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Analysis of Leukemia Cell Metabolism through Stable Isotope Tracing in Mice

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[Abstract] Once thought to be a mere consequence of the state of a cell, intermediary metabolism is now recognized as a key regulator of mammalian cell fate and function. In addition, cell metabolism is often disturbed in malignancies such as cancer, and targeting metabolic pathways can provide new therapeutic options. Cell metabolism is mostly studied in cell cultures *in vitro*, using techniques such as metabolomics, stable isotope tracing, and biochemical assays. Increasing evidence however shows that the metabolic profile of cells is highly dependent on the microenvironment, and metabolic vulnerabilities identified *in vitro* do not always translate to *in vivo* settings. Here, we provide a detailed protocol on how to perform *in vivo* stable isotope tracing in leukemia cells in mice, focusing on glutamine metabolism in acute myeloid leukemia (AML) cells. This method allows studying the metabolic profile of leukemia cells in their native bone marrow niche.

Keywords: Cell metabolism, Metabolic tracing, Cancer biology, Mouse models, Leukemia, Glutamine

[Background] Unraveling the molecular regulation of cancer cells is critical to understand their insurgence, functioning, and potential therapeutic vulnerabilities. A dysregulated cellular metabolism is now widely accepted as a hallmark of many cancers (DeBerardinis and Chandel, 2016). Cancer cells display considerably different metabolic requirements compared to most normal differentiated cells and reprogram pathways controlling nutrient acquisition and metabolism to meet their specific bioenergetic, biosynthetic, and redox demands. Emerging evidence, mostly from solid tumors, shows that several metabolic characteristics of cancer cells can also be linked to therapeutic resistance (Zhao *et al.*, 2013). Metabolic heterogeneity between cells, driven by genetic, cell state, or microenvironmental diversity, may further allow subpopulations of cells to escape therapy-related cell death (Martinez-Outschoorn *et al.*, 2016).

The importance of examining the metabolism of cells in their native environment is underscored by several studies that have shown that artificial *in vitro* conditions can create metabolic dependencies that do not exist *in vivo* (Davidson *et al.*, 2016; Cantor *et al.*, 2017; Muir *et al.*, 2017). However, studying metabolism in an *in vivo* setting brings several challenges. Gene or protein levels of metabolic enzymes may not always adequately reflect the metabolic state of a cell (Metallo and Vander Heiden, 2013). Measuring single metabolites in cells *in vivo* or on tissue sections is feasible, as is metabolic profiling of

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freshly-isolated cells through metabolomics analysis (Arnold *et al.*, 2015; Faubert and DeBerardinis, 2017). These approaches have proven extremely valuable, but they provide a snapshot of metabolite levels and no dynamic information or insight into the flow of metabolites. Metabolic tracer analysis is a complementary method that uses stable isotope-labeled metabolites, which are taken up and metabolized by the cells, after which the fate of the labeled atoms (usually carbon, nitrogen, or hydrogen) can be analyzed by mass spectrometry. In this way, the relative activity of one or more selected metabolic pathways can be studied. Metabolic tracer analysis, as well as metabolic flux analysis, which combines metabolic tracer analysis with tracer uptake data and computational modeling, has become a standard technique in the study of cancer cell metabolism, offering insights into the relations between cell biology and biochemistry (Antoniewicz, 2018). While metabolic tracing is mainly performed with cells in culture, it is also more and more used in *in vivo* settings, both in animal models and in humans (Faubert and DeBerardinis, 2017; Fernández -García *et al.*, 2020). Most *in vivo* metabolic tracing studies have focused on solid tumors, analyzing the tumor as a whole, without separating the cancer cells from other cells of the tumor microenvironment, such as immune cells, stromal cells, or blood vessel cells. The advantage of working with liquid tumors is that the cancer cells can be readily obtained from the bone marrow without the need for enzymatic tissue digestion and can be separated from the other cells by fluorescence-activated cell sorting (FACS), thus allowing specific analysis of cancer cell metabolism *in vivo*.

