Mitochondrial pyruvate metabolism and glutaminolysis toggle steady-state and emergency myelopoiesis

Hannah A Pizzato  
*Washington University School of Medicine in St. Louis*

Yahui Wang  
*Washington University in St. Louis*

Michael J Wolfgang  
*Johns Hopkins University*

Brian N Finck  
*Washington University School of Medicine in St. Louis*

Gary J Patti  
*Washington University School of Medicine in St. Louis*

See next page for additional authors

Follow this and additional works at: [https://digitalcommons.wustl.edu/oa_4](https://digitalcommons.wustl.edu/oa_4)

Part of the Medicine and Health Sciences Commons

Please let us know how this document benefits you.

**Recommended Citation**


This Open Access Publication is brought to you for free and open access by the Open Access Publications at Digital Commons@Becker. It has been accepted for inclusion in 2020-Current year OA Pubs by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Mitochondrial pyruvate metabolism and glutaminolysis toggle steady-state and emergency myelopoiesis

Hannah A. Pizzato1,2, Yahui Wang3, Michael J. Wolfgang4,5, Brian N. Finck6,7, Gary J. Patti3,7,8, and Deepta Bhattacharya1,2,9

To define the metabolic requirements of hematopoiesis, we examined blood lineages in mice conditionally deficient in genes required for long-chain fatty acid oxidation (Cpt2), glutaminolysis (Gls), or mitochondrial pyruvate import (Mpc2). Genetic ablation of Cpt2 or Gls minimally impacted most blood lineages. In contrast, deletion of Mpc2 led to a sharp decline in mature myeloid cells and a slower reduction in T cells, whereas other hematopoietic lineages were unaffected. Yet MPC2-deficient monocytes and neutrophils rapidly recovered due to a transient and specific increase in myeloid progenitor proliferation. Competitive bone marrow chimera and stable isotope tracing experiments demonstrated that this proliferative burst was progenitor intrinsic and accompanied by a metabolic switch to glutaminolysis. Myeloid recovery after loss of Mpc2 or cyclophosphamide treatment was delayed in the absence of GLS. Reciprocally, MPC2 was not required for myeloid recovery after cyclophosphamide treatment. Thus, mitochondrial pyruvate metabolism maintains myelopoiesis under steady-state conditions, while glutaminolysis in progenitors promotes emergency myelopoiesis.

Introduction

As hematopoietic stem cells (HSCs) differentiate, they gradually lose the potential to generate specific blood cell types until the commitment to a single lineage is achieved. Since HSCs are exceedingly rare, their differentiation occurs concomitantly with a progressive expansion of downstream progenitors to ensure sufficient production of mature cells (Bryder et al., 2006). Along with a numerical expansion, downstream progenitors possess both more proliferative and differentiative capacity than HSCs (Passegué et al., 2005; Sun et al., 2014). This progressive expansion of progenitors and mature lineages is known as transit amplification. Under homeostatic conditions, transit amplification matches the rate of new cell generation with the turnover of mature cells. Because each mature lineage has its own distinct lifespan, the mechanisms that maintain homeostasis are necessarily complicated. For example, neutrophils do not survive for more than a few days, while mature B lymphocytes persist for several months (Cronkite et al., 1959; Fulcher and Basten, 1997; Mauer et al., 1960; Pillay et al., 2010). The different arms of hematopoiesis must therefore be controlled in modular and separable ways.

Feedback triggered by an acute loss of downstream cells to upstream progenitors may require a distinct response to restore homeostasis. As one example, repeated bleeding of mice triggers HSCs to proliferate and self-renew at an increased rate, leading to the restoration of bone marrow cells and increased splenocytes (Cheshier et al., 2007). Upon transfusion of RBCs in these mice, HSC proliferation returns to normal. As another example, transplantation of a small number of WT HSCs into RAG2 and IL-2 receptor common γ chain–deficient mice, which lack B, T, and natural killer (NK) cells, results in a rapid repopulation of mature lymphocytes (Bhattacharya et al., 2006). This is driven by a pronounced and selective expansion of B and T cell progenitors despite very low levels of HSC chimerism. These data suggest that HSCs and progenitors respond to fill a void left by the loss of more differentiated downstream progenitors and mature cells in a process called emergency hematopoiesis. The intrinsic mechanisms that allow cells to shift from steady-state to emergency hematopoiesis still remain to be defined (Venezia et al., 2004).

Metabolic adaption might permit hematopoietic progenitors to adjust output in response to demand. For example, a decrease
in lymphocytes occurs within hours of streptozotocin treatment (Muller et al., 2011), which ablates pancreatic beta cells and induces diabetes and high glucose levels (Rakieten et al., 1963; Arison et al., 1967; Lenzen, 2008). Yet by 4 wk after treatment with streptozotocin, lymphocyte numbers return to normal (Nagareddy et al., 2013). Similarly, mouse lymphocytes decrease after 6 wk on a high-fat diet (Luo et al., 2015) but increase after 90 d (Trottier et al., 2012). Moreover, nutrient availability varies substantially throughout the course of the day (Fickel and Sung, 2020; Potter et al., 2016; Schiller and Dorow, 1973), yet there is relatively little impact on the abundances of mature cells (Born et al., 1997; Haus et al., 1983; Lawrence et al., 1933; Sabin et al., 1927). These examples represent independent studies and are not directly comparable to each other. Nonetheless, the data hint that while severe metabolic alterations may transiently alter hematopoietic composition, progenitors may be able to adapt to these changes to recover and maintain homeostasis. Defining whether and how such an adaptation occurs requires genetic tools and a dissection of progenitor-intrinsic metabolic pathways.

Much of the work performed to date on hematopoietic metabolism has focused on the relative dependence on glycolysis versus mitochondrial functions by HSCs and progenitors. The metabolic profile of HSCs is notably different than those of lineage-committed progenitors (Agathocleous et al., 2017). HSCs generally rely on glycolysis to remain quiescent, but as differentiation proceeds, a shift to increased oxidative phosphorylation occurs to ensure bioenergetic requirements are met (Takubo et al., 2013; Yu et al., 2013; Wang et al., 2014; Maryanovich et al., 2015; Ito and Suda, 2014; Nakamura-Ishizu et al., 2020; Simsek et al., 2010; Suda et al., 2011; Ho et al., 2017). Accordingly, lineage-committed progenitors produce more reactive oxygen species than do upstream HSCs, and increasing levels of reactive oxygen species hinders HSC function (Inoue et al., 2017). HSCs generally rely on glycolysis to remain quiescent, but as differentiation proceeds, a shift to increased oxidative phosphorylation occurs to ensure bioenergetic requirements are met (Takubo et al., 2013; Yu et al., 2013; Wang et al., 2014; Maryanovich et al., 2015; Ito and Suda, 2014; Nakamura-Ishizu et al., 2020; Simsek et al., 2010; Suda et al., 2011; Ho et al., 2017). Though HSCs do still rely on mitochondrial functions (Ansó et al., 2017; Bejarano-García et al., 2016; Chen et al., 2008; Gan et al., 2010; Gurumurthy et al., 2010; Luchsinger et al., 2016; Maryanovich et al., 2012; Mortensen et al., 2011; Nakada et al., 2010; Qi et al., 2021; Umamoto et al., 2018; Vannini et al., 2016), the preponderance of evidence suggests that hematopoietic progenitors depend more heavily on oxidative phosphorylation of carbon sources to generate ATP. The specific carbon sources that fuel oxidative phosphorylation in hematopoietic progenitors in vivo have not been genetically defined, though in a broad sense glucose is essential for M-CSF–driven myelopoiesis (Karmaus et al., 2017). Assuming distinct bioenergetic requirements, it is possible that each blood lineage preferentially relies on different carbon sources and metabolic pathways.

Here, we utilized in vivo genetic models to interrogate the roles of long-chain fatty acid oxidation, glutaminolysis, and mitochondrial pyruvate import in hematopoietic homeostasis. In a striking example of homeostatic maintenance, myeloid progenitors genetically deprived of mitochondrial pyruvate not only switched to glutaminolysis, but this switch was also accompanied by a transient and rapid proliferative burst to regenerate only themselves and their mature myeloid daughters, whereas other progenitors and lineages were unaffected. While glutaminolysis is not required for the homeostatic maintenance of myeloid cells, it is generally required for emergency myelopoiesis even when mitochondrial pyruvate metabolism is available. These data demonstrate that progenitors have intrinsic metabolic sensing abilities that promote lineage-specific hematopoietic homeostasis. Additionally, the metabolic pathways utilized to maintain myeloid cells under steady-state differ from those used under situations of acute insult.

