Assessing the impact of the nutrient microenvironment on the metabolism of effector CD8$^+$ T cells

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i Abstract

Immune cell function is tightly regulated by cellular metabolism, which in turn is strongly linked to the nutrient availability in the microenvironment surrounding the cells. This link is critical for effector CD8$^+$ T cells which, after activation, must migrate from nutrient-rich environments into nutrient-scarce regions such as the tumor microenvironment. Assessing how nutrient availability modulates the metabolism of effector CD8$^+$ T cells is thus key for understanding how harsh environments may impair their proliferation and effector function. Here, we describe an approach to systematically study the impact of the nutrient microenvironment on the metabolism of effector CD8$^+$ T cells, based on performing stable ^{13}C isotope labeling measurements on *in vitro*-differentiated murine effector CD8$^+$ T cells.

ii Keywords: CD8$^+$ T cells, immunometabolism, ^{13}C tracer analysis, nutrient microenvironment, custom media formulations

1 Introduction

$CD8^+$ T cells are the spearhead of the adaptive immune system. Upon emergence of a pathogen (e.g. a virus or transformed cells), naïve $CD8^+$ T cells in secondary lymphoid organs are stimulated into an active state via recognition of the foreign antigen presented via peptide/major histocompatibility complex I (pMHC-I) by antigen-presenting cells (e.g. dendritic cells) ***(1)***. The interaction between the T-cell receptor and pMHC-I activates $CD8^+$ T cells to undergo a rapid proliferation and differentiation process into an effector state, characterized by the ability to produce and release large amounts of cytotoxic cytokines (such as IFN-γ, TNF-α, and Granzyme B) ***(1)***. Effector $CD8^+$ T cells will then migrate to the site of infection, and deploy their cytotoxic activity against the specific pathogen.

Over the past years, the link between cellular metabolism and immune cell function has emerged as one of the hallmarks of immune regulation ***(2,3)***. As a result, the field of immunometabolism has become an increasingly active area of research ***(4,5)***, and a growing number of studies have aimed at characterizing the metabolism of $CD8^+$ T cells. In this sense, it has been established that the activation and differentiation of resting, naïve $CD8^+$ T cells into highly proliferative, cytotoxic effector $CD8^+$ T cells entail a major reprogramming in cellular metabolism, stemming from the different needs that T cells must meet in each of those different states ***(6,7)***. Indeed, it is now known that naïve $CD8^+$ T cells rely mainly on a catabolic metabolism (including oxidative phosphorylation and fatty acid oxidation), allowing them to efficiently support their resting state ***(8)***. Conversely, it has been shown that effector $CD8^+$ T cells are characterized by a switch to aerobic glycolysis and other anabolic pathways (e.g. amino acid and fatty acid metabolism), which they need to keep up with the high energetic and biosynthetic requirements imposed by rapid proliferation, and also in order to deploy their cytotoxic function ***(9)***.

Evidently, cellular metabolism, and particularly anabolism, is strongly linked to the availability of nutrients in the surrounding microenvironment. This link is of particular importance in the case of effector $CD8^+$ T cells, which must migrate from favorable, nutrient-sufficient environments (such as the spleen, lymph nodes and blood), into potentially inhospitable regions, such as the tumor microenvironment ***(10,11)***. It is thus critical to assess how nutrient availability modulates the metabolism (and consequently the proliferation and functionality) of effector $CD8^+$ T cells in these harsh environments, in order to understand what factors may potentially impair their metabolic fitness, preventing them from exerting their cytotoxic activity. In this regard, a number of groups have demonstrated how the nutrient microenvironment shapes the fitness of effector $CD8^+$ T cells, showing that the scarcity of nutrients such as glucose ***(9,12,13)***, arginine ***(14)***, and serine ***(15)***, and the acummulation of

metabolic waste products such as lactic acid ***(16,17)***, can all compromise T-cell proliferation and function. All these results highlight the importance of assesing the impact of the microenvironment on the metabolism and functionality of T-cells.

Here, we decribe a systematic approach to determine how changes in the availability of a wide variety of nutrients (relative to their physiological concentrations) modulate the metabolism of effector $CD8^+$ T cells. This approach is based on performing stable ^{13}C isotope labeling measurements on *in vitro* activated and effector-differentiated murine $CD8^+$ T cells (Figure 1). Stable ^{13}C isotope labeling measurements have long been used to study the metabolism of mammalian cells ***(18,19)***, and are the *de facto* standard for inferring alterations in intracellular metabolism resulting from changes in the extracellular environment ***(20,21)***. In these experiments, a nutrient of choice is replaced in the culture medium formulation by a labeled analog with some (or all) of its carbon atoms substituted by the stable isotope ^{13}C. Cells are then cultured in this medium to allow the incorporation of the ^{13}C label into the intracellular metabolites downstream of this nutrient. The resulting ^{13}C-label incorporation patterns, together with the intracellular levels for different metabolites, can then be determined by rapidly stopping (i.e. *quenching*) metabolism and analyzing the cell extracts using mass spectrometry (MS) ***(22)***. With this information, one can attain a qualitative description of the fate of the labeled nutrient, as well as of the activity of metabolic pathways connected to that nutrient ***(21)***. Furthermore, by combining the measured ^{13}C-labeling patterns and metabolite levels with nutrient uptake/secretion and cellular proliferation measurements, and incorporating this information into an appropriate reaction network model, quantitative information about the intracellular metabolic fluxes can be obtained, providing the most accurate picture of intracellular metabolism available ***(23,24)***.

A key feature of the described approach lies in the cell culture medium formulation, or Blood-Like Medium (BLM). The latter has been adapted from the literature ***(25)*** to match the concentrations found in human plasma for a variety of nutrients, in order to provide results more representative of physiological conditions. In addition, the described BLM formulation has the further advantage of being easily customizable, allowing to *pull out* a variety of individual nutrients (in particular, several non-essential amino acids, glucose and pyruvate) with minimum work. This enables replacing these nutrients with ^{13}C-labeled analogs of choice, or to investigate the impact of the full or partial depletion of any (or a combination) of them on the metabolism of effector $CD8^+$ T cells. Finally, we describe a protocol for the metabolic quenching of suspension cells, based on a recently

published method developed for spheroid quenching ***(26)*** (please refer also to Chapter 4 of this book), but adapted to the specific characteristics of T cells and other small suspension cells.