In our original study, we investigated the metabolic adaptations of acute myeloid leukemia (AML) cells *in vivo* during the course of induction chemotherapy treatment and identified transient changes in glutamine levels (van Gastel *et al.*, 2020). We performed *in vivo* stable isotope tracing, according to the protocol described here, to understand the metabolic fate of glutamine in control AML cells or cells persisting after chemotherapy. We used a transplant-based mouse model of human AML, driven by the MLL-AF9 fusion protein (Corral *et al.*, 1996). The AML cells also expressed GFP, which greatly facilitates cell sorting. The protocol described below should be compatible with any mouse leukemia model in which the leukemia cells express a fluorescent marker. It is also possible to perform metabolic tracing using our protocol in other hematopoietic cell populations, such as normal hematopoietic progenitor cells (Lineage^{-}cKit^{+}), as long as they can be readily isolated from bone marrow and separated from other cells by FACS.

Materials and Reagents

1. 5 ml round bottom polystyrene test tube with cell strainer snap cap (Corning, Falcon, catalog number: 352235)
2. 50 ml centrifuge tube, conical bottom (Corning, Falcon, catalog number: 352070)
3. 40 µm cell strainer (Corning, Falcon, catalog number: 352340)
4. 300 µl insulin syringe with 31 G needle (BD Biosciences, catalog number: 328440)
5. 1 ml syringe (VWR, catalog number: 612-0106)
6. Needle, 25 G × 5/8 (BD Biosciences, catalog number: 305122)

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7. K2-EDTA Microtainer tubes (BD Biosciences, catalog number: 365974)
8. 1.7 ml Posi-Click microcentrifuge tubes (Denville, catalog number: C-2170)
9. Alcohol preparatory pads (Covidien, WEBCOL, catalog number: 89029-964)
10. LC/MS sample vials (ThermoFisher, catalog number: C4000-11)
11. LC/MS vial caps (ThermoFisher, catalog number: C5000-54B)
12. ZORBAX Extend-C18, 2.1 × 150 mm, 1.8 µm (Agilent, catalog number: 759700-902)
13. L-glutamine, $^{13}C_5$, 99% (Cambridge Isotope Laboratories, catalog number: CLM-1822-H)
14. L-glutamine, $^{15}N_2$, 98% (Cambridge Isotope Laboratories, catalog number: NLM-1328)
15. Sodium chloride (Sigma-Aldrich, catalog number: S5886)
16. Water for UHPLC, for mass spectrometry (Sigma-Aldrich, catalog number: 900682)
17. Methanol for UHPLC, for mass spectrometry (Sigma-Aldrich, catalog number: 900688)
18. Dulbecco's phosphate-buffered saline (PBS), without calcium and magnesium (Corning, catalog number: 21-031-CV)
19. Fetal bovine serum (FBS), USDA tested (Cytiva, HyClone, catalog number: SH3091003)
20. ACK lysing buffer (ThermoFisher, Gibco, catalog number: A1049201)
21. 7-AAD (BD Biosciences, BD Pharmingen, catalog number: 559925)
22. Tributylamine, for liquid chromatography (Fisher Scientific, catalog number: 60-046-943)
23. Glacial acetic acid, for liquid chromatography (Fisher Scientific, catalog number: A35-500)
24. Saline (see Recipes)
25. Flow buffer (see Recipes)
26. Cell lysis buffer (see Recipes)
27. Mobile Phase Buffer A (see Recipes)
28. Mobile Phase Buffer B (see Recipes)

Equipment

1. Mouse restraining device (Braintree Scientific Inc., Tailveiner Restrainer, catalog number: TV-150 STD)
2. Micro dissecting scissors (Roboz Surgical, catalog number: RS-5906SC)
3. Narrow pattern forceps (Fine Science Tools, catalog number: 11002-12)
4. Mortar (VWR, catalog number: 89038-144)
5. Pestle (VWR, catalog number: 89038-160)
6. Refrigerated swinging bucket centrifuge 5910R with S-4xUniversal rotor (Eppendorf, catalog number: 5942000315)
7. Refrigerated microcentrifuge 5430R with FA-45-30-11 rotor (Eppendorf, catalog number: 5428000010)
8. Analytical balance (Mettler Toledo, catalog number: ME54)
9. BD FACSAria II Cell Sorter (BD Biosciences)
10. SpeedVac Vacuum Concentrator (ThermoFisher, catalog number: RVT450)