Results

Long-chain fatty acid oxidation and glutaminolysis are dispensable for most hematopoietic lineages

To define essential carbon sources that fuel the TCA cycle in hematopoietic lineages, we employed in vivo genetic ablation models. We focused on long-chain fatty acid oxidation, glutaminolysis, and mitochondrial pyruvate utilization as all these pathways are major contributors to ATP production in many other cell types. First, we used mice deficient in carnitine palmitoyltransferase 2 (Cpt2), which transports long-chain fatty acids into the inner matrix of the mitochondria for oxidation (Fig. 1 A; Houten et al., 2016; Lee et al., 2015). Because germline deletion of Cpt2 is lethal (Isackson et al., 2008; Ji et al., 2008; Longo et al., 2006; Nyman et al., 2005), we crossed Cpt2fl/fl;Rosa26 CreER animals, which constitutively express tamoxifen-inducible Cre recombinase (Ventura et al., 2007). WT CD45.1+ and Cpt2fl/fl;Rosa26 CreER+/− or+/− CD45.2+ bone marrow cells were mixed in equal proportions and transplanted into irradiated recipients (Fig. 1 B). Reconstitution was allowed to proceed for at least 8 wk before the administration of tamoxifen to ablate Cpt2 in CD45.2+ cells. This system avoids lethality following Cpt2 deletion and allows assessment of blood lineage-intrinsic phenotypes following the restoration of mature cells after irradiation.

After 2 wk of tamoxifen treatment, chimerism of hematopoietic populations in the spleen and bone marrow was assessed and normalized to the chimerism of either pre-tamoxifen blood lineages for splenic populations or HSCs for bone marrow progenitors. This normalization corrects for variability in chimerism between animals prior to tamoxifen-induced deletion, which typically ranged from 40 to 60%. Analysis of the spleen included B cells, dendritic cells (DCs), Gr1hi myeloid cells, Gr1lo myeloid cells, NK cells, and T cells (gating strategy in Fig. S1 A). Bone marrow populations included HSCs, Flk2+ multipotent progenitors (MPPs; Christensen and Weissman, 2001; Adolfsson et al., 2001), Flk2+ common myeloid progenitors (CMPs; Karsunky et al., 2003), granulocyte-macrophage progenitors (GMPs; Akashi et al., 2000; Becker et al., 2012), megakaryocyte-erythroid progenitors (MEPs; Pronk et al., 2007), and common lymphoid progenitors (CLPs; gating strategy in Fig. S1 B; Inlay et al., 2009). Despite efficient Cpt2 deletion (Fig. S1 C), we found no differences between Cpt2 KO cells and WT controls (Fig. 1 C; raw chimerism values in Fig. S2 A). This suggests that long-chain fatty acid oxidation is not required for the development or maintenance of lymphoid or myeloid cells and that other...
pathways such as glutaminolysis or pyruvate metabolism may be more important for these lineages. Prior studies have suggested an important role for glutaminolysis in human erythropoiesis (Oburoglu et al., 2014), but other lineages have not yet been examined. Glutaminase (GLS) is an aminohydrolase that catalyzes the conversion of glutamine to glutamate, which can then be converted into the TCA cycle intermediate \( \alpha \)-ketoglutarate (Fig. 1A; Curthoys and Watford, 1995). A potential compensatory enzyme, GLS2, is minimally expressed within the hematopoietic system (Heng et al., 2008). Since germline \( Gls \) deletion causes lethality (Masson et al., 2006), we generated competitive bone marrow chimeras using \( Gls^{fl/fl} \) mice (Mingote et al., 2016). Given that erythrocytes, megakaryocytes, and platelets lack CD45 expression, we generated mixed chimeras for \( Gls \) using \( \beta \)-actin GFP transgenic mice as WT competitors (Fig. 1B). GFP expression, though minimal in mature erythrocytes, can be detected in MEPs and in mature platelets (Forsberg et al., 2006; Wright et al., 2001). At the end of tamoxifen treatment, the same cell subsets examined in Cpt2-deleted chimeras were analyzed alongside platelets in the blood (gating strategy in Fig. S1, A–D). Deletion of \( Gls \) was efficient (Fig. S1 E) but did not affect lymphoid or myeloid cell chimerism (Fig. 1D; raw chimerism values in Fig. S2 B). GLS-deficient MEP chimerism was slightly reduced (about 10%, \( P < 0.05 \)) but platelets were unaffected (Fig. 1D and Fig. S2 B). Together these data demonstrate that long-chain fatty acid oxidation and glutaminolysis are largely dispensable for lymphoid and myeloid cells, while erythroid progenitors have a
Mitochondrial pyruvate is transiently required by both myeloid progenitors and mature cells

MPC1 and MPC2 are subunits of the heteromeric mitochondrial pyruvate carrier (MPC), both of which are necessary for the transport of pyruvate into the mitochondria (Fig. 1 A; Bricker et al., 2012; Herzig et al., 2012). As with Cpt2 and Gls, germline deletion of either subunit of MPC is lethal (Vigueira et al., 2014). During the course of prior work on the metabolism of antibody-secreting cells (Lam et al., 2016), we preliminarily observed that neutrophils were reduced upon Mpc2 ablation in mixed chimeras. Other lymphoid lineages were not obviously impacted, at least at short timepoints after Mpc2 deletion. To investigate further, we generated mixed bone marrow chimeras of WT CD45.1+ and CD45.2+ Mpc2+/+; Rosa26 CreER/− or CreER+/− genotypes (McComnis et al., 2015). A more comprehensive analysis was then performed compared with our earlier results to quantify other lineages, progenitors, and the kinetics of blood lineage chimerism changes following Mpc2 ablation.

After reconstitution, half of the chimeras were given tamoxifen and then sacrificed at least 10 wk later (prolonged KO; Fig. 2 A). This group allows for the assessment of longer-term effects on lymphoid lineages, which turn over infrequently. The other half was given tamoxifen 2 wk prior to euthanasia (recent KO). Consistent with our prior observations, the recent KO group was significantly reduced in neutrophil chimerism (Fig. 2, B and C). Ly6Chi and Ly6Clo monocytes were also substantially reduced immediately following Mpc2 deletion, while DCs were slightly reduced (Fig. 2, B and C). Mpc2-deficient neutrophils and monocytes were rescued by Mpc2-encoding retrovirus (Fig. S3, B–D), demonstrating that the phenotype was not due to Cre or tamoxifen toxicity. B cell chimerism was not affected in either group, whereas Mpc2-deficient T cell chimerism was diminished only in the prolonged KO group (Fig. 2 C). Yet contrary to our expectations, after prolonged deletion of Mpc2, neutrophils, Ly6Clo monocytes, and Ly6Chi monocytes all recovered to mirror WT controls (Fig. 2 C). We considered the possibility that the myeloid recovery was driven by the preferential expansion of cells that had incompletely deleted Mpc2. However, this seems unlikely given that this recovery would occur in competition against CD45.1+ WT cells. Indeed, Mpc2 remained fully deleted following prolonged deletion in both the spleen and bone marrow (Fig. S3 E).

In the recent KO group, there was a modest reduction in Mpc2-deficient GMPs and a more substantial reduction in common monocyte progenitors (CMoPs; Fig. 2, D and E; raw chimerism values in Fig. S3 F; gating strategy in Fig. S3 G; Hettinger et al., 2013). MFP and CMP chimerism remained unaffected, as did that of common DC progenitors (CDPs; Naik et al., 2007; Onai et al., 2007), MEPs, and CLPs (Fig. 2 E and Fig. S3 F). Consistent with what was observed in mature myeloid cells, progenitor chimerism was unaffected following prolonged Mpc2 deletion (Fig. 2 E and Fig. S3 F). These results suggest that MPC2 is required for the homeostasis of the myeloid lineage, but that these cells can somehow adapt over time to the loss of mitochondrial pyruvate metabolism.

