treated AMs than in glutamine-deprived, BPTES or CB839-treated AMs (Figure 2B,C). We next examined whether the mitochondrial glutamine-dependent metabolic pathways are influenced following CB839 treatment. We incubated these cells with [^{13}C5] glutamine and examined [^{13}C5] glutamine-derived carbon tracing of glutamate and TCA metabolites using GC/MS (gas chromatography/mass spectrometry). Metabolism of [^{13}C5] glutamine into glutamate results in the production of M+5-labeled glutamate and the following oxidative TCA cycle produces M+4-labeled citrate, succinate and fumarate (Figure 2D). After this process, two of unlabeled carbon incorporation from glucose oxidation further produces M+3 and M+1 glutamate as well as M+2-labeled citrate, succinate and fumarate (Figure 2D). Except for M+0-labeled, the fractional labeling values of glutamate, citrate, succinate, and fumarate in the CB839-treated AMs were lower than those in the control untreated AMs (Figure 2D). These data suggest glutamine metabolism can increase mitochondrial respiration along with oxidative TCA cycle in AMs.

3.3. Blockade of Glutamine Metabolism Attenuates the Activation of AMs via Multiple Pathways In Vitro

We next sought to further investigate the underlying molecular mechanisms by which glutamine metabolism in regulating AM proliferation. To this end, we treated AMs with or without GM-CSF, or GM-CSF with DON and performed RNA-sequencing in treated AM. As shown in Figure 3A, GM-CSF treatment altered the transcriptional profile of AMs with the upregulation of many cell-cycle-dependent genes (Figure 3A), which is consistent with the fact that the GM-CSF primes AM proliferation in vitro. DON treatment, however, potently altered the transcriptional program of GM-CSF treated AMs (Figure 3A). Gene set enrichment analysis (GSEA) was performed to uncover the pathways impacted by the effect of glutamine metabolism on the activated AMs. GSEA on the MSigDB hallmark gene sets and KEGG pathways indicated that DON treatment diminished gene sets associated with cell proliferative responses including E2F targets, Myc targets, mitotic spindle, mTORC1, and G2M checkpoint. In contrast, DON treatment promoted the expression of genes involved in inflammatory responses, IFN-α and IFN-γ responses in AMs (Figure 3B). Thus, the inhibition of glutamine metabolisms altered the balance of proliferative versus inflammatory genes in AMs. It has been reported that macrophage self-renewal requires the expression of genes involved in embryonic stem cell (ESC) function [27]. We found that ESC-associated genes were significantly down-modulated in DON treated AMs. We next examined the colony-forming ability (shown as colony-forming units (CFUs) of AMs following DON treatment. As Figure 3D demonstrated, DON treatment led to a reduction of colony size and numbers in AMs. Thus, these data suggest that glutamine metabolism is important in regulating AM self-renewal ability.