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11. LP Vortex Mixer (ThermoFisher, catalog number: 11676331)
12. 6470 Triple Quadrupole Mass Spectrometer (Agilent)

Software

1. BD FACSDiva Software, version 8.0.2 (BD Biosciences)
2. Excel (Microsoft)
3. Mass Hunter, version B.08.00 (Agilent)

Procedure

A schematic representation of the entire procedure is provided in Figure 1.

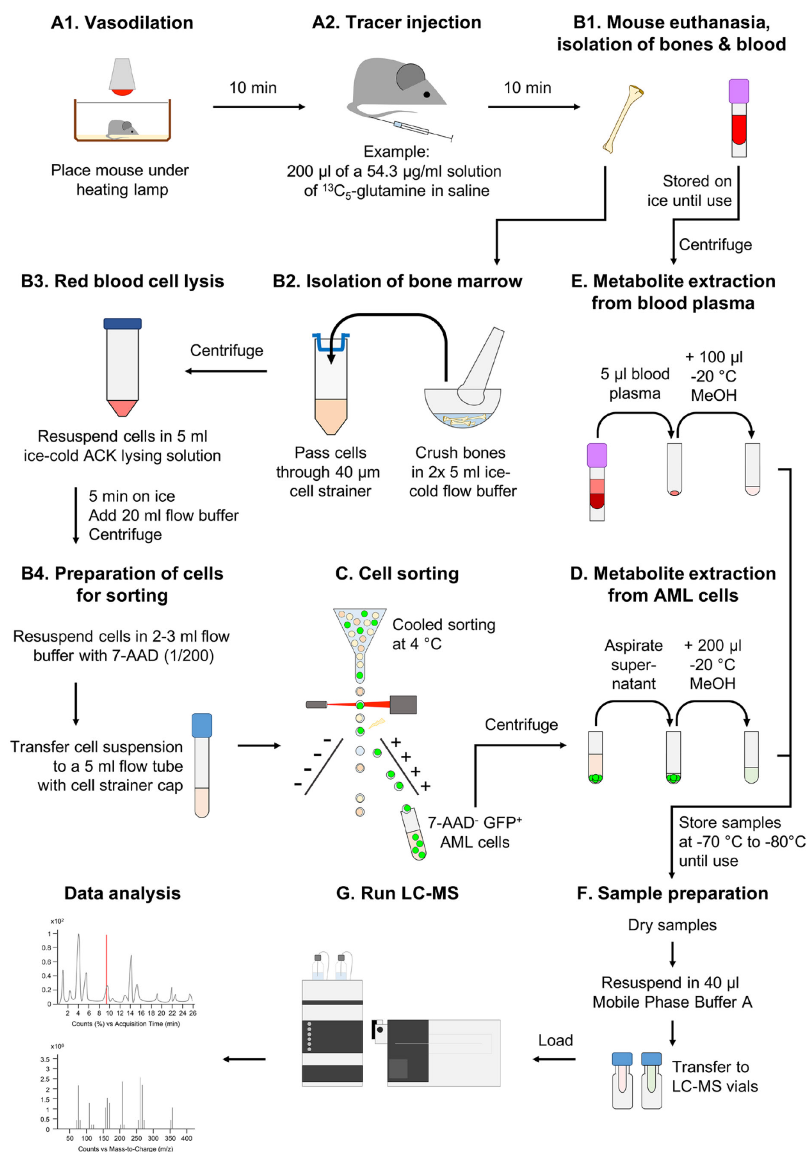

Figure 1. Schematic overview of the procedure

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A. Metabolic tracer preparation and injection