Mature myeloid cells diminish then rapidly recover after Mpc2 deletion

Given that myeloid cells are initially depleted upon Mpc2 deletion but recover by 10 wk, we performed experiments to define the kinetics of this recovery. Following over 8 wk of reconstitution, Mpc2 chimeras were administered tamoxifen and then were bled every 2–3 wk to monitor donor contribution over time relative to their pre-tamoxifen chimerism. Rather than a gradual recovery, we observed a sharp increase in Mpc2-deficient myeloid cells within 2 wk after the initial nadir (Fig. 3 A; raw chimerism values in Fig. S4 A). These cells then further expanded beyond their pre-tamoxifen levels. In contrast, the reduction in Mpc2-deficient T cell chimerism was observed at 2 wk after tamoxifen and remained relatively stable throughout the duration of the experiment (Fig. 3 A). This is consistent with the requirement of MPC1 for αβ T cell development in the thymus (Ramstead et al., 2020). Similarly, pyruvate dehydrogenase is also required for thymic T cell development (Jun et al., 2021). B cells were not affected by the loss of Mpc2 at any timepoint (Fig. 3 A). We additionally examined Cpt2 and Gls chimeras over time to determine if a defect would become apparent later after deletion. Yet no defects were observed for at least 60–80 d (Fig. 3, B and C). These data suggest that myeloid cells are dependent on mitochondrial pyruvate import yet can adjust to the absence of MPC2 over time.

GMPs and CMoPs proliferate rapidly immediately following Mpc2 deletion

Given the recovery we observed in myeloid cells following the deletion of Mpc2, we next performed experiments to determine if Mpc2-deficient cells were proliferating more and/or surviving longer to facilitate this recovery. To determine if the proliferation of myeloid progenitors was altered by the loss of Mpc2, we injected chimeras with BrdU 1 h prior to sacrifice (Fig. 4 A). We then compared BrdU incorporation in the WT CD45.1+ cells with control, recent KO, or prolonged KO CD45.2+ cells within the same animals. Recent Mpc2-deficient GMPs and CMoPs proliferated significantly more than did WT GMPs and CMoPs within the same mouse, while proliferation was similar between Mpc2-deficient and -sufficient CMoPs (Fig. 4, B and C). After prolonged deletion of Mpc2, proliferation of these populations was similar to that of their WT counterparts (Fig. 4, B and C). No changes were observed in the proliferation of Mpc2-deficient HSCs, MPPs, CLPs, or mature myeloid cells (Fig. S4 B). These data demonstrate that Mpc2 deficiency triggers an adaptation in GMPs and CMoPs that is accompanied by a transient burst in proliferation. This allows for rapid regeneration of themselves and mature myeloid populations. After prolonged Mpc2 deletion, this proliferative burst subsides.

Prior studies have shown that upstream hematopoietic progenitors can sense and respond to the loss of downstream mature lineages by increasing proliferation (Boettcher and Manz, 2017; Cheshier et al., 2007; Takizawa et al., 2012). We considered the possibility that Mpc2-deficient progenitors are better at...
sensing and proliferating in response to mature cell loss than their WT counterparts. Alternatively, the initial proliferative burst in MPC2-deficient progenitors may occur cell-intrinsically, perhaps due to a metabolic switch. To distinguish between these alternatives, we generated mixed bone marrow chimeras at a 90:10 ratio of WT to MPC2 KO cells. In this setting, the absolute loss in mature myeloid cells is minimal and would not be expected to trigger feedback to upstream progenitors. We observed a similar reduction in mature myeloid cells immediately following MPC2 deletion with a full recovery 2 wk
Figure 3. Mature myeloid cells diminish then rapidly recover after Mpc2 deletion. (A) CD45.2 peripheral blood chimerism of mature cell populations was assessed every 2–3 wk in Mpc2 chimeras. Values are normalized to pre-tamoxifen chimerism of each cell type. Data are pooled from three independent experiments. Mean values ± SEM are shown. ****, P < 0.0001 by paired two-way ANOVA with post-hoc Tukey’s multiple comparisons test.

(B and C) CD45.2 peripheral blood chimerism of mature cell populations was assessed every 2 wk in Cpt2 (B) or Gls (C) chimeras. Values are normalized to pre-tamoxifen chimerism of each cell type. Mean values ± SEM are shown. Data are pooled from two independent experiments. P values >0.05 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test are not depicted.
later (Fig. S4 C). This suggests that the recovery is not due to a heightened sensing of mature cell loss but rather proliferation triggered by a cell-intrinsic metabolic switch.

We next performed a BrdU pulse-chase experiment to determine if the recovery of MPC2-deficient cells is also fueled by enhanced survival. Due to the infrequency of mature myeloid cells following the recent deletion of \textit{Mpc2}, we focused on survival in the prolonged KO group. Chimeras were administered BrdU in their drinking water for 1 wk (Fig. 4 D). At the end of the week, mice were switched back to normal drinking water and were bled to establish baseline BrdU incorporation. The mice were then bled for three consecutive days following the cessation of BrdU administration to determine cell survival time and turnover. There were no differences observed in BrdU retention over time in peripheral Ly6C\textsuperscript{hi} monocytes or neutrophils between control mice or MPC2-deficient mice after prolonged deletion (Fig. 4 E). As anticipated, B and T cell survival was also unaffected by \textit{Mpc2} deletion (Fig. S4 D). Taken together, these data suggest that the MPC2-deficient myeloid recovery is due specifically to increased myeloid progenitor proliferation fueled by a cell-intrinsic metabolic switch.
by an intrinsic metabolic switch and is not due to enhanced mature cell survival.

**GMPs use glutamine in vitro to generate TCA cycle intermediates**

The rapid proliferation of GMPs and CMoPs immediately following the loss of the MPC suggests that these progenitors switch to another carbon source to survive and expand to meet the sudden demand to produce more mature myeloid cells. Such metabolic switches have been observed in other cell types and model organisms (Bensard et al., 2020; Schell et al., 2014; Vanderperre et al., 2016; Yang et al., 2014). To begin to define an alternate carbon source for MPC2-deficient myeloid progenitors and to determine whether the switch is driven by a transcriptional adaptation, we performed RNA sequencing (RNA-seq) on GMPs from control or \( \text{Mpc2}^{\text{KO}} \) chimeras. Immediately following the deletion of \( \text{Mpc2} \) in GMPs, the expression of 149 genes was significantly different relative to WT GMPs (Fig. 5 A). Notably, \( \text{Glul} \), which converts glutamate and ammonia into glutamine (Deuel et al., 1978; Tate and Meister, 1971), was upregulated \( \sim 1.5 \)-fold in MPC2-deficient GMPs relative to control GMPs (Fig. 5 A). \( \text{Bcat1} \), which transfers \( \alpha \)-amino groups from branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) to \( \alpha \)-ketoglutarate to generate \( \alpha \)-ketoacids and glutamate (Hutson et al., 1988; Ichihara and Koyama, 1966), was also modestly upregulated in MPC2-deficient GMPs (Fig. 5 A). Yet only 17 genes, including \( \text{Mpc2} \), were significantly altered in GMPs following prolonged deletion of \( \text{Mpc2} \) (Fig. 5 B). Of the differentially expressed genes, 10, including \( \text{Bcat1} \), were shared between both recent and prolonged deletion groups (Fig. 5 C). KEGG pathway analyses were performed for both RNA-seq datasets but no pathways were identified with \( q < 0.05 \) and more than five gene hits.

Given the transcriptional upregulation of \( \text{Bcat1} \) and \( \text{Glul} \) in MPC2-deficient GMPs, we employed an in vitro differentiation assay to determine whether these progenitors switch to oxidation of glutamine, BCAAs, or other carbon sources. GMPs were sorted from WT mice and cultured with stem cell factor (SCF) and GM-CSF to induce proliferation and granulocyte and myeloid differentiation. In contrast to our in vivo data, which show a clear requirement for mitochondrial pyruvate, treatment of cultures with the MPC inhibitor UK5099 (Halestrap and Denton, 1975) had no impact on cell survival over a 7-d period (Fig. 6 A). Treatment with UK5099 did increase the amount of pyruvate excreted in the cell culture media (Fig. 6 B), demonstrating successful inhibition of MPC. For genetic confirmation of these results, we sorted GMPs from \( \text{Mpc2}^{\text{fl/fl}}; \text{CreER}^{+} \) and \( \text{CreER}^{+} \) mice and cultured them with 4-hydroxytamoxifen (4-OHT) to induce...
Mpc2 deletion in floxed cells. No differences in cell counts were observed between Mpc2-deficient and control cells despite efficient deletion of Mpc2 (Fig. 6, C and D). These results are consistent with work showing marked differences between in vivo and in vitro metabolism in other systems (Cheng et al., 2011; Davidson et al., 2016; DeBerardinis et al., 2007; Faubert and DeBerardinis, 2017; Sellers et al., 2015). Though these in vitro results did not align with our in vivo data, the system provides an opportunity to define potential energy sources used by GMPs when mitochondrial pyruvate is not used.