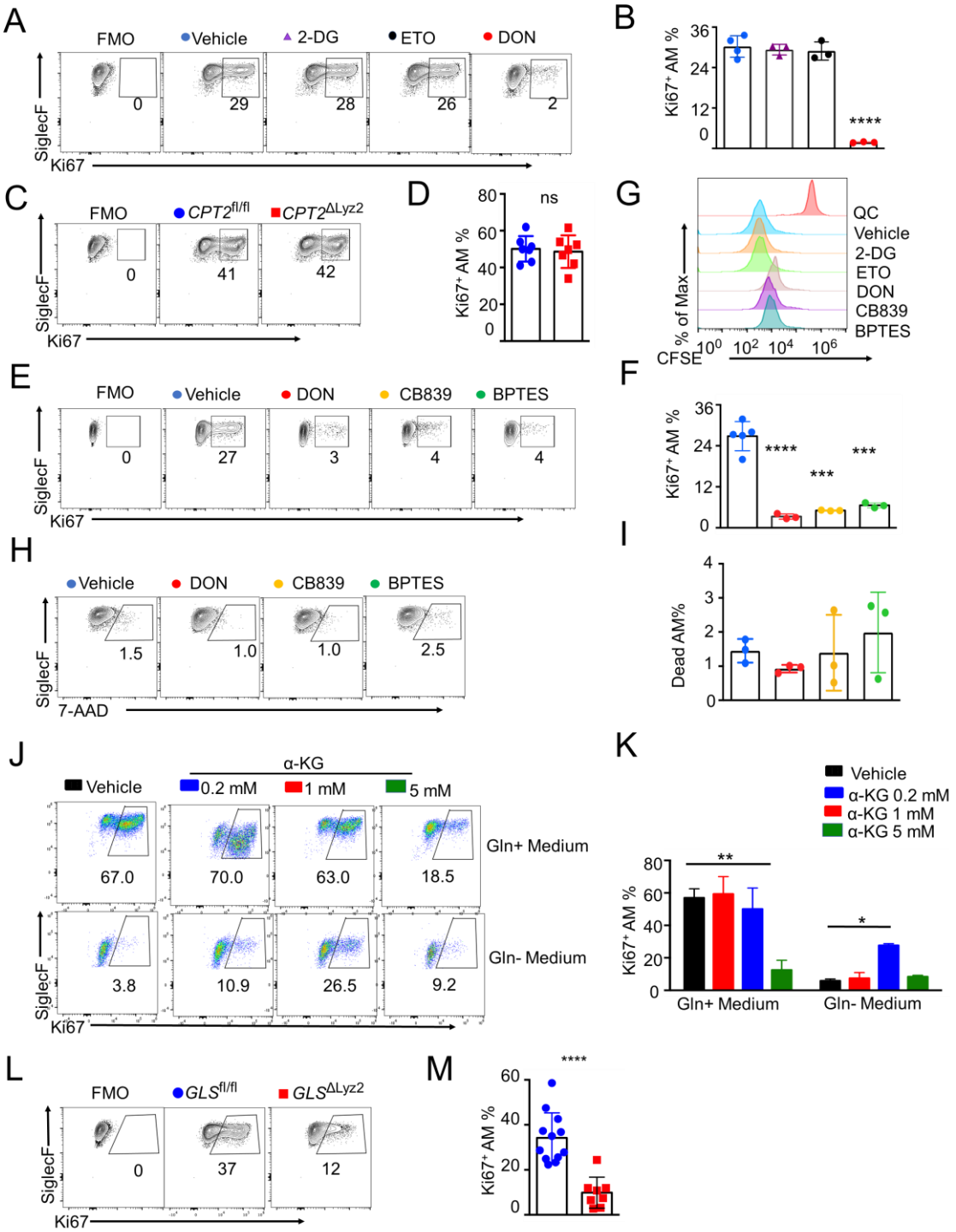

Figure 1. Glutamine regulates the proliferation of alveolar macrophages *in vitro*. (**A**) Flow cytometry analysis of Ki67 expression in alveolar macrophages (AMs) following *in vitro* culture treated by 2-Deoxyglucose (2-DG), CPT1a inhibitor etomoxir (ETO) or 6-diazo-5-oxo-L-norleucine (DON). (**B**) Percentage of Ki67-positive AMs with 2-DG, ETO or DON treatment (n = 3–4) *in vitro*. (**C**) Flow cytometry analysis of Ki67 expression in *CPT2*$^{fl/fl}$ or *CPT2*$^{\Delta Lyz}$ AMs *in vitro*. (**D**) Percentage of Ki67-positive *CPT2* $^{fl/fl}$ or *CPT2*$^{\Delta Lyz}$ AMs (n = 7) *in vitro*. (**E**) Flow cytometry analysis of Ki67-positive AMs treated with BPTES or CB-839 *in vitro*. (**F**) Percentage of Ki67-positive AMs in BPTES or CB-839 treatment conditions (n = 3–5) *in vitro*. (**G**) CFSE staining of AMs treated with 2-DG, ETO, DON, BPTES or CB-839 (n = 3) *in vitro*. (**H**) Flow cytometry analysis of 7-AAD-positive AMs with BPTES or CB-839 treatment *in vitro*. (**I**) Percentage of 7-AAD-positive AMs with BPTES or CB-839 treatment (n = 3) *in vitro*. (**J**) Flow cytometry analysis of Ki67 expression in AMs treated by α-Ketoglugarate (α-KG) in presence or absence of glutamine *in vitro*. (**K**) Percentage of Ki67-positive AMs after treatment by α-KG in the presence or absence of glutamine (n = 3) *in vitro*. (**L**) Flow cytometry analysis of Ki67 expression in *GLS* $^{fl/fl}$ or *GLS*$^{\Delta Lyz}$ AMs *in vitro*. (**M**) Percentage of Ki67-positive *GLS* $^{fl/fl}$ or *GLS*$^{\Delta Lyz}$ AMs (n = 8–12) *in vitro*. Representative or pooled data (**D**, **K** and **M**) from at least two independent experiments. Data are the mean ± SEM. ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$ between the indicated groups or compared to the vehicle group or control group.