1. Prepare the tracer by dissolving $^{13}C_5$-glutamine or $^{15}N_2$-glutamine powder in saline at a concentration of 54.3 mg/ml. Heat gently in a water bath at 37°C until dissolved. Per mouse, 200 µl of tracer solution is needed.
2. Place the mouse in a cage under a heating lamp for 5 to 10 min prior to injection (or use an alternative to cause vasodilation, such as a heating pad or by soaking the tail in warm water). Ensure the animal does not overheat.
3. Place the animal in the restraining device.
4. Swab the tail with an alcohol preparatory pad.
5. Aspirate 200 µl of the tracer solution into a 300 µl insulin syringe with 31 G needle.
6. Slowly inject the tracer solution in the lateral tail vein.
7. Start a timer set for 10 min.

B. Cell isolation and preparation

1. Exactly 10 min after tracer injection, euthanize the mouse.
2. Obtain peripheral blood by cardiac puncture using a 1 ml syringe with 25 G needle.
3. Transfer blood to a K2-EDTA tube and place on ice.
4. Isolate the femora and tibiae, clean them well to remove the muscle tissue, and place the bones in a pre-chilled mortar containing 5 ml of ice-cold flow buffer.
   Note: The mortar should be pre-chilled by placing it in an ice bucket for 15 min before euthanizing the mouse.
5. Remove the mortar containing the bones from the ice bucket and crush the bones using a pestle by applying force in a vertical motion. Return the mortar to the ice bucket.
   Note: Avoid grinding the bones as this will create small debris that can interfere with cell sorting.
6. Break up any visible cell clumps by pipetting the cell solution up and down several times using a 1 ml micropipette.
7. Transfer the cell suspension to a 50 ml conical tube with 40 µm cell strainer, placed in an ice bucket.
8. Add another 5 ml of flow buffer to the mortar containing the bone fragments, crush again using a pestle until the bone fragments are completely white.
9. Break up any visible cell clumps by pipetting the cell solution up and down several times using a 1 ml micropipette.
10. Transfer the cell suspension to the 50 ml conical tube with 40 µm cell strainer containing the cells from the first crush.
11. Centrifuge the cell suspension in a refrigerated swinging bucket centrifuge for 5 min at 350 × *g*, 4°C.
12. Decant or aspirate the supernatant.
13. Resuspend the cell solution in 5 ml of ice-cold ACK lysing buffer (to remove red blood cells) and

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incubate on ice for 5 min.

14. Add 20 ml of flow buffer.
15. Centrifuge in a refrigerated swinging bucket centrifuge for 5 min at 350 × *g*, 4°C.
16. Decant or aspirate the supernatant.
17. Resuspend the cells in 2-3 ml flow buffer containing 7-AAD at a 1/200 dilution, and transfer the cell solution to a 5 ml flow tube with cell strainer cap.
    Note: In the original study (van Gastel et al., 2020), we only used GFP (marking the leukemia cells) and 7-AAD for cell sorting. It is possible to also stain the cells with fluorescently-labeled antibodies against cell surface markers. In that case, it is preferred to resuspend the cells first in a smaller volume (500 µl) containing the antibodies, incubate them on ice in the dark for 10 min, and then add 1.5-2.5 ml of flow buffer containing 7-AAD. If an appropriate antibody dilution is used, it is usually not necessary to remove unbound antibody. It is recommended to perform pilot experiments to ensure adequate staining is achieved with a 10-min staining time and no washing.
18. Proceed immediately with cell sorting

C. Cell sorting

1. Ensure the sample input and sample collector of the cell sorter are cooled to 4°C and that laser voltage set up and compensation are performed prior to starting the experiment. This can be done before the tracer injection in the mice or by a different person during sample preparation.
2. Identify the target population (Figure 2), and sort at least 10,000 cells into a microcentrifuge tube containing 500 µl of ice-cold saline. Ideally, 50,000-200,000 cells are sorted. Limit sorting time to a maximum of 30 min. Be sure to write down the cell number obtained for each sample.
3. Place cells on ice immediately after sorting is completed.