To define the alternate carbon sources GMPs use in place of pyruvate, we performed stable isotope 13C tracing experiments. WT GMPs were cultured in media containing different 13C carbon sources for 24 h, and liquid chromatography/mass spectrometry (LC/MS) was used to analyze mass shifts in TCA cycle intermediates. 13C-glucose, which yields pyruvate through glycolysis, did not detectably contribute to the TCA cycle of in vitro GMPs (Fig. 7), consistent with our observations that UK5099 did not affect these cultures. We then traced 13C-labeled leucine, isoleucine, and valine to determine if GMPs utilize BCAAs in the TCA cycle given the upregulation of Bcat1 in both recent and prolonged Mpc2 KO GMPs relative to WT (Fig. 5, A–C). Labeling of TCA cycle intermediates was not observed (Fig. 7). With the upregulation of Glut in Mpc2 recent KO GMPs relative to WT (Fig. 5 A), we also wanted to examine the usage of glutamine. Additionally, in a mouse model of colon cancer, Mpc1 ablation in intestinal stem cells leads to the preferential use of the long-chain fatty acid palmitate in intestinal crypts, while adenomas utilize glutamine (Bensard et al., 2020). To determine if GMPs could be utilizing either of these carbon sources, we followed 13C-labeled glutamine and palmitate. While palmitate did not detectably contribute to the TCA cycle, carbons from glutamine did contribute to the intermediates malate and aspartate (Fig. 7). No contributions were observed from extracellular pyruvate directly or from lactate or alanine, which are also sources of pyruvate (Fig. 7). Additionally, no labeling of TCA cycle intermediates was observed following culture with 13C-acetate or the remaining amino acids (Fig. 7). These data indicate that myeloid progenitors use glutamine to drive the TCA cycle when not using pyruvate, at least in vitro.

**Figure 6.** In vitro GMPs do not require the MPC for survival. (A) WT GMPs were cultured with DMSO or 10 μM UK5099 for 7 d. On each day of the culture, cells were harvested, and live cells were counted. One experiment with 10 technical replicates from two independent experiments. P values >0.05 by two-tailed t test are not depicted. (B) Pyruvate excretion rate in the media of GMPs cultured with DMSO or UK5099 over a 48-h culture. ***, P < 0.0001 by Student’s two-tailed t test. Mean values ± SEM are shown for four replicates from two independent experiments. (C) GMPs from Mpc2fl/fl;Rosa26 CreER1/− or Rosa26 CreER1/− mice were treated with 0.5 μM 4-OHT. On each day of the culture, cells were harvested, and live cells were counted. One experiment with 10 technical replicates from two mice per group is depicted as a representative of two independent experiments. P values >0.05 by Student’s two-tailed t test are not depicted. (D) Quantitative PCR analysis of genomic Mpc2 deletion in GMPs from Mpc2fl/fl;Rosa26 CreER1/− or Rosa26 CreER1/− mice. GMPs were treated with 4-OHT for 2 d prior to DNA extraction. DNA quantification within loxP sites was quantified relative to WT cells and GAPDH. Mean values ± SEM are shown for four replicates from two independent experiments. ****, P < 0.0001 by one-way ANOVA with post-hoc Tukey’s multiple comparisons test.
glutamine, whereas myeloid cells specifically require pyruvate but switch to glutaminolysis in its absence. Several DKO chimeras did not display myeloid cell loss (Fig. 8 A), likely due to inefficient deletion of \textit{Mpc2} in these animals (Fig. S5, B and C). In contrast, \textit{Gls} deletion was consistently efficient in DKO chimeras, as observed in genomic DNA from bone marrow cells and splenocytes after recent deletion (Fig. S5 B), and in RNA-seq of GMPs after prolonged deletion (Fig. S5 C).

To determine if the proliferation of myeloid progenitors was altered by the loss of both \textit{Mpc2} and \textit{Gls}, we performed a similar experiment as outlined in Fig. 4 A. Chimeras were injected with BrdU 1 h prior to sacrifice, and we assessed BrdU incorporation in both the WT CD45.1+ cells and control or recent DKO CD45.2+ cells within the same animals. Only chimeras in which \textit{Mpc2} alleles were more than 90% deleted relative to WT controls were analyzed. The proliferation of WT and DKO CMPs, GMPs, and CMoPs was similar (Fig. 8 C). Similar levels of proliferation between DKO and WT cells are consistent with the minimal recovery of mature cells following the deletion of \textit{Mpc2} and \textit{Gls} (Fig. 8 A). These data demonstrate that glutamine is required for the proliferative burst of MPC2-deficient GMPs and CMoPs.

Deletion of \textit{Gls} delays emergency myelopoiesis

We next performed experiments to determine if glutaminolysis is only utilized in the absence of mitochondrial pyruvate or if it is also important in other more physiological settings of emergency myelopoiesis, such as that induced by cyclophosphamide, a chemotherapeutic drug. After cyclophosphamide treatment,
mature myeloid cells are rapidly depleted and then recover through emergency myelopoiesis (Jacquelin et al., 2013; Park et al., 2012; Duggan et al., 2017). Control, Glu KO, and Mpc2 KO chimeras were injected with cyclophosphamide, and mature cells were measured in the periphery for up to 7 d after treatment (Fig. 9 A). We then compared the mature cell recovery in WT CD45.1+ cells with control, Mpc2 KO, or Glu KO CD45.2+ cells within the same animal. We observed a delayed recovery by GLS-deficient Ly6Chi monocytes and neutrophils relative to WT controls (Fig. 9 B). MPC2-deficient Ly6C hi monocytes and neutrophils demonstrated a similar recovery to their WT counterparts. B and T cells were also reduced following cyclophosphamide treatment, but their recovery was not affected by Glu or Mpc2 deletion (Fig. 9 B). The involvement of glutaminolysis in emergency myelopoiesis was also examined in vitro. GMPs were sorted from WT or Glu KO chimeras and cultured with SCF, GM-CSF, IL-3, and IL-6 to induce emergency myelopoiesis (Lantz et al., 1998; Weber et al., 2015; Zhan et al., 1998; Metcalf, 1980; Metcalf et al., 1986; Walker et al., 1985; Walker et al., 2008; Cutler et al., 1985; Ihle et al., 1983; Kindler et al., 1986; Metcalf et al., 1987; Romani et al., 1996; Pojda et al., 1992; Peters et al., 1997; Bernard et al., 1994; Patchen et al., 1991). Cells were additionally treated with DMSO or UK5099 to examine the role of pyruvate in emergency myelopoiesis. While UK5099 did not alter CD45.2 chimerism or cell counts in either WT or GLS-deficient cells, there was a significant decrease in GLS-deficient cells relative to WT that slightly recovered over time (Fig. 9 C). These data demonstrate glutaminolysis promotes emergency but not steady-state myelopoiesis (Fig. 1 D and Fig. 3 C).