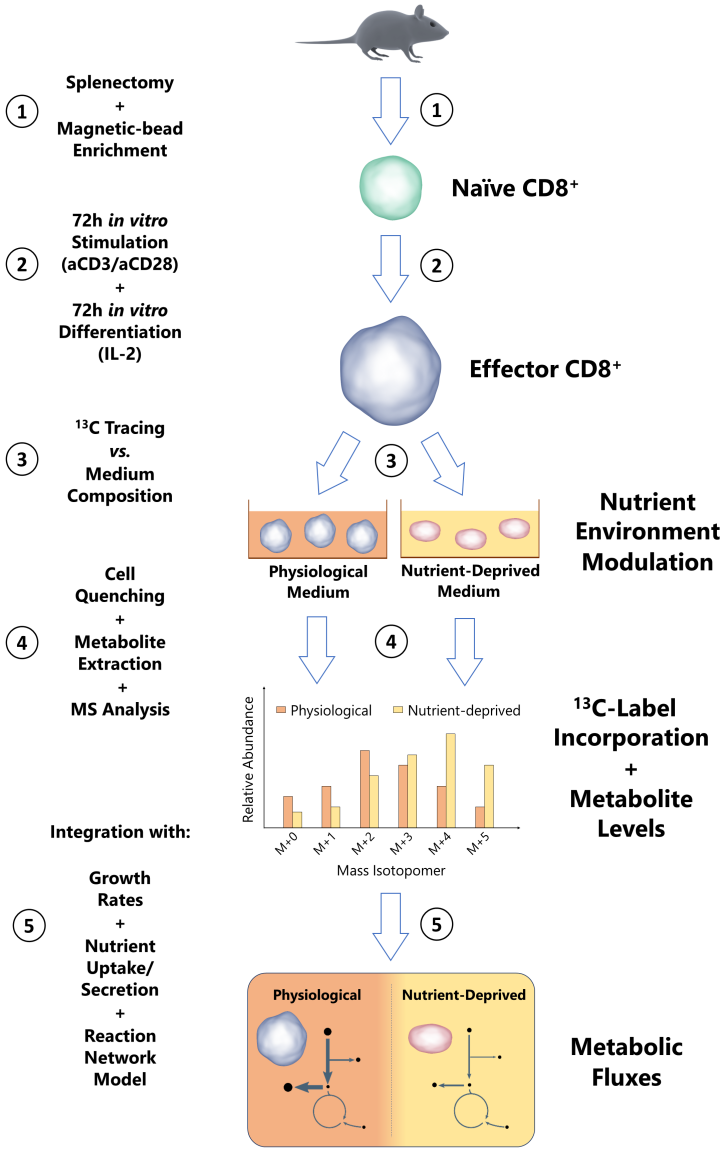

Figure 1: Outline of the approach described for studying the impact of the nutrient microenvironment on the metabolism of effector CD8+ T cells. Naïve CD8+ T cells are isolated from mouse spleens (Step 1; see 3.2), and further activated and differentiated into an effector state *in vitro* (Step 2; see 3.3 and 3.4). These effector CD8+ T cells are then cultured under different medium conditions, in the presence of stable ^{13}C isotope tracers (Step 3; see 3.1 and 3.5). Intracellular metabolites are then extracted from these cells, and ^{13}C-label incorporation patterns and metabolite levels are determined via MS-based analysis (Step 4; see 3.6, 3.7, and 3.8). Coupling this information with growth rates, metabolite uptake and secretion rates, and an appropriate metabolic flux model (Step 5; see 3.8), a quantitative assessment of the impact of the different nutrient conditions investigated on the metabolism of effector CD8+ T cells can be obtained.

2 Materials

Prepare and store all reagents at room temperature unless otherwise noted. For commercial reagents, follow the manufacturers' storage and handling guidelines unless specified. Diligently follow local waste disposal regulations. Use a lab coat, gloves, and if required safety glasses at all times. Work if necessary under a chemical

fume hood (e.g. when handling hazardous/volatile substances) or under a biological safety cabinet (e.g. when handling cells or cell culture material). Familiarize yourself with the chemical/biological-safety and ethical regulations applying to each of the materials listed below before proceeding.

2.1 Blood-Like Medium (BLM) preparation

1. Clean 500 mL mixing bottle (glass or plastic).
2. Milli-Q water
3. **20x Inorganic Salt stock**: dissolve the components in Table 1 in 1 L Milli-Q water:

Table 1: Composition of 20x Inorganic Salt stock

Component	Amount in 1 L (g)
Sodium Chloride (NaCl)	116.88
Calcium Chloride dihydrate ($CaCl \cdot 2H_2O$)	7.35
Potassium Chloride (KCl)	3.91
Magnesium Sulfate ($MgSO_4$)	3.01
Monosodium Phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$)	2.34

4. **30x Sodium Bicarbonate stock**: 63.01 g $NaHCO_3$ dissolved in 1 L Milli-Q water.
5. **200x Ferric Nitrate stock**: 242.4 mg $Fe[NO_3]_3 \cdot 9H_2O$ dissolved in 100 mL Milli-Q water.
6. **100x joint Amino Acid stock**: dissolve the components in Table 2 in 200 mL Milli-Q water + 150 mM hydrochloric acid (HCl; see Note 1). Prepare 5.25 mL aliquots and store at -20°C.

Table 2: Composition of 100x joint Amino Acid stock

Component	Amount in 200 mL (mg)
L-Asparagine	237.8
L-Citrulline	192.7
L-Cystine	312.4
L-Histidine	372.4
L-Isoleucine	262.4
L-Leucine	446.0
L-Lysine (monohydrochloride)	803.7
L-Methionine	89.5
L-Ornithine (monohydrochloride)	269.8
L-Phenylalanine	224.7
L-Threonine	571.8
L-Tryptophan	318.6
L-Tyrosine	268.2
L-Valine	538.9

7. **308x L-Glutamine stock**: L-Glutamine 200 mM (commercially available). Prepare 1.7 mL aliquots and store at -20°C.

8. **1000x individual Non-Essential Amino Acid stocks**: weigh the components in Table 3 and dissolve each of them **separately** (see Note 2) in 40 mL Milli-Q water. Prepare separate 525 µL aliquots of each 1000x stock and store at -20°C.

Table 3: Composition of 1000x individual Non-Essential Amino Acid stocks

Component	Amount in 40 mL (mg)
L-Alanine	1818
L-Aspartic acid (see Note 3)	31.9
L-Arginine	446
L-Glutamic acid (monosodium salt monohydrate)	577
Glycine	991
L-Proline	1658
L-Serine	589

9. **40x D-Glucose stock** (200 mM): 504.4 mg D-Glucose dissolved in 14 mL Milli-Q water (prepare fresh before every experiment; see Note 4).
10. **100x BLM Vitamin stock**: mix 90 mL 100x MEM Vitamin Solution (commercially available) and 10 mL 100x RPMI 1640 Vitamin Solution (commercially available). Prepare 5.25 mL aliquots and store at 4°C.
11. **500x Taurine stock**: 406.7 mg Taurine dissolved in 50 mL Milli-Q water.
12. **500x Phenol Red stock**: 235.2 mg Phenol Red sodium salt dissolved in 50 mL Milli-Q water (see Note 5).
13. **1000x Sodium Pyruvate stock**: Sodium Pyruvate 100 mM (commercially available). Store at 4°C.
14. **1100x β-Mercaptoethanol stock**: β-Mercaptoethanol 55 mM (commercially available). Store at 4°C.
15. Penicillin-Streptomycin 5,000 U/mL. Prepare 5 mL aliquots and store at -20°C.
16. Heat-inactivated fetal bovine serum (hi-FBS): thaw a bottle of FBS, then heat-inactivate by warming up to 50°C for 45 minutes. Prepare 50 mL aliquots and store at -20°C.
17. Dialyzed hi-FBS: using 3,500 Da cutoff MW dialysis cassettes, dialyze 30 mL hi-FBS against 4 L Milli-Q water + 0.15 M NaCl for 72h (replacing dialysis buffer daily). Prepare 5 mL aliquots and store at -20°C.
18. ^{13}C-labeled tracer stock solution at the appropriate concentration (e.g. 200 mM $^{13}C_5$-L-Glutamine for glutamine tracing experiments at standard BLM concentration).
19. OPTIONAL: additional *treatment* compounds (e.g. sodium L-lactate, see 3.1.2).
20. pH meter.
21. Aqueous 12 M HCl and 5 M NaOH (for pH adjustment).
22. Magnetic stirring plate and magnetic stirrer.
23. 0.22 µm vacuum filtration units.