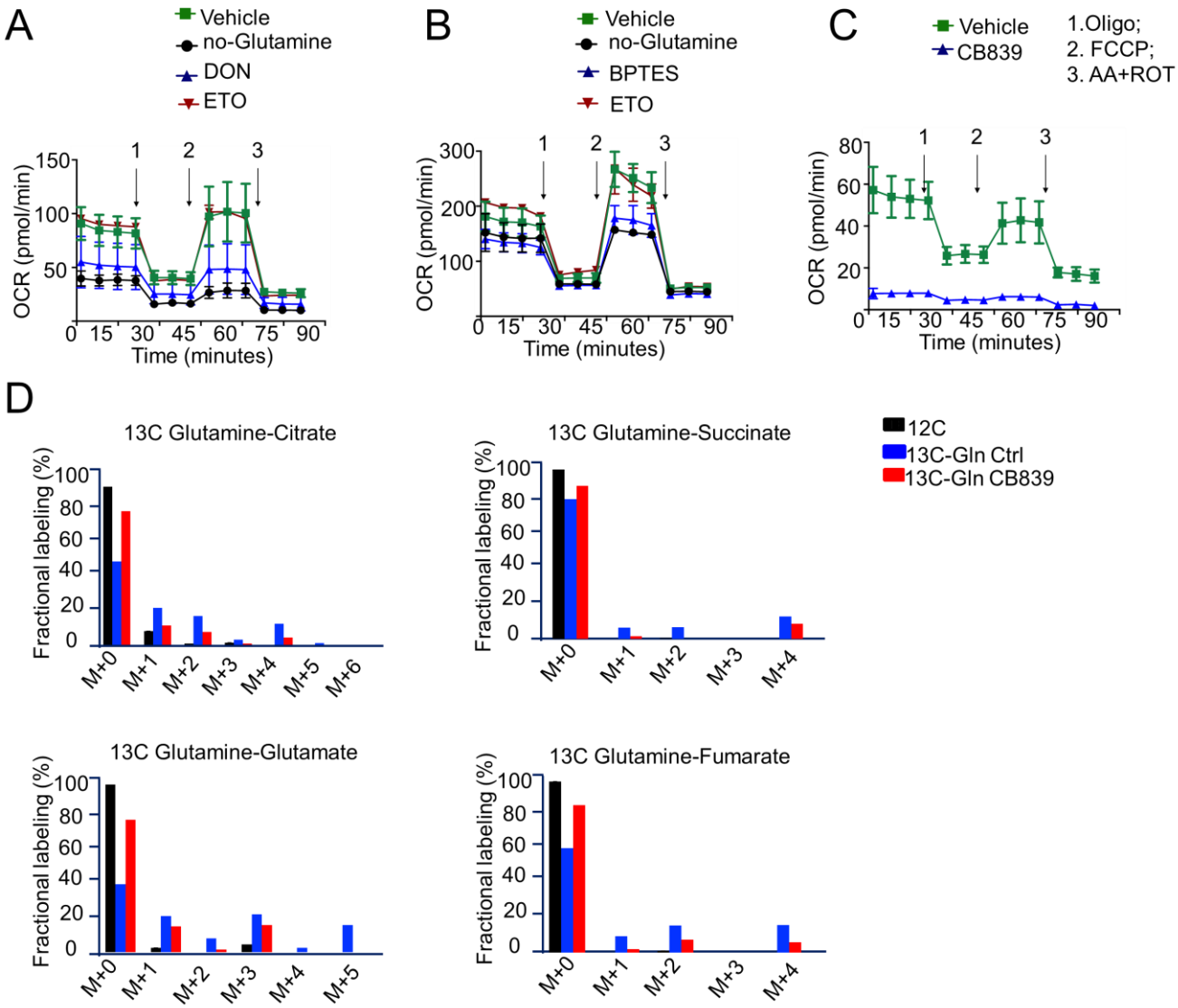

Figure 2. Glutaminolysis regulates metabolic reprogramming of AMs. (**A**) Determination of oxygen-consumption rates (OCR) of AMs untreated or treated with glutamine, vehicle, DON or ETO. (**B**) Determination of OCR of AMs untreated or treated with glutamine, vehicle, BPTES or ETO. (**C**) Determination of OCR of AMs treated with vehicle or CB839. (**D**) Labeling and detection of fractional flux in CB839-treated AMs (n = 3). Representative data from at least two independent experiments.

3.4. Blockade of Glutamine Metabolism Attenuates AM Proliferative Responses In Vivo

We next sought to examine the roles of glutamine metabolisms in the regulation of AM proliferation *in vivo*. To do so, we first intranasally treated WT mice with recombinant GM-CSF to stimulate robust AM proliferation *in vivo*. Then we treated the mice with PBS or DON and analyzed AM proliferation through Ki67 expression and BrdU incorporation assay (Figure 4A). As shown in Figure 4B,C, GM-CSF treatment resulted in increased Ki67 expression and BrdU incorporation in AMs, suggesting GM-CSF triggered AM proliferation as expected. DON treatment significantly but not completely abrogated AM proliferation in responses to GM-CSF treatment (Figure 4B,C). These data suggest that optimal AM proliferation *in vivo* requires glutamine uptake. Previously, we have shown that influenza infection