To further test the importance of Mpc2 and/or Glu on steady-state and emergency myelopoiesis, we generated non-competitive chimeras with WT, Mpc2 KO, Glu KO, or Glu Mpc2 DKO donor bone marrow. Mice were provided tamoxifen chow to induce deletion and were monitored for signs of moribundity and survival. Severe neutropenia can cause mortality due to the inability to maintain microbial homeostasis at barrier surfaces. All Glu Mpc2 DKO chimeras showed severe signs of moribundity warranting euthanasia or died between days 7 and 9 of tamoxifen administration (Fig. 10). Mpc2 KO chimeras survived...
Figure 9. Deletion of Gls delays emergency myelopoiesis. (A) Schematic representation of cyclophosphamide experiment using Gls KO, Mpc2 KO, and Cre only control mixed bone marrow chimeras. Chimeric mice were injected with cyclophosphamide and then were bled on days 2 through 7 after treatment. (B) Peripheral blood cell counts of both CD45.1+ (WT) and CD45.2+ (WT, Gls KO, or Mpc2 KO) Ly6C<sup>hi</sup> monocytes, neutrophils, B cells, and T cells from cyclophosphamide-treated chimeras. Values are normalized to pretreatment counts of each cell type. Data are pooled from two independent experiments. Mean values ± SEM are shown. *, P < 0.05; **, P < 0.01 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test. (C) GMPs from WT or Gls KO chimeras were cultured with DMSO or 10 μM UK5099 in the presence of IL-3 and IL-6 for 7 d. On each day of the culture, cells were harvested and CD45.2<sup>+</sup> chimerism (left) and live cell counts (right) were obtained. Chimerism is normalized to the chimerism of the sorted GMPs. Cell counts are normalized to the total number of CD45.2<sup>+</sup> cells plated on day 0. Data are pooled from two independent experiments with a total of 14 replicates from four mice per group. Mean values ± SEM are shown. ***, P < 0.001 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test.
slightly longer, with animals persisting between 8 and 10 d. These data provide additional support for the importance for MPC2 in steady-state myelopoiesis. Unexpectedly, Gls KO chimeras also started dying at day 10, with just one mouse surviving at least 75 d following tamoxifen administration (Fig. 10). These data suggest that either emergency myelopoiesis is engaged relatively frequently even without intentional acute insults or that GLS is potentially required for hematopoietic effector functions that we did not test here.

**Discussion**

Hematopoietic stem and progenitor cells maintain homeostasis by matching blood lineage replenishment to the rate of mature cell loss. A failure to maintain homeostasis can lead to myeloproliferative disorders and cancer, lymphopenia and susceptibility to infection, and anemia from reduced RBCs. To maintain homeostasis, stem and progenitor cells must continuously replenish mature cells, whether due to normal turnover of downstream lineages or from a significant loss of cells due to acute insults. In these two examples, the rates of replenishment required to maintain or restore homeostasis are markedly different. The extreme loss of mature cells necessitates the rapid proliferation and differentiation of progenitors (Bhattacharya et al., 2006; Cheshier et al., 2007). However, during a steady state, distinct pathways are presumably engaged to maintain but not overproduce cells. Our results here provide support for this concept, as mitochondrial pyruvate metabolism and glutaminolysis are toggled during steady-state and emergency myelopoiesis. Ensuring that only affected lineages are expanded and restored adds an additional level of specificity and complexity to this problem.

Under steady-state conditions, a progressive switch from glycolysis to oxidative phosphorylation promotes ATP production to support transit amplification (Takubo et al., 2013; Yu et al., 2013; Wang et al., 2014; Maryanovich et al., 2015; Ito and Suda, 2014; Nakamura-Ishizu et al., 2020; Simsek et al., 2010; Suda et al., 2011; Ho et al., 2017). Here, we demonstrated that the genetic ablation of pyruvate import into mitochondria sharply reduced mature myeloid cells, but these cells recovered rapidly due to a transient proliferative burst specifically by myeloid progenitors. Interestingly, this burst was observed in GMPs and CMoPs, but not in upstream CMPs. Perhaps, earlier progenitors developmentally closer to HSCs have not fully switched to oxidative phosphorylation and can continue to rely on glycolysis for energy production. The recovery of MPC2-deficient myeloid cells occurred in competition with WT cells, demonstrating that this proliferative burst was intrinsic to cells devoid of mitochondrial pyruvate import. The burst was lineage specific and only transient. Thus, MPC2-deficient myeloid cells did not continue to outcompete their WT competitors at later time points, which otherwise would have led to a myeloproliferative disorder. Moreover, an accompanying burst in multipotent and lymphoid progenitors, which might have led to an overproduction of irrelevant lineages, was not observed. These findings are consistent with prior studies showing relatively stable numbers of circulating mature cells despite marked changes in nutrient availability, altered by circadian rhythms and diet (Pickel and Sung, 2020; Potter et al., 2016; Schlief and Dorow, 1973). These data further highlight the precise control progenitors have in maintaining homeostasis.

While the proliferative burst in progenitors promotes myeloid homeostasis after Mpc2 ablation, stopping this burst following myeloid recovery would seem equally as important. One possible mechanism is that MPC2-deficient progenitors briefly switch to lactate production due to increased cytosolic pyruvate. This has been shown to occur in tumors as part of the Warburg effect, leading to the regeneration of nicotinamide adenine dinucleotide (NAD\(^+\)), which in turn enables repeated cycles of glycolysis and biomass production to enable rapid proliferation (DeBerardinis et al., 2008; Vander Heiden et al., 2009; Warburg, 1925). Yet once MPC2-deficient progenitors switch to glutaminolysis, feedback mechanisms such as citrate-mediated inhibition of phosphofructokinase activity (Garland et al., 1963; Poorman et al., 1984) might shut down this increased glycolysis and normalize the proliferative rate. These changes are difficult to directly observe in vitro, as demonstrated by our experiments in which mitochondrial pyruvate was minimally utilized even by untreated WT myeloid progenitors. Yet consistent with this possible mechanism, deletion of lactate dehydrogenase A permanently impairs proliferation of hematopoietic progenitors in vivo, as does loss of the M2 isoform of pyruvate kinase (PKM2), which limits the initial formation of pyruvate (Wang et al., 2014). When we ablated both Gls and Mpc2, the recovery of mature myeloid cells was significantly impaired, even though deletion of Gls alone had no discernable effect. This suggests that glutaminolysis is functionally required following acute loss of myeloid cells and/or for rapid progenitor proliferation but not steady-state myelopoiesis. Reciprocally, MPC2 is required for steady-state but not emergency myelopoiesis. Relevant to this point, many malignancies engage Warburg metabolism, which promotes conversion of pyruvate to lactate rather than oxidation in the mitochondria. Indeed, the removal of glutamine from

**Figure 10.** Full chimeras do not survive deletion of Mpc2 and/or Gls. Full chimeras were generated with bone marrow from WT (ROSAs26 CreER\(^{+/−}\); Mpc2 KO (Mpc2\(^{−/−}\); ROSAs26 CreER\(^{+/−}\)), Gls KO (Gls\(^{−/−}\); ROSAs26 CreER\(^{+/−}\)), or Gls Mpc2 DKO (Gls\(^{−/−}\); Mpc2\(^{−/−}\); ROSAs26 CreER\(^{+/−}\)) donors. Chimeras were provided tamoxifen chow for 14 d, and severe moribundity and survival were monitored over time. The single WT mouse that died was found in a flooded cage, suggesting an unrelated death. Data are pooled from two independent experiments. ***, P < 0.01; ****, P < 0.0001 by log-rank test.
cultures, knocking down glutamine transporters, and pharmacologically inhibiting GLS in acute myeloid leukemia reduce leukemic cell proliferation while increasing apoptosis (Gallipoli et al., 2018; Jacque et al., 2015; Willems et al., 2013). Our genetic data here demonstrate that glutaminolysis inhibition does not impact normal progenitors under steady-state conditions, suggesting that such treatments are worth exploring for the treatment of disorders such as acute myeloid leukemia.

While our data demonstrate an important role for glutamine in the absence of mitochondrial pyruvate in myeloid cells, the ablation of both Gls and Mpc2 did not eliminate these populations and a slight recovery from the initial decline was observed. Additionally, in cyclophosphamide-induced myeloablation experiments, GLS-deficient myeloid cells had a delayed recovery relative to controls but still did return to pretreatment levels. Thus, there might be additional carbon sources that promote recovery. For example, while MPC1 deficiency in mice leads to lethality around embryonic day 13.5, administering pregnant dams a ketogenic diet restores embryonic development (Vanderperre et al., 2016). Additionally, ablation of Mpc1 in the intestinal epithelium of mice leads to increased palmitate oxidation relative to WT controls in ex vivo intestinal crypts (Bensard et al., 2020). Similarly, although myeloid progenitors primarily switch to glutaminolysis in the absence of mitochondrial pyruvate, we have previously observed that MPC2-deficient neutrophils can be rescued by retroviral transduction with a long-chain fatty acid transporter (Lam et al., 2016; Schaffer and Lodish, 1994). Although we did not observe contributions from palmitate in vitro, preliminary experiments showed a selection against cells that had deleted both Cpt2 and Mpc2 in DKO chimeras. These data suggest a possible role for long-chain fatty acid oxidation to complement MPC2 deficiency in vivo. Additionally, while we did not trace carbons from BCAAs to TCA cycle intermediates in vitro, RNA-seq revealed an upregulation of Bcat1 in MPC2-deficient GMPs relative to WT, suggesting BCAAs may also be utilized.