2.2 Naïve CD8$^+$ T cell isolation

1. C57BL6/N mouse, typically 8–12 weeks old (see Note 6).
2. Sterile phosphate-buffered saline (PBS).
3. **Separation Buffer**: PBS with 3% hi-FBS and 10 mM EDTA. Prepare 200 mL. Sterilize using a 0.22 µm membrane vacuum filtration unit. Store at 4°C, place on ice 5 minutes before using.
4. Red Blood Cell Lysis Buffer (Roche, Cat. No. 11-814-389-001). Store at 4°C, place on ice 5 minutes before using.
5. Pentobarbital (Nembutal®), 50 mg/mL.
6. Sterilized forceps and surgical scissors (disinfect with 70% ethanol).
7. 15 and 50 mL sterile conical centrifuge tubes.
8. 70 µm sterile nylon cell strainers compatible with 50 mL tubes.
9. 10 mL sterile syringe with rubber-tipped plunger.
10. Mouse Naïve CD8$^+$ T cell enrichment kit. Store at 4°C.
11. Ice.
12. Cell counting apparatus.
13. Centrifuge fitting 15 and 50 mL tubes.
14. Biological safety cabinet.

2.3 CD8$^+$ T cell activation

1. **Standard unlabeled BLM** (see 3.1.1). Store at 4°C, warm up to room temperature before use.
2. Sterile PBS.
3. Anti-mouse CD3ε. Store at 4°C.
4. Anti-mouse CD28. Store at 4°C.
5. Mouse recombinant IL-2. Store at -80°C (see Note 7).
6. 15 mL sterile conical centrifuge tubes.
7. Tissue-culture treated, round-bottom 96-well plates.
8. 20 to 200 µL multichannel pipette.
9. Sterile 50 mL reagent reservoir.
10. Cell counting apparatus.

11. Centrifuge able to fit 15 mL tubes.
12. Biological safety cabinet.
13. Humidified, temperature and CO_2-controlled cell culture incubator.

2.4 Effector $CD8^+$ T cell differentiation

1. **Standard/customized unlabeled BLM** (see 3.1.2). Store at 4°C, warm up to room temperature before use.
2. Sterile PBS.
3. Mouse recombinant IL-2. Store at -80°C (see Note 7).
4. 15 and 50 mL sterile conical centrifuge tubes.
5. Tissue-culture treated, round-bottom 96-well plates.
6. 20 to 200 µL multichannel pipette.
7. Sterile 50 mL reagent reservoirs.
8. Cell counting apparatus.
9. Centrifuge fitting 15 and 50 mL tubes.
10. Biological safety cabinet.
11. Humidified, temperature and CO_2-controlled cell culture incubator.

2.5 ^{13}C-tracer labeling

1. **Standard/customized ^{13}C-labeled BLM** (see 3.1.3). Store at 4°C, warm up to room temperature before use.
2. Mouse recombinant IL-2. Store at -80°C (see Note 7).
3. 15 and 50 mL sterile conical centrifuge tubes.
4. Tissue-culture treated 6-well plates.
5. 20 to 200 µL multichannel pipette.
6. Sterile 50 mL reagent reservoirs.
7. Cell counting apparatus.
8. Centrifuge fitting 15 mL tubes.
9. Biological safety cabinet.
10. Humidified, temperature and CO_2-controlled cell culture incubator.

2.6 Cell quenching and media sampling

Prepare the quenching buffer under a chemical fume hood using MS-grade solvents and high-purity solutes, and store in a clean glass bottle (see Note 8). Have all materials listed below ready before the start of this step.

1. **Quenching Buffer**: 10 mM ammonium acetate in 60:40 methanol-water (90 mL per culture plate). To prepare 1L, weigh 771 mg ammonium acetate, dissolve in 400 mL Milli-Q water, add 600 mL MS-grade methanol, and mix. Store at -20°C.
2. 96% Ethanol (for quenching bath, re-usable).
3. 2 styrofoam boxes, one filled with dry ice.
4. One 50 mL conical centrifuge tube (precool in the dry ice box).
5. 15 mL sterile conical centrifuge tubes (3 per culture plate).
6. 1.5 mL Eppendorf SafeLock tubes (6 per culture plate).
7. Low-temperature thermometer (capable of measuring down to -50°C).
8. Plastic tube-rack fitting 3 x 15mL conical centrifuge tubes.
9. Serological pipette controller and 50 mL serological pipettes (2 per culture plate).
10. Vacuum aspirator (set at low speed) and 2 glass Pasteur pipettes per culture plate (see Note 9).
11. Cell counting apparatus.
12. Centrifuge fitting 15 mL tubes, set at 1 minute spin time and 2,000 g (see Note 10).
13. 200–1000 µL pipette and a box of 1 mL tips.
14. A container for medium waste.

2.7 Metabolite extraction from cell and medium samples

Work under a chemical fume hood both when preparing and handling all extraction reagents. Unless otherwise noted, prepare all extraction reagents using MS-grade solvents and high-purity solutes, store these reagents in clean glass bottles (see Note 8), and use only clean glass pipets (see Note 8) or inert-plastic pipette tips when handling them.

1. **Internal Standard stock 1**: 1 mg/mL norvaline/glutarate in water. Weigh 50 mg each norvaline/glutaric acid, and dissolve in 50 mL Milli-Q water. Aliquot in Eppendorf SafeLock tubes. Store at -20°C for up to one year.
2. **Internal Standard stock 2**: 1 mg/mL heptadecanoic acid (C_{17}) in chloroform. Weigh 10 mg C_{17} and dissolve in 10 mL MS-grade chloroform. Store at -80°C for up to one month.

3. **Extraction Buffer 1**: 0.94 µg/mL norvaline/glutarate in 3:5 water-methanol. For every sample to be extracted, mix 300 µL Milli-Q water, 500 µL MS-grade methanol, and 0.75 µL internal standard stock 1. Store at -20°C until extraction.
4. **Extraction Buffer 2**: 10 µg/mL C_{17} in chloroform. For every sample to be extracted, mix 500 µL MS-grade chloroform and 5 µL internal standard stock 2. Store at -80°C until extraction.
5. 2 mL Eppendorf SafeLock tubes (1 per sample to be extracted).
6. 1.5 mL Eppendorf SafeLock tubes (2 per sample to be extracted).
7. One large styrofoam box filled wit dry ice.
8. 200–1000 µL pipette and filterless inert-plastic tips.
9. Refrigerated micro-centrifuge fitting 1.5 and 2 mL tubes.
10. Refrigerated vortexer.
11. Refrigerated centrifugal vacuum concentrator.

3 Methods

The following protocol is designed for the isolation of $CD8^+$ T cells from a single mouse spleen, which will typically yield enough cells for studying metabolic alterations among at least 6–8 different nutrient conditions (including a standard control condition). For larger studies, scale up the protocol accordingly.