caused partial AM depletion and was followed by subsequent AM proliferation to regenerate the AM compartment [7]. We, therefore, examined whether glutamine metabolism could regulate AM proliferative burst following IAV infection. To do so, we infected WT mice with influenza and then treated the mice with PBS or DON at day 6 and 7 post-infection (d.p.i.). We then examined Ki67 expression and BrdU incorporation at 8 d.p.i. (Figure 4D). Host morbidity was monitored and there was no significant difference between these two groups (Figure 4E). As shown in Figure 4F,G, DON treatment significantly diminished Ki67 expression and BrdU+ AMs following IAV infection. Thus, these data demonstrated that glutamine metabolism is required for maximal AM proliferation following GM-CSF treatment or viral-mediated AM depletion.

3.5. Genetic Ablation of Glutaminase in AMs Impaired Optimal AM Proliferation In Vivo

We next examined whether glutaminase function is required for AM proliferation *in vivo*. To do so, control and *GLS*$^{\Delta Lyz2}$ mice were treated with rGM-CSF as in Figure 4A (Figure 4H). AM Ki67 expression and BrdU incorporation were then examined by flow cytometry. As shown in Figure 4I,J, myeloid glutaminase deficiency resulted in moderate but significant impairment in Ki67 expression and BrdU incorporation. These data suggest that optimal AM proliferation requires glutaminase during homeostasis.