When we depleted mature myeloid cells with cyclophosphamide in chimeras to observe the role of metabolic pathways in emergency myelopoiesis, GLS-deficient myeloid cells had a delayed recovery relative to controls. Given that glutaminolysis is dispensable for homeostatic myelopoiesis, these data suggest that different metabolic pathways are required to maintain cells at a steady state and to promote recovery following an acute insult. This difference in metabolic pathways utilized could be connected to factors that differ between steady-state and emergency myelopoiesis, such as cytokines. GM-CSF, G-CSF, and M-CSF promote survival, proliferation, and myeloid differentiation. Following systemic infection, which commonly induces emergency hematopoiesis or in the context of myeloproliferative disorders, the concentrations of these cytokines in the serum are up to 100-fold higher than those found in the steady state (Hamilton, 2008; Kawakami et al., 1990; Selig and Nothdurft, 1995; Watari et al., 1989). Thus, the in vitro experiments we performed with progenitors may have been more representative of emergency myelopoiesis, thereby explaining why we did not observe any functional contribution of mitochondrial pyruvate metabolism. With the addition of IL-3 and IL-6 to cultures to further induce emergency myelopoiesis, we again did not observe a phenotype with MPC inhibition. We did, however, see reduced proliferation of GLS-deficient cells relative to controls. Differences in the levels of these cytokines might mediate metabolic switches during steady-state and emergency myelopoiesis. Similarly, proliferation-terminating cytokines, such as transforming growth factor β1 (TGFβ1), might act through metabolic pathways to enforce relative quiescence (Hérault et al., 2017).

Nonetheless, the proliferation of myeloid progenitors triggered by the loss of MPC2 was initiated cell-intrinsically rather than by feedback from loss of mature cells. These data suggest the ability of progenitors to adjust in anticipation of the loss of their mature progeny. In the example we provide here, this adaptation is triggered by a metabolic switch that more dramatically impacts mature cells than the upstream progenitors. In this setting, glutaminolysis is again required for rapid recovery. Together, the data suggest that pyruvate metabolism and glutaminolysis serve as general metabolic toggles to switch between steady-state and emergency myelopoiesis.

Materials and methods

Mice

All animal procedures used in this study were approved by the Animal Care and Use Committees at Washington University in St. Louis and the University of Arizona. C57BL6/N and B6.Ly5.2 (CD45.1) mice were obtained from Charles River Laboratories. β-Actin GFP transgenic mice were obtained from The Jackson Laboratory (stock number 006567). Mpc2fl/fl mice were kindly provided by B.N. Finck (McCommis et al., 2015; currently available from The Jackson Laboratory, stock number 032118). Cpt2fl/fl mice were kindly provided by M.J. Wolfgang (Lee et al., 2015). Glsfl/fl mice were obtained from The Jackson Laboratory (stock number 01894; Mingote et al., 2016). All floxed mouse lines were crossed to ROSA26 CreER mice obtained from The Jackson Laboratory (stock number 008463). Littermates were used when possible.

Mixed bone marrow chimeras

To generate mixed bone marrow chimeras, bone marrow cells from a CD45.2 donor mouse carrying floxed alleles were mixed 1:1 or 1:9 with bone marrow cells from B6.Ly5.2 CD45.1 mice (or β-actin GFP transgenic mice for Gls chimeras). Full chimeras were generated using only bone marrow cells from CD45.2 donor mouse. 10 × 10⁶ total mixed bone marrow cells were injected retro-orbitally into 800 cGy-irradiated B6.Ly5.2 CD45.1 mice. Recipient mice were administered Sulfatrim (40 mg/ml sulfamethoxazole and 8 mg/ml trimethoprim) via drinking water for 2 wk. Chimeras were rested for at least 8 wk to allow reconstitution. Peripheral blood chimerism was assessed through tail venipuncture prior to tamoxifen administration. To induce deletion, tamoxifen was administered in their diet (400 mg ta-moxifen citrate/kg diet; Envigo) for 2 wk.

For Mpc2 retroviral chimeras, bone marrow cells were c-kit–enriched using CD117 microbeads (Miltenyi Biotec), and 50,000–100,000 cells were cultured in a 96-well plate in
DMEM/F12 supplemented with 10% FBS, HEPEs, non-essential amino acids, sodium pyruvate, glutamine, 50 µM β-mercaptoethanol (Gibco), 50 ng/ml SCF (Peprotech), and 50 ng/ml thrombopoietin (Peprotech) at 37°C with 5% CO₂ (Lam et al., 2016). The following day, 5 µl of Mpc2 retrovirus was added to each well. A day after infection, cells were mixed 1:1 with WT CD45.1 cells (similarly cultured but not infected) and transplanted as described above. For retrovirus production, Mpc2 constructs were cloned into a murine stem cell virus–IREs plasmid. Lenti-X 293Ts (Clontech) were transfected at about 60% confluency in a 10 cm² tissue culture dish using 30 µl FuGene HD (Promega), 1.75 µg pMD2.G vector, 3.25 µg pBS CMV-gag-pol (Addgene), and 5 µg Mpc2 retroviral vector. 4–6 h after transfection, the medium was changed. At both 48 and 72 h later, the supernatant was collected and filtered through 0.45 µm syringe filters. 3 ml of 1× PBS/25% polyethylene glycol 8000 (Sigma-Aldrich) was mixed with 12 ml of the harvested viral supernatant and then incubated at 4°C. The following day, the mixture was spun down at 3,000 ×g at 20°C until the time of use. Aliquots were prepared and then stored at −80°C until the time of use.

**Mouse tissue processing**

Spleens were harvested and dissociated using frosted glass microscope slides. Femurs, tibiae, and pelvic bones were isolated and crushed with a mortar and pestle. RBCs were lysed using a croscope slides. Femurs, tibiae, and pelvic bones were isolated and suspended the pellet. Aliquots were prepared and then stored at −80°C until the time of use. DAPI and propidium iodide were purchased from Sigma-Aldrich. Cells were analyzed on a BD LSR II, BD Fortessa, BD FacsVerse, or Cytake Aurora. All fluorescence-activated cell sorting was performed on a BD FACS Aria II or III. Data were analyzed using Flowjo software (FlowJo Enterprise).

**Quantitative PCR analysis**

To quantify the deletion of Cpt2, Gls, and Mpc2, 50,000–150,000 CD45.2+ cells were sorted from the spleen, bone marrow, or peripheral blood of chimeras into PBS with 5% adult bovine serum. DNA was isolated using the Genomic DNA Mini Kit (IBI Scientific). Quantitative PCR amplification was performed using a region of GAPDH (forward primer: 5'-AACCTTGGGATTGAGAACG-3' and reverse primer 5'-GATGAGGATGATGTGCTT-3'). Primers for Cpt2, Gls, and Mpc2 amplified a region within the loxP sites. The Cpt2 forward primer was 5'-GATGGTGAAGTGTCCTAAC-3' and the reverse was 5'-GCGAGACCGATGTGTTCT-3'. The Gls forward primer was 5'-ATCTCCTGCGCTGCTGCTG-3' and the reverse was 5'-GGAGACTGGATCTTCTT-3'. The Mpc2 forward primer was 5'-CCCCACCGTGTCTTAAATGTGC-3' and the reverse was 5'-CAGACTGAGCTAGCTGAGTGC-3'. PCR reactions were performed in 20 µl containing SYBR Green PCR Master Mix (Applied Biosystems) and 10 µl of each primer. Reactions were performed in a StepOnePlus real time qPCR machine (Applied Biosystems) with 40 cycles of the following program: 94°C, 30 s; 59.1°C, 30 s; 72°C, 30 s; followed by a melt-curve analysis. Quantification was calculated using normalized ΔCt values. An average from WT samples was used to estimate a value of 1.