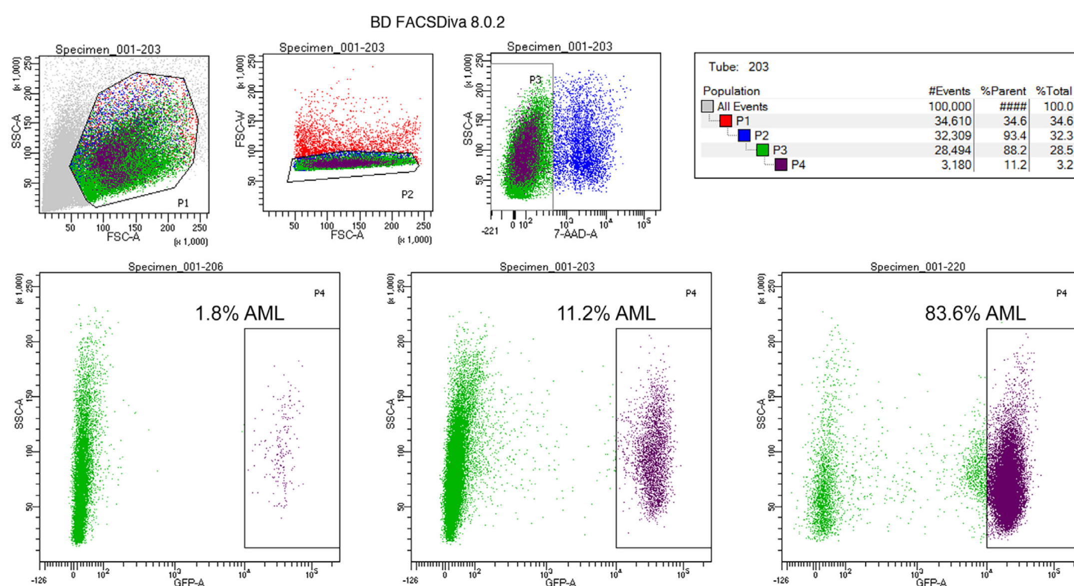

Figure 2. Representative FACS experiment. Gating strategy for sorting viable AML cells (7-

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AAD$^-$GFP$^+$) from the bone marrow of mice. Top graphs show the gating strategy for viable cells; bottom graphs show gating of AML cells in a mouse with low (left), medium (middle), or high (right) leukemic burden. P1 gate, leukocytes; P2 gate, single cells; P3 gate, viable cells; P4 gate, AML cells that are sorted.

D. Metabolite extraction from cells

1. Centrifuge cells in a refrigerated table-top microcentrifuge for 2 min at 500 × *g*, 4°C.
2. Gently aspirate the supernatant, leaving <20 µl behind. To avoid aspirating the cell pellet, which may be nearly invisible if the number of sorted cells is low, place the aspirator tip at the side of the tube that was nearest to the center of the centrifuge (thus farthest from the cell pellet).
3. Add 200 µl of cell lysis buffer, cooled to -20°C. Pipet up and down to mix well.
4. Store the samples at -70°C to -80°C.

E. Metabolite extraction from peripheral blood plasma

1. Centrifuge blood samples in a refrigerated table-top microcentrifuge for 5 min at 2,500 × *g*, 4°C.
2. Transfer supernatant to a microcentrifuge tube.
3. Transfer 5 µl of the plasma to a separate microcentrifuge tube (the rest of the plasma can be stored at -70°C to -80°C).
4. Add 100 µl of cell lysis buffer, cooled to -20°C. Pipet up and down to mix well.
5. Store the samples at -70°C to -80°C.

F. Sample preparation for liquid chromatography-mass spectrometry (LC-MS)

1. Prepare Mobile Phase Buffer A and Buffer B.
2. Remove samples from -70°C to -80°C storage and dry down in a SpeedVac Vacuum Concentrator set to 4°C.
3. Resuspend dried samples in 40 µl Mobile Phase Buffer A.
4. Vortex at 4°C for 10 min.
5. Centrifuge samples at 10,000 × *g* at 4°C for 10 min.
6. Move supernatant into LC-MS vials and load into the autosampler.