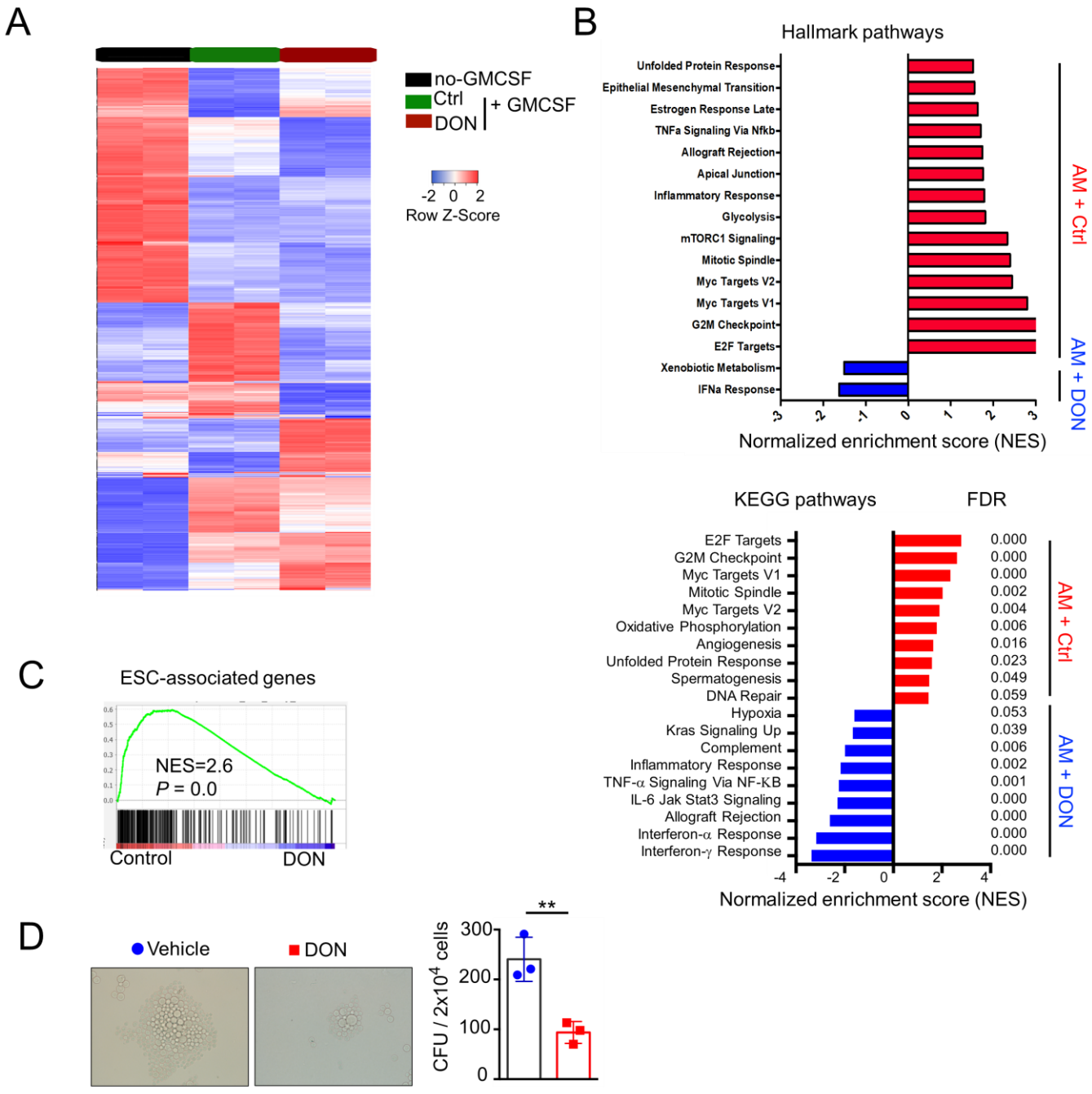

Figure 3. Blockade of glutamine metabolism attenuates the activation of AMs via multiple pathways *in vitro*. (**A**) Heatmap showing the gene expression profile from RNA sequencing of AMs treated with GM-CSF and the combination of GM-CSF and DON (n = 3). (**B**) Normalized enrichment scores (NES) of hallmark pathways and Gene Ontology (GO) pathways were obtained from the GSEA analysis of samples from DON-treated and control samples. (**C**) Gene set enrichment analysis (GSEA) enrichment score curve of ESC-associated genes obtained from the GSEA of control and DON samples. (**D**) Clonogenic assay indicating the effect of DON on the proliferation of AMs (n = 3). Representative data from two independent experiments (except A, B, and C). Data are the mean ± SEM. ** $p < 0.01$ between the indicated groups.