Prepare and store all reagents at room temperature unless otherwise noted. For commercial reagents, follow the manufacturers' storage and handling guidelines unless specified. Diligently follow local waste disposal regulations. Use a lab coat, gloves, and if required safety glasses at all times. Work if necessary under a chemical fume hood (e.g. when handling hazardous/volatile substances) or under a biological safety cabinet (e.g. when handling cells or cell culture material). Familiarize yourself with the chemical/biological-safety and ethical regulations applying to each of the materials used in the protocol before proceeding.

3.1 Blood-Like Medium (BLM) preparation

The following guidelines are based on the preparation of a total of 500 mL of base Blood-Like Medium (BLM) and final working aliquots of 50 mL. Adjust the protocol accordingly to prepare smaller/larger media volumes.

3.1.1 Standard BLM preparation

This medium formulation will be used for the activation (see 3.3) and initial differentiation (see 3.4) of all $CD8^+$ T cells, as well as for their final effector differentiation in control (i.e. non-customized) environment conditions (see 3.4). A summary of the final concentrations of the different components in this standard formulation is given in Table 5.

1. Add the components in Table 4 (in the order listed, see Note 11) in a clean 500 mL mixing bottle. Shake gently after every addition to improve mixing.

Table 4: Component volumes needed to prepare a final volume of 500 mL BLM

Component	Volume needed for 500 mL BLM (mL)
Milli-Q water	365
20x Inorganic Salt stock	25
30x Sodium Bicarbonate stock	16.7
200x Ferric Nitrate stock	2.5
200 mM (40x) D-Glucose stock	12.5
100x joint Amino Acid stock	5
200 mM (308x) L-Glutamine stock	1.63
1000x individual Non-Essential Amino Acid stocks	0.5 each (3.5 total)
100x BLM Vitamin stock	5
500x Taurine stock	1
500x Phenol Red stock	1
100 mM (1000x) Sodium Pyruvate stock	0.5

2. Add a clean magnetic stirrer to the mixing bottle, place on top of a stirring plate, and immerse the pH meter in the liquid. While stirring, adjust the pH to 6.9 by dropwise addition of either 12M HCl or 5M NaOH (see Notes 12 and 13). Proceed immediately with step 3.
3. Filter using a 0.22 µm membrane filter unit and store at 4°C for up to 10 days. This is your base BLM (see Note 14).
4. Right before the applicable phase of the experiment, prepare a 50 mL working BLM aliquot by mixing together 44 mL of base BLM, 5 mL of hi-FBS (for final concentration of 10% v/v), 1 mL of Penicillin-Streptomycin 5,000 U/mL (for final concentration of 100 U/mL), and 45.5 µL of 55 mM (1100x) β-Mercaptoethanol stock (see Note 15). Proceed immediately with step 5.
5. Filter using a 0.22 µm membrane filter unit and store at 4°C, ready to use, for up to 3 days. This is your standard BLM (see Note 14).

Table 5: Composition of standard BLM formulation

Compound	Concentration (µM)
Amino Acids	
L-Alanine	510
L-Arginine	64
L-Asparagine	90
L-Aspartic acid	6
L-Citrulline	55
L-Cystine	65
L-Glutamic acid	98
L-Glutamine	650
Glycine	330
L-Histidine	120
L-Isoleucine	100
L-Leucine	170
L-Lysine	220
L-Methionine	30
L-Ornithine	80
L-Phenylalanine	68
L-Proline	360
L-Serine	140
L-Threonine	240
L-Tryptophan	78
L-Tyrosine	74
L-Valine	230
Other Nutrients	
D-Glucose	5000
Sodium Pyruvate	100

Compound	Concentration (µM)
Vitamins and Other Supplements	
Biotin	0.08
Choline Chloride	8.53
D-Calcium Pantothenate	1.94
Folic Acid	2.3
Niacinamide	8.2
Para-Aminobenzoic Acid	0.73
Pyridoxine Hydrochloride	4.9
Riboflavin	0.32
Thiamine Hydrochloride	3
i-Inositol	29.43
Vitamin B12	0.0004
Taurine	130
Inorganic Salts	
Calcium Chloride	2500
Ferric Nitrate	30
Magnessium Sulfate	1250
Potassium Chloride	5000
Sodium Bicarbonate	25000
Sodium Chloride	100000
Sodium Phosphate	750
Cell Culture Supplements	
β-Mercaptoethanol	50
Phenol Red (Sodium Salt)	25
FBS	10% v/v
Penicillin-Streptomycin	100 U/mL

3.1.2 Customized BLM preparation

These media formulations will be used in the final 24 h of effector differentiation of $CD8^+$ T cells in customized (e.g. nutrient-deprived) environment conditions (see 3.4). To prepare a customized BLM formulation, follow identical steps as those outlined in section 3.1.1 for preparing standard BLM, with the following modifications:

1. For nutrient deprivation studies, appropriately decrease the added volume(s) of the corresponding nutrient stock(s) during step 1 of 3.1.1 above, and compensate the volume deficit by addition of Milli-Q water.

   Example A: to assess the impact of glucose deprivation down to 20% of physiological blood levels (i.e. 1 mM), add only 2.5 mL of the 40x D-Glucose stock in step 1 of 3.1.1, and compensate the volume deficit by adding an extra 10 mL of Milli-Q water to the mixture (see Note 16).

2. For compound treatment (e.g. waste-product accumulation) studies, prepare a concentrated stock of the compound of interest, and add appropriately to the mixture as part of step 1 of 3.1.1 above, compensating for the volume excess by reducing the starting volume of Milli-Q water accordingly.

Example B: to assess the impact of environmental lactate accumulation at supraphysiological levels (e.g. 20 mM), prepare a concentrated 100x (i.e. 2M) stock of sodium L-lactate in Milli-Q water, and add 5 mL of it to the mixture. To compensate for the volume excess, add only 360 mL of Milli-Q water in step 1 of 3.1.1.

3.1.3 ^{13}C-labeled BLM preparation

These media formulations will be used for the ^{13}C labeling of effector-differentiated CD8^{+} T cells in either control or customized (e.g. nutrient-deprived) conditions (see 3.5). To prepare a ^{13}C-labeled BLM formulation, follow identical steps as those in sections 3.1.1 and 3.1.2 for preparing standard/customized BLM, with the following modifications:

1. Replace the metabolite(s) to be used as tracer(s) with the appropriate ^{13}C-labeled analog(s).

   Example C: for glutamine tracing experiments, prepare a 200 mM stock solution of $^{13}C_5$-L-Glutamine in Milli-Q water, and use it in place of its unlabeled analog in step 1 of 3.1.1 above.
2. Use dialyzed hi-FBS instead of standard hi-FBS in step 4 of 3.1.1 above (see Note 17).

3.1.4 OPTIONAL: A recommendation for increased reproducibility and efficiency in BLM preparation

In general, and especially when a wide number of conditions will be explored, it is most convenient to start by preparing a base BLM formulation (steps 1–3 in 3.1.1) common to all those conditions (including both labeled/unlabeled and customized/standard control conditions), and only add the appropriate amounts of all differential compounds upon preparing working aliquots (step 4 in 3.1.1). This ensures maximum reproducibility among the underlying basic media composition (and pH) for all different conditions explored, and reduces the time invested in medium preparation.