G. LC-MS analysis

1. Create a compound database on the Agilent MassHunter Acquisition software and include the compounds you wish to analyze, including information on the compound name, chemical formula, precursor ion mass, product ion mass, fragmentation energy, collision energy, and retention time. All this information can be found on the Agilent dMRM metabolomics database. For all new compounds, a standard must be run to optimize all of these parameters. When adding stable isotopes of metabolites to the list (such as ^{15}N-UMP), use the same retention fragmentation energy, collision energy, and retention time as the light metabolite (^{14}N-UMP).
2. Set up the liquid chromatography method as follows: Set up a ZORBAX Extend-C18, 2.1 × 150

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mm, 1.8 µm column, and equilibrate the column for 15 min with Buffer A at 0.25 ml/min. Begin each run with a flow rate of Buffer A at 0.25 ml/min for 2.5 min, followed by a linear gradient from 100% Buffer A to 80% Buffer A: 20% Buffer B for 5 min. Follow with linear gradients of 80% Buffer A: 20% Buffer B to 55% Buffer A: 45% Buffer B for 5.5 min; 55% Buffer A: 45% Buffer B to 1% Buffer A: 99% Buffer B for 7 min; and 1% Buffer A: 99% Buffer B for 4 min. After the run is done, a backwash of 100% acetonitrile should be used to clean the column for 10 min followed by 8 min of re-equilibration with 100% Buffer A.

3. Ensure the instrument is cleaned and calibrated prior to use. Turn on the Agilent 6470 Triple Quadrupole mass spectrometer and let it warm up for at least 30 min prior to use. Set up the mass spectrometry method as follows: Agilent Jet Spray ionization; nebulizer, 45 psi; capillary voltage, 2,000 V; nozzle voltage, 500 V; sheath gas temperature, 325°C; and sheath gas flow, 12 L/min. Set the instrument to collect mass data for the duration of the first 24 min of the liquid chromatography method in which the metabolites are expected to elute.
4. Write a sequence applying the above compound database, liquid chromatography, and mass spectrometry protocols to each sample that will be run. Include in the sequence a 15 µl injection from the autosampler into the LC-MS for each sample and a subsequent needle wash to ensure that there is no cross contamination between samples.

Data analysis

1. Import the data into the Agilent Mass Hunter Software.
2. Click on each metabolite in each sample to ensure that the peak elutes at the correct retention time, has the appropriate parent and product ion masses, and that the peak is fully integrated.
3. Export the ion counts for each peak integrated.
4. Open the results in an Excel worksheet.
5. Calculate the fractional abundance of each isotopologue by dividing the ion count (IC) of each isotopologue by the sum of the ICs of all isotopologues for a particular metabolite. For example, for calculating the M+2 isotopologue fractional abundance of glutamine (where M denotes the mass of the unlabeled metabolite), use the following formula:

$$\frac{IC^{M+2}}{IC^{M+0} + IC^{M+1} + IC^{M+2} + IC^{M+3} + IC^{M+4} + IC^{M+5}}$$

An example of representative data obtained from a typical *in vivo* metabolic tracing experiment is given in Table 1.

6. The fractional abundance of M+5 glutamine (when using $^{13}C_5$-glutamine) or M+2 glutamine (when using $^{15}N_2$-glutamine) in the peripheral blood plasma is used to exclude samples with insufficient labeling. A successful experiment will show a fractional abundance of fully labeled glutamine in the plasma of 0.40-0.60. If the fractional abundance of fully labeled glutamine in

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the plasma is below 0.40 all samples of that mouse are excluded.

Table 1. Example of data obtained for an *in vivo* metabolic tracing experiment. Mice were injected with $^{15}N_2$-glutamine, and contribution of glutamine-derived nitrogen to the synthesis of other amino acids was investigated in peripheral blood and AML cells isolated from bone marrow. As starting material, 5 µl of peripheral blood plasma or 50,000 AML cells were used. Ion counts, obtained in MassHunter, were exported to Excel, where the fractional abundances of the isotopologues of interest, the averages, and standard deviations (STDEV) were calculated. Care should be taken when interpreting the isotopologue fractional abundances for low abundant metabolites (see the results for proline in the AML cells for example), which tend to give a large variability between samples.