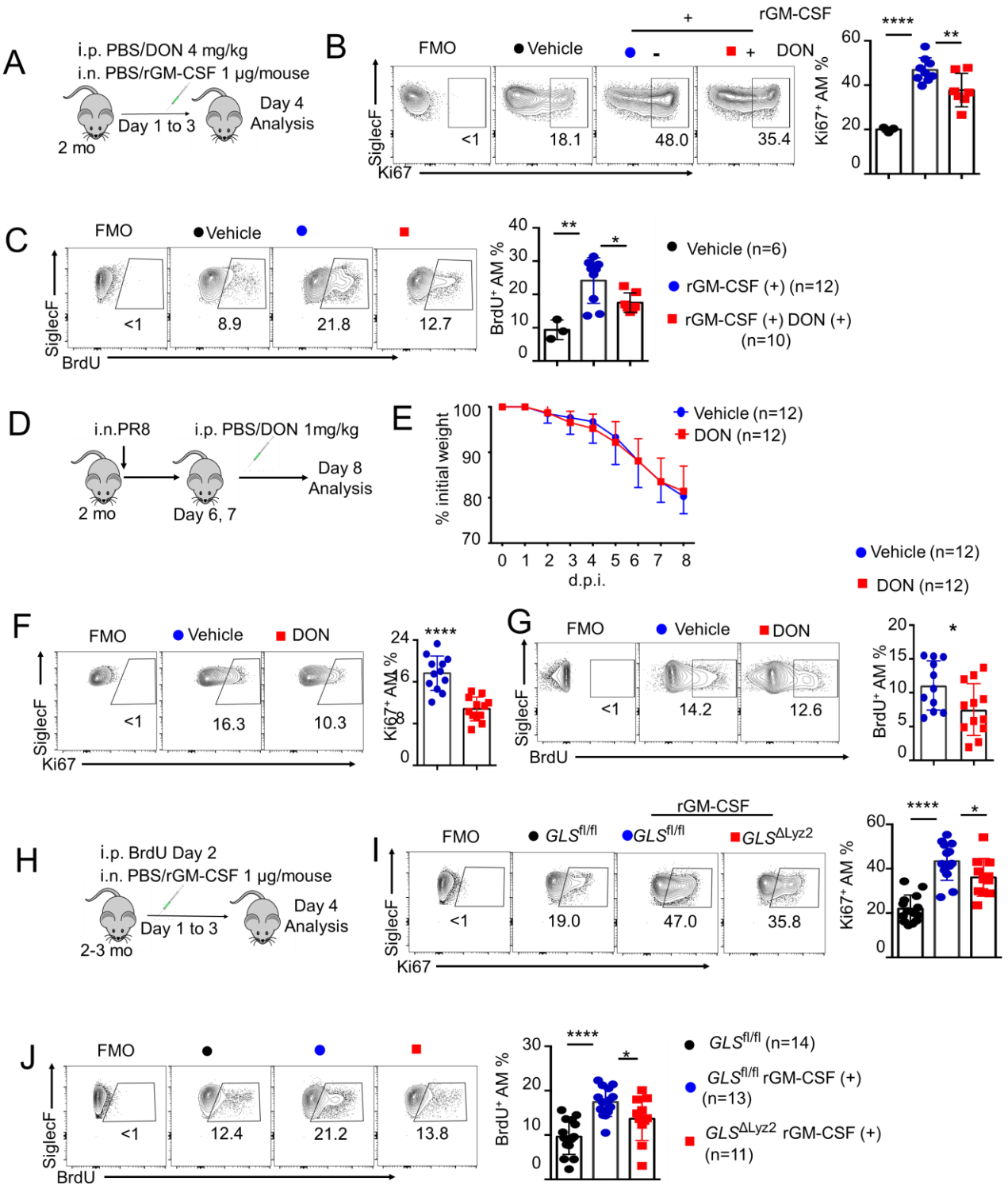

Figure 4. Blockade of glutamine metabolism or genetic ablation of glutaminase in AMs impaired optimal AM proliferation *in vivo*. (**A**) Schematic showing experimental design. (**B**) Determination of the proliferation of AMs by Ki67 staining and flow cytometry after treatment with rGM-CSF in combination with or without DON (n = 6–12) *in vivo*. (**C**) Determination of the proliferation of AMs by BrdU staining and flow cytometry after treatment with rGM-CSF in combination with or without DON (n = 6–12) *in vivo*. (D–G) WT mice were infected with IAV. (**D**) Schematic showing experimental design. (**E**) Host morbidity was monitored. Determination of the proliferation of AMs by Ki67 (**F**) or BrdU (**G**) staining and flow cytometry (n = 12) *in vivo*. (H-J) *GLS* $^{fl/fl}$ or *GLS*$^{\Delta Lyz}$ mice were subjected to rGM-CSF treatment for 3 days *in vivo* followed by BrdU assay of AM proliferation at day 4 post injection. (**H**). Schematic showing experimental design. (**I**) Percentage of Ki67-positive AMs isolated from *GLS* $^{fl/fl}$ or *GLS*$^{\Delta Lyz}$ mice with or without rGM-CSF treatment (n = 11–14) *in vivo*. (**J**) Percentage of BrdU-positive AMs isolated from *GLS* $^{fl/fl}$ or *GLS*$^{\Delta Lyz}$ mice with or without rGM-CSF treatment (n = 11–14) *in vivo*. Representative or pooled data (B, C, E, F, G, I and J) from two or three independent experiments. Data are the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 between the indicated groups.

4. Discussion

AMs can proliferate and self-renewal to maintain their compartment throughout adulthood during homeostasis. Additionally, infection with respiratory viruses such as influenza and SARS-CoV-2 often leads to partial to severe depletion of the AM compartment [7,38,39] and AM proliferation is important in reconstituting the AM compartment [7,38]. Given the important roles of AM in maintaining lung homeostasis, promoting lung repair and protecting against microbial infection, it is