Example D: to investigate changes in glutamine metabolism under conditions of partial glucose deprivation (a combination of *Examples A* and *C* above), proceed to step 3 in 3.1.1 skipping the addition of both glucose and glutamine in step 1, to obtain a common glucose/glutamine-free base BLM formulation (which can be stored at 4°C for 10 days), and then, when needed, to make a 50 mL working aliquot of:

1. **Standard, unlabeled** BLM (for 3.3.4, 3.4.1, 3.4.10): mix 1.25 mL of the 200 mM D-Glucose stock + 163 µL of the 200 mM L-Glutamine stock into 42.59 mL of this glucose/glutamine-free base BLM, and use the resulting 44 mL as the base BLM in step 4 of 3.1.1. Proceed with the remaining steps as indicated.
2. **Glucose-deprived, unlabeled** BLM (for 3.4.10): proceed identically as in the former case, but using 0.25 mL (instead of 1.25 mL) of the 200 mM D-Glucose stock, and compensating with 1 mL Milli-Q water.

3. **Standard, ^{13}C-labeled** BLM (for 3.5.1): proceed identically as in the standard, unlabeled case, but using the 200 mM U$^{13}C_5$-L-Glutamine stock (instead of the unlabeled one), and dialyzed hi-FBS in step 4 of 3.1.1.
4. **Glucose-deprived, ^{13}C-labeled** BLM (for 3.5.1): proceed identically as in the former case, but using 0.25 mL (instead of 1.25 mL) of the 200 mM D-Glucose stock, and compensating with 1 mL Milli-Q water.

3.2 Naïve CD8$^+$ T cell isolation

Always work in sterile conditions under a biological safety cabinet.

1. Sacrifice the C57BL/6N mouse with an overdose of pentobarbital (3 µL per gram of animal weight of a 50 mg/mL solution). Using sterilized scissors and forceps, open the abdominal cavity of the mouse, harvest the spleen, and immediately transfer into a tube with 10 mL ice-cold Separation Buffer. Dispose of the animal carcass following the appropriate regulations.
2. Place a 70 µm strainer on top of a second 50 mL conical tube, and prime its membrane with 5 mL Separation Buffer.
3. Using sterilized scissors and forceps, hold the spleen over the strainer and cut it into 3–4 small pieces. When done, immerse the tips of forceps and scissors in the 10 mL Separation Buffer left in the first tube, to wash the tissue remaining in them into the liquid and recover more cells. After that, pour the liquid over the cut spleen on the strainer.
4. Remove the plunger from the 10 mL syringe, and use its rubber tip to mash the spleen pieces over the strainer. Use grinding circular movements to homogenize the tissue. Periodically, draw up 5–10 mL Separation Buffer with a disposable pipette and wash down the cells from within the strainer (see Note 18). Continue mashing until only the white connective tissue of the outer spleen membrane remains. Wash the strainer one last time with Separation Buffer and discard it.
5. Top the conical tube containing the mashed, filtered cells with Separation Buffer up to 45 mL, spin down (5 min at 500 g), and remove the supernatant. The resulting cell pellet will be red in color.
6. Resuspend the pellet thoroughly in 5 mL ice-cold Red Blood Cell Lysis Buffer (see Note 19). Bring the tube into ice for 5 minutes, shaking twice during this period to prevent clump formation.

7. Add 25 mL Separation Buffer to neutralize the Red Blood Cell Lysis Buffer, spin down (5 min at 500 g), and remove the supernatant. Add an extra 25 mL of Separation Buffer for a second wash and repeat. The pellet should now be clearer, of a whitish color.
8. Resuspend the pellet thoroughly in 5 mL Separation Buffer, place a new 70 µm strainer on top of a new 50 mL conical tube, and transfer the 5 mL cell suspension into this tube through the strainer (see Note 18). Wash down the strainer with an extra 5 mL of Separation Buffer, to recover more cells.
9. Homogenize the resulting 10 mL single-splenocyte suspension and count (see Notes 20 and 21).
10. Continue as described in the guidelines for your specific naïve $CD8^+$ T cell enrichment kit. Finish by collecting the isolated naïve $CD8^+$ T cells in a 15 mL conical tube (see Note 22), and immediately proceed to next step (see 3.3.5).

3.3 $CD8^+$ T cell activation

Always work in sterile conditions under a biological safety cabinet. The present protocol requires preparing the $CD8^+$ T cell activation culture plate one day in advance. Therefore, plan the experiment accordingly.

1. One day before starting the experiment, prepare a 10 µg/mL anti-CD3ε solution in sterile PBS. Using the multichannel pipette and a reagent reservoir, dispense 50 µL of this solution (i.e. 0.5 µg anti-CD3ε) into each of the 60 inner wells of a tissue-culture treated, round-bottom 96-well plate (see Notes 23 and 24).
2. Using the multichannel pipette and a reagent reservoir, fill the remaining 36 wells (i.e. those forming the outer rim) of the 96-well plate with 200 µL PBS each, and fill also the 77 inter-well spaces (the holes between every set of 4 contiguous wells) with 100 µL PBS each (see Note 24).
3. Cover the plate, seal with Parafilm® and incubate at 4°C overnight.
4. On the day of the experiment, before harvesting the spleen, prepare a 50 mL working aliquot of standard BLM (see 3.1.1).
5. Homogenize and count the isolated naïve $CD8^+$ T-cell suspension (see Note 25) in the tube used for collection (see 3.2.10). Top the tube up to 12 mL with standard BLM, spin down (5 min at 300 g), and remove the supernatant.

6. Based on the prior cell count, resuspend the cell pellet at a density of 0.5 x 10^6 cells/mL in standard BLM (see Note 26). Add IL-2 and anti-CD28 to final concentrations of 10 ng/mL and 0.5 µg/mL, respectively (see Note 27).
7. Bring the 96-well plate to the flow and, using the multichannel pipette, aspirate the 50 µL anti-CD3ε solution out of all 60 inner wells. Remove the excess uncoated antibody by washing twice, each time adding 200 µL of sterile PBS to each well, letting sit for 1 minute, then removing them with the multichannel pipette.
8. Homogenize the cell suspension in the 15 mL tube, transfer into a reagent reservoir and, using the multichannel pipette, seed 200 µL/well (i.e. 10^5 cells/well) into as many wells as possible out of the inner 60 wells (see Notes 25 and 28). Fill all remaining empty wells with 200 µL PBS. Incubate at 37°C and 5% CO2 for 24 h.
9. After 24 h, take the plate out of the incubator and into the flow and, using the multichannel pipette, carefully remove 100 µL (i.e. 50%) of the medium in each well. Immediately after, carefully add 100 µL of fresh standard BLM with 10 ng/mL IL-2 and 0.5 µg/mL anti-CD28 to each well using the multichannel pipette and a reagent reservoir (see Note 29). Incubate at 37°C and 5% CO2 for an extra 24 h. After that, perform an identical 50% medium replacement (see Note 30), and incubate for a final 24 h, for a total activation of 72 h.

3.4 Effector $CD8^+$ T cell differentiation

Always work in sterile conditions under a biological safety cabinet.