**BrdU pulse and pulse-chase experiments**

For BrdU pulse experiments, mice were injected with 1 mg BrdU intraperitoneally 1 h prior to sacrifice. For BrdU pulse-chase experiments, mice were provided with 0.8 mg/ml BrdU in their drinking water, replaced daily for 7 d. At the end of the 7 d, mice were returned to normal drinking water and were bled that day and the following 3 d. Cells were then processed and
stained for BrdU incorporation with the FITC BrdU Flow Kit (BD) per the manufacturer’s instructions.

**RNA-seq**

Single-cell suspensions of bone marrow cells were generated as per the tissue processing section. CD27<sup>+</sup> cells were enriched using APC microbeads (Miltenyi Biotec). CD45.2<sup>+</sup> GMPs were sorted from WT (ROSA26 CreER<sup sesso</sup>), Mpc2, Gls, and Gls Mpc2 KO chimeras at the end of tamoxifen treatment or about 10 wk after tamoxifen treatment into Buffer RA1 (Macherey-Nagel). RNA was isolated using the NucleoSpin RNA XS kit (Macherey-Nagel). Sequencing libraries were generated by Novogene using the SMARTseq V4 kit (Takara Bio). Paired-end 150 bp reads (100 bp) were acquired by Novogene using Illumina NovaSeq. For the SMARTseq V4 kit (Takara Bio). Paired-end 150 bp reads (100 bp) were acquired by Novogene using Illumina NovaSeq. For Reads were mapped to Gencode’s M27 annotation files, and transcript abundances were calculated using Salmon (Patro et al., 2017). DESeq2 was used to quantify differentially expressed genes (Love et al., 2014). To analyze exon usage, fastq files were first aligned to the mm10 genome using HISAT2 (Kim et al., 2015). HISAT2 bam files were then visualized using Integrative Genomics Viewer (Thorvaldsdóttir et al., 2013) and used to quantify transcripts per kilobase million (TPM) for floxed exons via DEXSeq (Anders et al., 2012). RNA-seq data are available in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO; recent KO accession number GSE225578 and prolonged KO accession number GSE184548). Venn diagrams were generated at https://www.meta-chart.com/venn/#/data.

**GMP cultures**

Single-cell suspensions of bone marrow cells were generated as per the tissue processing section. C-kit<sup+</sup> cells were enriched using CD117 microbeads (Miltenyi Biotec). GMPs were sorted into RPMI containing 10% FBS, glutamine, and penicillin/streptomycin. 50,000–100,000 GMPs were cultured for pyruvate excretion analysis or 13C tracing experiments, while 1,000 vehicle control or to inhibit MPC. When GMPs were obtained for metabolic requirements of myelopoiesis https://doi.org/10.1084/jem.20221373

For palmitate, 100 μM UK5099 (Sigma-Aldrich) was added to the medium as a control. Palmitate-BSA was added. For pyruvate, 100 μM 13C-palmitate-BSA and 100 μM oleate-BSA was added. For pyruvate, 100 μM 13C-pyruvate was supplemented. Concentrations used for all other 13C tracing experiments were as follows: 3 mM 13C-lactate, 400 μM 13C-alanine, 500 μM 13C-acetate, 430 μM 13C-asparagine, 382 μM 13C-leucine, 382 μM 13C-isoleucine, 171 μM 13C-valine, 220 μM 13C-lysine, 91 μM 13C-phenylalanine, 60 μM 13C-tyrosine, 336 μM 13C-cysteine, 133 μM 13C-methionine, 286 μM 13C-serine, 136 μM 13C-glutamate, 1.15 mM 13C-arginine, 97 μM 13C-histidine, 174 μM 13C-proline, 101 μM 13C-methionine, 168 μM 13C-threonine, 91 μM 13C-phenylalanine, 110 μM 13C-tyrosine, and 150 μM 13C-aspartate.

**Isotope labeling and metabolite extraction**

GMPs were sorted and cultured as described above. GMPs were sorted and cultured as described above. Cells were collected on day 4 of culture, washed with PBS, and replated in the appropriate media (listed below) for 24 h. Following incubation, cells were collected, washed with PBS, washed with high-performance liquid chromatography (HPLC)–grade water, and quenched with HPLC-grade methanol. Pellets were dried using a SpeedVac. Dried cell pellets were extracted with 1 ml methanol/ACN/water (2:2:1). Samples were vortexed for 30 s, incubated for 1 min in liquid nitrogen, and then sonicated for 10 min. This process was repeated three times. After a 1-h incubation at −20°C, samples were centrifuged at 14,000 rpm for 10 min. The supernatant was collected and dried via SpeedVac and reconstituted in ACN/water (1:1) for LC/MS analysis.

All U-13C carbon sources were obtained from Cambridge Isotope Laboratories. For all 13C experiments, the base medium was RPMI containing 10% FBS, glutamine, penicillin/streptomycin, 10 ng/ml GM-CSF, and 10 ng/ml SCF. When GMPs were obtained from Mpc2fl/fl;ROSA26 CreER<sup sesso</sup>/− Gls, and ROSA26 CreER<sup sesso</sup>/+ mice, 0.5 μM 13C-alanine, 500 μM 13C-acetate, 430 μM 13C-asparagine, 382 μM 13C-leucine, 382 μM 13C-isoleucine, 171 μM 13C-valine, 220 μM 13C-lysine, 91 μM 13C-phenylalanine, 60 μM 13C-tyrosine, 336 μM 13C-cysteine, 133 μM 13C-methionine, 286 μM 13C-serine, 136 μM 13C-glutamate, 1.15 mM 13C-arginine, 97 μM 13C-histidine, 174 μM 13C-proline, 101 μM 13C-methionine, 168 μM 13C-threonine, 91 μM 13C-phenylalanine, 110 μM 13C-tyrosine, and 150 μM 13C-aspartate.

**LC/MS analysis**

Media samples and cell extracts were analyzed with Agilent 1290 UHPLC system coupled to an Agilent 6540 quadrupole time-of-flight mass spectrometer. Polar metabolites were separated on a
Cyclophosphamide experiments
Mice were injected with 175 mg/kg cyclophosphamide (Sigma-Aldrich) in PBS intraperitoneally (Duggan et al., 2017). Prior to treatment and on days 2–7 after treatment, mice were bled as described above into dipotassium EDTA-coated tubes (BD). Blood samples were analyzed on an Element HTS (Heska) to obtain white blood cell counts. Flow cytometry was used as described above to determine the frequency of each cell population. Cell frequencies were applied to white blood counts to obtain a cell count for each population of interest. Cell counts for each day were then normalized to pretreatment cell counts.

Statistical analysis
All statistical analyses were conducted using GraphPad Prism and are detailed in each figure legend. Figures were created with BioRender.com.

Online supplemental material
Fig. S1 shows the general flow cytometry gating strategy for cell type identification used throughout the article and confirms the deletion of Cpt2 and Gls. Fig. S2 shows the raw chimera values for Cpt2 and Gls KO chimeras. Fig. S3 shows the flow cytometry gating strategy used for additional myeloid subsets, demonstrates Mpc2 retroviral rescue of Mpc2-deficient myeloid cells, confirms deletion of Mpc2, and includes raw chimera values for Mpc2 KO chimeras. Fig. S4 includes raw chimera data for Mpc2 KO chimera bleeds over time, BrdU pulse, and pulse-chase data for additional cell types, as well as the kinetics of mature myeloid cell loss and recovery in mixed bone marrow chimeras with 90% WT and 10% Mpc2 KO cells. Fig. S5 shows raw chimera values for Gls Mpc2 DKO chimeras and the extent of deletion of Gls and Mpc2 in these chimeras.

Data availability
RNA-seq data from Fig. 5 are available in the NCBI GEO. Data for recent and prolonged MPC2-deficient GMPs are under accession numbers GSE225578 and GSE184548, respectively. All other data are available in the article or upon reasonable request to the corresponding author.

Acknowledgments
This work was funded by the National Institutes of Health grants RO1AI29945 (D. Bhattacharya), R3ES2028365 (G.J. Patti), and RO1DK116746 (M.J. Wolfgang). The development of Mpc2-floxed mice was supported by National Institutes of Health grant R01 DK104735 (B.N. Finck). Open Access funding provided by the University of Arizona.