thus of critical importance to elucidate the underlying molecular and metabolic mechanisms regulating AM proliferation and self-renewal during homeostasis and/or disease conditions. Here we have deepened our understanding of the mechanisms of AM self-renewal process by revealing the requirement of glutamine metabolism in optimal AM proliferation in vitro and in vivo.

The roles of cellular metabolism in macrophage function and polarization have been intensively studied [40], but the function of metabolism in modulating macrophage self-renewal is less clear. Previously, it was suggested that mTOR dependent metabolic program is vital for AM proliferation *in vitro* and *in vivo* [41]. More recently, we found that mitochondrial metabolism is required for AM proliferation and self-renewal *in vitro* [7]. However, the specific nutrient pathways fueling mitochondrial energy metabolism required for AM proliferation were not yet identified. In this study, we found that the inhibition of lipid mitochondrial oxidation or glucose uptake minimally affected AM proliferation *in vitro*, but the suppression of glutamine uptake or the inhibition of the enzymatic activity of glutaminase diminished AM proliferation following GM-CSF treatment. Furthermore, glutaminase deficiency diminished AM proliferation and self-renewal *in vitro* and *in vivo*. Together, these data suggest that compared to lipid or glucose, glutamine is likely preferentially required for fueling the mitochondrial energy metabolism for AM proliferation and self-renewal. Of note, the inhibition of glutamine metabolism markedly suppressed AM proliferation *in vitro*, but the effects were less dramatic *in vivo*. These data are in contrast to the phenotypes of TFAM deficiency in AMs, which greatly impaired mitochondrial fitness and AM self-renewal *in vivo* [35]. It is possible that other metabolic pathways such as glucose and/or lipid oxidation may compensate for the requirement of glutamine metabolism *in vivo* when glutamine metabolism is impaired.