1. On the day of the experiment, prepare a 100 mL working aliquot of standard BLM (see 3.1.1).
2. Prepare 2 round-bottom 96-well plates for seeding: using the multichannel pipette and a reagent reservoir, fill the 36 outer-rim wells and the inter-well spaces of each plate with 200 µL and 100 µL sterile PBS each, respectively (see Note 24).
3. After the 72 h activation, bring the plate to the flow and harvest the activated cells (see Note 31). One row at a time, pipet the contents of each well up and down 10–12 times using the multichannel pipette, in order to detach and resuspend the cells, and then transfer into a sterile reagent reservoir. Repeat until all cells have been harvested, and then transfer the pooled cell suspension into a 50 mL conical centrifuge tube.

4. Homogenize the contents of the tube and count (see Note 32). Top the tube up to 20 mL with standard BLM, spin down (5 min at 300 g), and remove the supernatant.
5. Based on the prior cell count, resuspend the cell pellet in standard BLM, at a density of 0.5 x 10^6 cells/mL (see Note 33). Add IL-2 to a final concentration of 10 ng/mL (see Note 27).
6. Based on the volume of your cell suspension, calculate the total number of wells that will be seeded and decide their arrangement (see Note 28). Fill the remaining wells with 200 µL PBS. Homogenize the cell suspension in the 50 mL tube, transfer into a reagent reservoir and, using the multichannel pipette, seed 200 µL/well into the chosen wells. Incubate at 37°C and 5% CO2 for 24 h.
7. Before the 24 h time point, prepare 4 round-bottom 96-well plates for seeding as in 3.4.2.
8. After 24 h, bring both plates to the flow and harvest the cells: proceed as in 3.4.3, but this time pipetting gently and only for 6–8 times, since cells will not be attached to the wells. Transfer the pooled cell suspension from both plates into a 50 mL conical centrifuge tube, homogenize, and count (see Note 34).
9. Spin down (5 min at 300 g), remove the supernatant and, based on the prior cell count, resuspend the cell pellet at a density of 0.5 x 10^6 cells/mL in standard BLM. Add IL-2 to a final concentration of 10 ng/mL (see Note 27), and seed the cells into the new plates as in 3.4.6. Incubate at 37°C and 5% CO2 for an extra 24 h (for a total of 48 h).
10. Before the 48 h time point, prepare 25 mL working aliquots of each of the customized BLM formulations corresponding to the conditions to be investigated (see 3.1.2), as well as of standard BLM (see 3.1.1), to use as a control. Prepare also one round-bottom 96-well plate per condition for seeding, as in 3.4.2.
11. At the 48 h time point, bring the plates to the flow and harvest the cells: proceed as in 3.4.3, transferring the pooled cell suspension from all plates into a 50 mL conical centrifuge tube. Homogenize and count (see Note 34).
12. Based on the counted cell density, transfer 5 x 10^6 cells per condition to be investigated into a separate 15 mL conical centrifuge tube. Top each tube up to 12 mL with the corresponding BLM formulation, spin down (5 minutes at 300 g), and remove the supernatants.
13. Resuspend each cell pellet in 10 mL of the corresponding BLM formulation, for a density of 0.5 x 10^6 cells/mL. Add IL-2 to each cell suspension at a final concentration of 10 ng/mL (see Note 27). Then,

working one condition at a time, seed the cells into each new plate as in 3.4.6. Incubate all plates at 37°C and 5% CO2 for an extra 24 h, for a total effector differentiation of 72 h (see Note 35).

3.5 ^{13}C-tracer labeling

Always work in sterile conditions under a biological safety cabinet. Starting from step 2, work one plate at a time, especially if many different conditions will be investigated.

1. On the day of the experiment, prepare 50 mL ^{13}C-labeled (see 3.1.3) aliquots of each of the customized BLM formulations that will be investigated, including standard BLM (to be used as control).
2. After 72 h differentiation, harvest the cells in each of the 96-well plates seeded in the previous section (see 3.4.13): proceed as in 3.4.8, transferring the pooled cell suspension from each plate into a separate 50 mL conical centrifuge tube. Homogenize the contents of the tube and count (see Note 36).
3. Based on the counted cell density, transfer 5 x 10^6 cells for the current condition into a separate 15 mL conical centrifuge tube. Top the tube up to 12 mL with the corresponding ^{13}C-labeled BLM formulation, spin down (5 minutes at 300 g), and remove the supernatant.
4. Resuspend the cell pellet in 10 mL of the corresponding ^{13}C-labeled BLM formulation, for a density of 0.5 x 10^6 cells/mL. Add IL-2 at a final concentration of 10 ng/mL (see Note 27).
5. Seed 3 mL of the cell suspension into each of the top 3 wells (i.e. in triplicate) of a 6-well plate (see Note 37). Right after seeding the triplicates for each condition, determine and write down their exact, common seeding density, by counting twice on the remaining 1 mL of the corresponding cell suspension (see Note 38). Then, fill the 3 bottom wells (i.e. also in triplicate) of the plate with 3 mL each of cell-free medium corresponding to that condition (see Note 39). Bring the 6-well plate to the incubator, and proceed again from step 2 for the next 96-well plate. Incubate all plates at 37°C and 5% CO2 for 24 h.

3.6 Cell quenching and media sampling

Prepare all materials listed in section 2.6 ahead of time. Starting from step 3, work as fast as possible and one plate at a time (see Note 40). At the end of the quenching, empty the vacuum aspirator container into an appropriate waste container for methanol, and dispose of it following the appropriate regulations.

1. Prepare cold ethanol bath: Place the plastic tube-rack into one of the styrofoam boxes and fill the latter with ethanol, until the liquid height is enough to cover the bottom 10 mL of a 15 mL conical tube. Introduce the low-temperature thermometer in the ethanol, and gradually add blocks of dry ice until the temperature reaches -30°C. Then, immerse the glass bottle with the Quenching Buffer into the bath, and control the temperature by adding dry ice as needed, so that it remains in the -30°C to -40°C range.
2. Label one set of 3 x 15 mL tubes (see Notes 41 and 42), in correspondence with the three cell-containing wells in the plate to be quenched. Label also 2 sets of 3 x 1.5 mL Eppendorf tubes, analogously to the former, to store medium samples from the cell-free and cell-containing wells.
3. Take a plate out of the incubator, and sample 1 mL of the cell-free medium in each of the bottom 3 wells into the appropriate 1.5 mL Eppendorf tubes.
4. Resuspend the contents of each of the top 3 cell-containing wells (see Note 43), and transfer into the corresponding 15 mL tubes (see Note 44). Right after transferring each well, sample cells twice (see Note 38) into cell-counting slides, and set aside for counting later.
5. Once all 3 wells have been transferred, spin down all three 15 mL tubes (1 min at 2,000 g). During the spin-down time, use a serological pipette to transfer 40–45 mL of cold Quenching Buffer into the 50 mL tube, and bring the latter back to dry ice (see Note 45).
6. Right after spinning, transfer 1 mL of the supernatant (i.e. the cell-conditioned medium) in each 15 mL tube into the appropriate 1.5 mL Eppendorf tube, discard the other 2 mL into the waste container (see Note 46), pour 10–15 mL of cold Quenching Buffer from the 50 mL tube into the 15 mL tube (see Note 47), and move the 15 mL tube to the rack in the cold ethanol bath.
7. Once all 3 tubes have been processed, move them to the centrifuge and spin down (1 min at 2,000 g). During the spin-down time, refill the 50 mL tube up to 40–45 mL with cold Quenching Buffer, and attach a glass Pasteur pipette to the vacuum aspirator.
8. Right after spinning, carefully move the 3 tubes to the rack in the ethanol bath (see Note 48), and uncap them (see Note 49). Using the vacuum aspirator and attached Pasteur pipette, aspirate the quenching buffer from each of the tubes. Bring each tube back into the bath immediately after aspiration, and leave open for next step.