		Peripheral blood plasma							AML cells						
		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Average	STDEV	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Average	STDEV
Glutamine	M+0	30830	25502	26762	27454	29612			20	25	86	36	22		
	M+1	7786	17595	14660	13673	5201			34	24	10	13	0		
	M+2	54684	34448	55789	50440	24197			39	56	114	90	31		
	M+4	411	126	351	318	211			48	20	45	67	22		
	M+5	39	22	12	13	20			10	3	7	3	6		
	M+2 (%)	58,3	44,3	57,2	54,9	40,8	51,1	8,0	25,5	43,7	43,5	43,1	38,5	38,9	7,8
Glutamate	M+0	102826	117575	141882	103621	124702			43682	43570	61282	40755	47633		
	M+1	30579	37968	50749	36823	22678			6774	19726	24256	16268	5010		
	M+5	93	51	85	59	86			18	4	34	13	27		
	M+1 (%)	22,9	24,4	26,3	26,2	15,4	23,0	4,5	13,4	31,2	28,3	28,5	9,5	22,2	10,0
Aspartate	M+0	57401	60341	76049	45436	68752			48226	46951	64433	42069	50249		
	M+1	9363	12971	17571	9110	6558			5232	15293	18345	11843	3067		
	M+4	13	13	2	6	12			2	1	8	7	2		
	M+1 (%)	14,0	17,7	18,8	16,7	8,7	15,2	4,0	9,8	24,6	22,2	22,0	5,8	16,8	8,5
Proline	M+0	514	488	445	578	335			30	11	17	35	8		
	M+1	25	61	43	57	43			14	3	19	8	15		
	M+5	5	5	1	4	1			0	1	1	5	2		
	M+1 (%)	4,5	11,0	8,8	8,9	11,4	8,9	2,7	31,4	17,9	50,9	16,4	59,9	35,3	19,5
Asparagine	M+0	2646	1984	2284	2279	2471			63	137	41	261	239		
	M+1	183	208	218	223	187			4	3	13	19	3		
	M+2	119	104	186	327	134			39	615	254	611	332		
	M+1 (%)	6,2	9,1	8,1	7,9	6,7	7,6	1,1	3,5	0,4	4,2	2,2	0,6	2,2	1,7
	M+2 (%)	4,0	4,5	6,9	11,5	4,8	6,4	3,1	36,5	81,4	82,4	68,5	57,8	65,3	19,0

Notes

1. The number of biological replicates needed depends on the variability between samples. For mouse leukemia transplant-based models, the variability between individual mice is usually smaller than for more complex systems such as primary mouse leukemias or human PDX lines. In our studies, we start with 10 mice per group, split over 2-3 separate experiments. Depending on the skill of the person performing the tail vein injections, 10-40% of samples will need to be excluded due to insufficient tracer levels in the blood.
2. This protocol requires three researchers to work together during Procedure A-E. We usually inject mice with tracer in a staggered fashion, injecting 1 mouse every 30 min. Person A injects the mouse, performs euthanasia, isolates the blood and bones, and crushes the bones (until Step B10). This sequence can be completed by a trained researcher in 30 min. Person B then takes over, centrifuges the cells, performs red blood cell lysis, prepares the cells for FACS, and brings them to the cell sorter in an ice box (Steps B11 to B18; takes about 20 min), where they

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hand over the samples to person C, who oversees the cell sorting. Person B then takes the previous sample, which should be finished after 30 min of sorting, and performs metabolite extraction from the cells (Procedure D; takes less than 10 min). Metabolite extraction from the peripheral blood (Procedure E) is usually performed by person B while waiting during the centrifugation and incubation steps (Steps B11, B13, and B15), although this can also be performed by an additional person. If performing additional cell surface staining, the sequence is adapted depending on the required staining time, but normally can still be done by three people. A well-trained team will be able to isolate and process samples of 10 mice in an afternoon.