We found that glutaminase deficient mice had regular AM compartments under steady-state, suggesting that glutaminase in AMs may not be required for the basal proliferation required for AM maintenance under steady-state. However, glutaminase deficiency impaired AM proliferation following GM-CSF treatment or after influenza infection. The difference may be explained by the difference in the rate of division of AM under variable conditions such as in homeostasis, GM-CSF treatment and/or following AM depletion following influenza infection. [7,38]. To this end, glutamine metabolism was selectively required for cells undergoing rapid division, as they are more metabolically active. Notably, as we showed previously that viral infection impaired AM mitochondrial function and AM proliferation, partially due to the Wnt-β-catenin signaling [7,35], whether acute viral infection can also modulate AM glutamine metabolism warrants further investigations.

Regarding the mechanisms of glutamine metabolism controlling AM proliferation, we found that the inhibition of glutamine metabolism potently impaired the expression of cell cycle and self-renewal-associated genes. Glutamine metabolism can regulate chromatin status through its downstream metabolite, α-ketoglutarate, which serves as a co-substrate for a class of histone demethylases including Jmjd3 (Jumonji domain-containing protein-3). Indeed, glutamine metabolism has been shown to regulate M2 macrophage differentiation through α-ketoglutarate dependent Jmjd3 function and the demethylation of H3K27 in the promoter region of M2-specific marker genes [42]. Further, α-ketoglutarate supplementation partially rescued AM proliferation defects in the absence of glutamine. Together, these data indicate that a-ketoglutarate-dependent epigenetic control of cell cycle-associated gene expression likely underlies the control AM proliferation by glutamine metabolism. Additionally, glutamine is a critical nitrogen donor for de novo nucleotide biosynthesis. It is thus also possible that glutamine regulates AM self-renewal through DNA replication and/or RNA synthesis during cell division. The relative contributions of different downstream glutamine metabolic pathways in AM proliferation and self-renewal need further investigation.

Altogether, our findings indicated that glutamine metabolism is required for optimal AM proliferation and self-renewal. Our study broadens the understanding of cellular metabolism in regulating pulmonary immune cell function and indicates that targeting glutamine metabolism may be promising to intervene in pulmonary diseases associated with impaired AM compartment and/or function such as the pulmonary alveolar proteinosis (PAP) and/or respiratory viral infection.

Limitations of the Study

One limitation of our study is that we did not measure glutamine levels in AMs in the presence or absence of DON following GM-CSF treatment. Such an experiment could help to clarify whether GM-CSF functions to increase glutamine uptake to facilitate AM proliferation.

Supplementary Materials

The following supporting information can be found at: https://www.sciepublish.com/article/pii/156.

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Author Contributions

Conception, M.W. and J.S.; Data acquisition, M.W., B.Z., T.H., R.Z.; Data analysis, M.W., C.L., C.Z., T.H., W.C., H.L and J.S; Manuscript writing, M.W. and J.S.; Reagents and manuscript editing, J.C.R., T.H., W.C., H.L., C.Z. Funding acquisition, J.S.

Ethics Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Mayo Clinic Institutional Animal Care and Use Committees (IACUC) (protocol # A00002027-16-R19).

Informed Consent Statement

Not applicable.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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