9. Once all 3 tubes have been aspirated, pour 10–15 mL of cold Quenching Buffer from the 50 mL tube into each of them, then quickly close the tubes, move them to the centrifuge, and spin down (1 min at 2,000 g). During the spin-down time, replace the glass Pasteur pipette in the vacuum aspirator.
10. Right after spinning, carefully move the 3 tubes back to the rack in the ethanol bath, and uncap them. Aspirate the quenching buffer from each of the tubes, and bring each tube back into the bath immediately after.
11. Once all 3 tubes have been aspirated, quickly close them and move them into the dry ice in the styrofoam box. Move also the six 1.5 mL Eppendorf tubes into dry ice.
12. Before proceeding with the next plate, determine and write down the final cell densities in each of your triplicates, by counting the cell-counting slides previously set aside for this purpose.
13. Proceed with the next plate. After quenching all plates, proceed to the next step of the protocol (metabolite extraction), or move all samples (15 mL tubes and 1.5 mL tubes) to a -80°C freezer until ready to extract.

3.7 Metabolite extraction from cell and medium samples

Always work under a chemical fume hood when handling extraction buffers.

3.7.1 Metabolite extraction from cell samples

1. Label one 2 mL Eppendorf per sample for protein-layer storage (PROT), and two 1.5 mL Eppendorf tubes per sample for polar metabolite (PM) and fatty acid (FA) storage. Label also three extra sets of 1 x 2 mL and 2 x 1.5 mL tubes, for 3 control *mock extraction* samples. Bring all tubes into a chemical fume hood.
2. Bring a box with dry ice containing the 15 mL tubes with the quenched cell samples (see 3.6.13) and the bottles with Extraction Buffers 1 and 2 into the chemical fume hood.
3. Working one 15 mL tube (i.e. one sample) at a time, pipet 800 µL of Extraction Buffer 1 into the tube, resuspend the contents thoroughly (see Note 50), transfer into the corresponding PROT-labeled 2 mL tube, and bring into dry ice. Repeat the process identically for all samples and an extra 3 empty 15 mL tubes, which will constitute your *mock* samples.
4. When done with all samples, add 500 µL of Extraction Buffer 2 to each of the 2 mL tubes.
5. Vortex all tubes for 10 minutes at 4°C and, immediately after, centrifuge for 10 minutes at 4°C and maximum speed. After centrifugation, three distinct phases will be distinguishable in each tube: an

upper (less dense) phase consisting of Extraction Buffer 1 (containing polar metabolites), a lower (denser) phase consisting of Extraction Buffer 2 (containing non-polar metabolites, i.e. fatty acids), and a middle, whitish layer containing proteins and nucleic acids (absent in *mock* samples).

6. Working one tube at a time, transfer the upper phase to the corresponding PM-labeled tube (see Note 51) then the lower phase to the corresponding FA-labeled tube (see Note 52). Keep the protein layer in the PROT-labeled tube, and move all three tubes into dry ice.
7. When done processing all samples, dry them in a centrifugal vacuum concentrator (see Note 53), and either proceed directly with sample preparation for MS-based analysis, or store at -80°C until ready to do so.

3.7.2 Metabolite extraction from medium samples

Proceed analogously to the extraction of cell samples, replacing steps 2 and 3 with the following:

2. After labeling all tubes, thaw and add 20 µL or 200 µL (for the analysis of polar-metabolite or fatty-acid uptake/secretion, respectively) of each of the medium samples collected in section 3.6 into the corresponding PROT-labeled 2 mL tubes (do not bring into dry ice). For *mock* samples, add instead an identical volume of Milli-Q water.
3. Add 800 µL of Extraction Buffer 1 into each of the tubes, then proceed with steps 4–7 above.

3.7.3 Metabolite extraction from standard-curve samples

To determine absolute metabolite abundances in the extracted samples (e.g. for the further determination of metabolic fluxes, see 3.8), prepare a concentrated standard stock, including all metabolites of interest at their maximum expected concentration in an equivalent 20 µL volume. Then, perform 7 serial 2x dilutions, for a total of 8 standard curve points, and extract 20 µL of each of these samples identically to polar-metabolite medium samples (see 3.7.2).

3.8 Mass spectrometric analysis of extracted metabolites and further interpretation

A wide variety of mass spectrometry (MS)-based approaches have been previously described, both in the literature ***(26-28)*** and in other chapters of this book (see for instance Chapters 1-6,8,10), that are applicable for analyzing the cell and medium extracts obtained in section 3.7. These involve coupling MS with different

chromatographic techniques (gas or liquid chromatography), with or without prior chemical derivatization of the metabolite extracts. Each of these techniques is more or less appropriate for analyzing certain groups of metabolites, and therefore the final choice depends on the particular metabolic pathways being investigated. The end goal here should be, regardless of the chosen method, to extract the following information for the metabolites of interest: First, their intracellular levels, which can be normalized (after internal-standard normalization and background correction from the *mock* samples) to cell mass based on the protein content of the samples (determined by analyzing the protein layers using a protein quantification assay). Second, their ^{13}C-label distributions which, after appropriate correction of natural ^{13}C abundance, contain the information about the incorporation of tracer metabolites and the activity of metabolic pathways connected to them. Last, their extracellular levels in the cell-containing and cell-free medium samples which, combined with the cell densities at the start and end of the labeling experiment, can be used to infer net metabolite uptake and secretion rates. All the above information can be used to extract a semi-quantitative description of the metabolism of effector $CD8^+$ T cells under the different nutrient conditions investigated, and can be compared among those different conditions to assess how the availability of specific nutrients modulate the metabolism of effector $CD8^+$ T cells. Furthermore, integrating these data into a proper metabolic flux model, one can perform formal metabolic flux analysis (MFA) ***(24)***, providing a fully quantitative description of the differential activity of the intracellular metabolic pathways of interest under the various conditions investigated. Evidently, the readouts of these metabolic measurements can further be complemented with those of other measurements (e.g. the proliferation readout itself, or cytokine production, measured using ELISA and/or flow cytometry), to relate these changes in metabolism to changes in $CD8^+$ T-cell effector function.

4 Notes

1. The addition of HCl (either in water or conjugated to the amino acids) is needed to enable dissolving, at these high concentrations, some amino acids with limited pure-water solubilities, such as L-Cystine, L-Lysine, L-Ornithine, L-Tryptophan, and L-Tyrosine.
2. Having separate stocks for these 7 non-essential amino acids, as well as for L-Glutamine, D-Glucose, and Pyruvate, facilitates *pulling out* these metabolites from the standard BLM formulation. This enables studying the impact of the full/partial deprivation of these nutrients, as well as using these metabolites as ^{13}C-labeled tracers.