3. For some conditions, the number of cells that can be obtained from a single mouse may be too low for adequate analysis (for example, in some leukemic mice after chemotherapy). In that case, it is possible to lyse cells in a smaller adjusted volume of 80% methanol (Step D3) and pool samples of different mice before drying the samples.
4. Our protocol uses intravenous bolus delivery of labeled glutamine to study its metabolic fate in leukemia cells in mice, while other studies have used repeated bolus delivery or continuous infusion of tracers (Broekaert and Fendt, 2019; Muir *et al.*, 2017). Metabolite labeling in the leukemia cells will depend on several factors, including the enrichment of the precursor pool in the blood, metabolism of labeled glutamine in other organs complicating the ways in which the stable isotope can enter the leukemia cells, and the time required to establish complex labeling patterns (Faubert and DeBerardinis, 2017). The major advantages of administering a single bolus of tracer are the ease of delivery and the relatively small amount of tracer required, reducing the cost of the experiment. Also, if the leukemia is sampled soon after the bolus, the complicating effects of metabolism in other tissues may be minimized. In our original study, where we determined the optimal time point for leukemia cells isolation after tracer injection, we detected, for example, several other labeled metabolites in the peripheral blood of mice as soon as 15 min after tracer injection (van Gastel *et al.*, 2020).

   Disadvantages of bolus injection include a lower degree of overall labeling and the absence of some specific labeling patterns that take longer to develop. Another concern is that bolus injection may increase the total precursor pool in the blood and that this by itself may alter the metabolism of the leukemia cells. While we found that total glutamine levels in peripheral blood indeed almost doubled with injection (50% unlabeled and 50% labeled), the levels of glutamine in AML cells in the bone marrow did not substantially change as a consequence of the bolus injection (see Figure 3). Glutamate, aspartate, and oxidized glutathione (GSSG) levels in AML cells also did not change.

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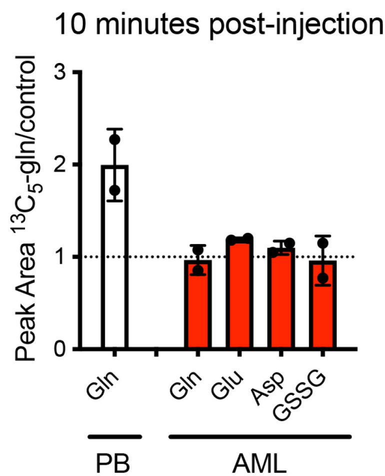

Figure 3. Effects of bolus injection of labeled glutamine on metabolite levels. Metabolite levels in mouse peripheral blood (PB) plasma and bone marrow AML cells after bolus injection of $^{13}C_5$-glutamine, compared to non-injected control mice. Gln, glutamine; Glu, glutamate: Asp, aspartate; GSSG, oxidized glutathione.

Recipes

1. Saline
   0.9% NaCl in water (LC-MS grade)
2. Flow buffer
   2% FBS in PBS
   Note: EDTA can be added to the flow buffer at 2 mM to prevent cell-cell interactions and reduce doublet events in FACS.
3. Cell lysis buffer
   80% methanol (LC-MS grade)
   20% water (LC-MS grade)
4. Mobile Phase Buffer A
   97% water (LC-MS grade)
   3% MeOH (LC-MS grade)
   10 mM Tributylamine (LC-MS grade)
   15 mM Glacial acetic acid (LC-MS grade)
   pH 5.5
5. Mobile Phase Buffer B
   MeOH (LC-MS grade)
   10 mM Tributylamine (LC-MS grade)
   15 mM Glacial Acetic Acid (LC-MS grade)

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Competing interests

D.T.S. is a director and equity holder of Agios Pharmaceuticals, Magenta Therapeutics, Editas Medicines, Clear Creek Bio and LifeVaultBio; he is a founder of Fate Therapeutics and Magenta Therapeutics and a consultant to FOG Pharma, VCanBio, and Flagship Pioneering. N.v.G. and D.T.S. are inventors on patents related to this work.

Ethics

Mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Arts and Sciences of Harvard University.

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