Author contributions: H.A. Pizzato and D. Bhattacharya conceptualized this study with D. Bhattacharya being the project administrator. The bulk of experiments were performed by H.A. Pizzato with assistance in both methodology and formal analysis by D. Bhattacharya in addition to supervision. Mass spectrometry experiments were performed by Y. Wang with assistance in methodology and formal analysis as well as supervision by G.J. Patti. Resources and funding were acquired by M.J. Wolfgang, B.N. Finck, G.J. Patti, and D. Bhattacharya. The original draft was written by H.A. Pizzato and D. Bhattacharya, with all other authors reviewing and editing the manuscript.

Disclosures: H.A. Pizzato reported “other” from Sana Biotechnology outside the submitted work. B.N. Finck reported “other” from Cirius Therapeutics during the conduct of the study. G.J. Patti reported a collaborative research agreement with Agilent Technologies and Thermo Fisher Scientific and a financial relationship with Panome Bio. D. Bhattacharya reported personal fees from Clade Therapeutics, GlaxoSmithKline, Gilead Sciences, and Sana Biotechnology outside the submitted work. No other disclosures were reported.

Submitted: 10 August 2022
Revised: 23 March 2023
Accepted: 10 May 2023

References
and carbamyl phosphate. *Proc. Natl. Acad. Sci.* USA. 68:781-785. [https://doi.org/10.1073/pnas.68.4.781](https://doi.org/10.1073/pnas.68.4.781)


Figure S1. Gating strategy and quantification of deletion of Cpt2 and Gls. (A) Representative flow cytometry gating strategy for splenic populations. (B) Representative flow cytometry gating strategy for bone marrow populations. (C) Quantitative PCR analysis of genomic Cpt2 deletion. CD45.2+ cells from the bone marrow or spleen were sorted for genomic DNA extraction. DNA quantification within loxP sites was quantified relative to WT cells and GAPDH. Mean values ± SEM are shown. Data are pooled from two independent experiments. ****, P < 0.0001 by Student’s two-tailed t test. (D) Representative flow cytometry gating strategy for platelets in the blood. (E) Quantitative PCR analysis of genomic Gls deletion. CD45.2+ GFP− cells from the bone marrow or spleen were sorted for genomic DNA extraction. DNA quantification within loxP sites was quantified relative to WT cells and GAPDH. Mean values ± SEM are shown. Data are pooled from two independent experiments. ****, P < 0.0001 by Student’s two-tailed t test.
Figure S2.  **Raw chimerism frequencies for Cpt2 and Glu chimeras.** (A) Raw chimerism values for Cpt2 chimeras shown in Fig. 1 C. Each symbol represents an individual mouse. Mean values ± SEM are shown, and data are pooled from three independent experiments. P values >0.05 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test are not depicted.  (B) Raw chimerism values for Glu chimeras shown in Fig. 1 D. Each symbol represents an individual mouse. Mean values ± SEM are shown, and data are pooled from two independent experiments. P values >0.05 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test are not depicted.
Figure S3. **Gating strategy, quantification of deletion of Mpc2, and Mpc2 retroviral rescue of MPC2-deficient myeloid cells.** (A) Representative flow cytometry gating strategies for splenic Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes and neutrophils. (B) Schematic representation of Mpc2 retroviral bone marrow chimera experiments to assess ability to rescue Mpc2 KO (+tam) or control (no tam) Ly6C<sup>lo</sup> monocytes and neutrophils. C-kit<sup>+</sup> cells were enriched from the bone marrow of Mpc2<sup>fl/fl;ROSA26 CreER<sup>+</sup>−</sup> mice and transduced with Mpc2 retrovirus prior to transplantation alongside bone marrow from WT mice into irradiated recipients. (C) Representative flow cytometry plots of Mpc2 retrovirus (RV) expression in HSCs, Ly6C<sup>hi</sup> monocytes, and neutrophils of an individual control or KO mouse. (D) Mpc2 transduction in HSCs paired with Ly6C<sup>hi</sup> monocytes (top) or neutrophils (bottom) from the same mouse (n = 6 for untreated and n = 4 for tamoxifen). Data are pooled from two independent experiments. *, P < 0.05; **, P < 0.01 by Student’s two-tailed paired t test. (E) Quantitative PCR analysis of genomic Mpc2 deletion in Mpc2 chimeras. CD45.2<sup>+</sup> cells from the bone marrow or spleen were sorted for genomic DNA extraction. DNA quantification within loxP sites was quantified relative to WT cells and GAPDH. Mean values ± SEM are shown. Data are pooled from two independent experiments. ****, P < 0.0001 by one-way ANOVA with post-hoc Tukey’s multiple comparisons test. (F) Raw chimerism values for Mpc2 chimeras shown in Fig. 2E. Each symbol represents an individual mouse. Mean values ± SEM are shown, and data are pooled from three independent experiments. ****, P < 0.0001 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test. (G) Representative flow cytometry gating strategies for CDPs and CMoPs in the bone marrow.
Figure S4. Proliferation, survival, and recovery of MPC2-deficient cells. (A) Raw chimerism values for peripheral myeloid cells of Mpc2 chimeras in Fig. 3A. Data are pooled from three independent experiments. Mean values ± SEM are shown. ****, P < 0.0001 by paired two-way ANOVA with post-hoc Tukey’s multiple comparisons test.

(B) BrdU incorporation was measured in both CD45.1+ (WT) and CD45.2+ (WT, recent Mpc2 deletion, or prolonged Mpc2 deletion) HSCs, MPPs, CLPs, Ly6C+ monocytes, and neutrophils following a 1-h pulse of BrdU. Each line connects CD45.1+ and CD45.2+ cells within the same mouse (n = 11 for WT chimeras and n = 19 for recent and prolonged KO chimeras). Data are pooled from two independent experiments. P values >0.05 by paired two-way ANOVA with post-hoc Sidak’s multiple comparisons test are not depicted.

(C) Mpc2 chimeras were setup at a ratio of 90% WT to 10% Mpc2fl/fl;ROSA26CreER−/− or CreER−/− bone marrow cells (as opposed to the 1:1 ratio in Fig. 3). CD45.2 peripheral blood chimerism of Ly6C+ monocytes and neutrophils was assessed every 2 wk in Mpc2 chimeras. Values are normalized to pre-tamoxifen chimerism of each cell type. Mean values ± SEM are shown. ****, P < 0.0001 by paired two-way ANOVA with post-hoc Tukey’s multiple comparisons test.

(D) BrdU incorporation was measured in peripheral CD45.2+ B and T cells for 3 d following a week of BrdU water administration. Mean values ± SEM are shown. Data are pooled from two independent experiments. P values >0.05 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test are not depicted.
Figure S5. **Quantification of efficiency of Gls and Mpc2 deletion.** (A) Raw chimerism frequencies for Gls Mpc2 chimeras in Fig. 8 B. Each symbol represents an individual mouse. Mean values ± SEM are shown. Data are pooled from two independent experiments. ***, P < 0.001; ****, P < 0.0001 by two-way ANOVA with post-hoc Tukey’s multiple comparisons test. (B) Quantitative PCR analysis of genomic Gls and Mpc2 deletion in recent Gls Mpc2 KO chimeras. CD45.2+ cells from the bone marrow or spleen were sorted for genomic DNA extraction. DNA quantification within loxP sites was quantified relative to WT cells and GAPDH. Mean values ± SEM are shown. Data are pooled from two independent experiments. ****, P < 0.0001 by Student’s two-tailed t test. (C) RNA-seq reads across exons 1–3 of Gls (top) and 2–5 of Mpc2 (bottom) of CD45.2+ GMPs from ROSA26 CreER+/- (WT), Mpc2 KO, Gls KO, or Gls Mpc2 DKO chimeras about 10 wk after tamoxifen treatment. Exon 1 of Gls and exon 3 of Mpc2 are floxed in the appropriate genotyped mice. One representative trace for each genotype is shown using Integrative Genomics Viewer (left). TPMs were quantified for the floxed exon of Gls (top right) or Mpc2 (bottom right). TPMs were calculated from reads obtained using DEXSeq. Each data point represents a mouse (n = 5 for each genotype). Mean values ± SEM are shown. ***, P < 0.001; ****, P < 0.0001 by one-way ANOVA with post-hoc Tukey’s multiple comparisons test.