3. Aspartic acid is particularly hard to dissolve in room-temperature water. Therefore, pre-warm the Milli-Q water up to 37°C before adding the required amino acid amount, and vortex vigorously afterwards, until the mixture does not present any clear precipitates.
4. The glucose stock is prepared fresh to minimize the risk of yeast-contamination upon long term storage.
5. Phenol red is not present in the blood, it is only added here as a pH indicator, to visually monitor the integrity of the medium. Note that Phenol red has some estrogenic activity, and therefore its supplementation can have an impact on the culture of estrogen-sensitive cells ***(29)***.
6. Other mouse strains may also be used, provided that they do not inherently lack $CD8^+$ T cells.
7. If provided lyophilized, reconstitute at 100 µg/mL in sterile Milli-Q water + 0.1% BSA, aliquot, and store at -80°C. Once thawed, working IL-2 aliquots can be stored at 4°C for up to one week.
8. Clean all glassware thoroughly before using for the preparation/storage of solutions for downstream mass spectrometry (MS)-related applications. To do this, working in a chemical fume hood, thoroughly rinse every piece of glassware (i.e. glass bottles and pipets) once with the following solvent sequence (in the order listed): Milli-Q water, Isopropanol, MS-grade methanol, MS-grade hexane. After that, rinse one last time with the solvent that will be used with/stored in this glassware item and air-dry. Dispose of all solvent waste appropriately.
9. The use of narrow-tipped Pasteur pipettes (rather than conventional aspiration tips) and low-speed aspiration is meant to prevent accidental suction of the T-cell pellet upon aspiration, since the pellet will be more loosely attached than usual due to the addition of cold Quenching Buffer.
10. The use of a high centrifugal force of 2,000 g (cf. the typical 300 g for pelleting live T cells) is meant not only to speed up the pelleting process, but also to increase the compactness of the pellet (which, as mentioned above, will be more loosely attached than usual due to the addition of cold Quenching Buffer). In our experience, this centrifugal force is optimal to form a strong pellet after 1 minute centrifugation, while preserving the integrity of the cells.
11. Here it is key to start by adding the Milli-Q water and only then diluting in the inorganic salt and bicarbonate stocks, otherwise a precipitation of inorganic salts may occur upon mixing such highly concentrated stocks.
12. The target pH of 6.9 compensates for the fact that the pH will increase upon the forthcoming two rounds of filtering to which the media will be subject, which will bring it to physiological (pH 7.4) values. As a rule of

thumb, and based on the Phenol Red indicator, the medium should look slightly orange (similar in color to RPMI 1640) right after pH adjustment to 6.9, and bright red after those two rounds of filtering.

13. Typically, the medium will be slightly basic (pH ~ 7.8) before pH adjustment. Therefore, addition of a small volume of 12M HCl will usually be needed. Add this volume slowly and dropwise, in 20–50 µL increments, to prevent over-acidification and splashing, and wait for pH reading equilibration after each addition. Always work under a chemical fume hood, and wear a lab coat, gloves, and protective glasses, when handling concentrated HCl and NaOH solutions.
14. Here, we denote by *base BLM* a medium formulation lacking supplements such as serum, antibiotics, and β-Mercaptoethanol, while we reserve the designation *standard* (or *customized*) *BLM* for the final medium used for cell culture. Our *base* formulation can be seen as an analog to commercially purchased media (e.g. RPMI 1640), where the above supplements are typically added just prior to use in culture. The different storage recommendations for the *base* and *standard* (or *customized*) *BLM* formulations are due to the presence of FBS in the latter but not in the former. It is known that interaction with FBS components may lead to a faster degradation of certain metabolites (e.g. L-Glutamine), even at 4°C, so long-term storage of FBS-containing media is not suggested for experiments where a stable medium composition is important.
15. β-Mercaptoethanol is not present in the blood, but is traditionally supplemented in T-cell cultures due to its antioxidant features, which are required for the appropriate maintenance of these cells *in vitro*.
16. The joint concentration of sodium and potassium salts (the most abundant components) in standard BLM is around 130 mM (see Table 5), corresponding to an osmolarity of 260 mOsm/L. Therefore, even a full depletion of glucose (the most abundant component after those salts, at 5 mM) will only alter the osmolarity of the medium by less than 2%. Hence, potential osmolarity changes due to medium customization are minor and therefore are ignored.
17. The use of dialyzed FBS is needed here in order to remove all non-labeled sources of the tracing metabolite of interest from the medium.
18. Make sure the strainer does not make a perfect seal with the walls of the tube, otherwise the liquid will not go through it due to overpressure inside the tube.
19. Make sure the cells are properly resuspended, to avoid clumps that will hinder the red blood cell lysis process.
20. To homogenize a cell suspension, pipet up and down a few times with a 200–1000 µL pipette set to 1 mL.

21. You should expect to get between 100–150 x 10^6 splenocytes from an 8–12 week-old C57BL/6N spleen.
22. If using a magnetic bead-based enrichment kit, the last step consists in collecting the enriched naïve $CD8^+$ T-cell fraction in a tube for further downstream use. For this, we recommend collecting in a 15 mL conical centrifuge tube, since this will facilitate the next steps in the protocol. Also, we suggest to avoid pooling cell fractions obtained from subsequent isolation steps performed on the same splenocyte sample, since in our experience this does not provide substantial gains in cell yield, while presenting the risk of reducing the purity of the collected cells. Instead, go with a single isolation step, and discard the cells remaining in the isolation tube after it.
23. Here we coat the plate with 0.5 µg/well of anti-CD3. Since cells will be later seeded at 200 µL/well, the effective concentration of anti- CD3ε during stimulation is 2,5 µg/mL, 5 times higher than for anti-CD28. Note that the use of non-treated culture plates will lead to a decreased efficiency in the anti-CD3ε coating of the plate, thus it is important to work with tissue-culture treated plates in this step.
24. Whenever possible, avoid using the 36 wells in the outer rim of 96-well plates, in order to prevent the so-called “edge effect”, by which medium will evaporate faster from these outer wells, leading to inconsistent results for the cells present in them. Filling both the outer rim wells and the inter-well spaces with PBS will decrease medium evaporation, further helping in preventing this effect from affecting other wells.
25. You should expect to get between 4–5 x 10^6 naïve $CD8^+$ T cells from an 8–12 week-old C57BL/6N spleen. This should be enough to fill up to 40–50 wells of a 96-well plate at a density of 10^5 cells/well.
26. We find a seeding density of 0.5 x 10^6 cells/mL to be optimal for activation in a round-bottom 96-well plate when using BLM as the activation medium. Higher densities (e.g. 10^6 cells/mL) may lead to the cells running out of nutrients during the 72 h activation (despite the daily 50% medium replacement). Lower densities may in turn reduce cell-cell interactions needed for an optimal activation.
27. Mix by gently flipping the tube upside-down a few times, and keep the tube capped in the flow until seeding.
28. Depending on the exact number of cells, choose the wells for the seeding so that they are arranged in a rectangular fashion (to facilitate the next steps in the protocol) and are located as far as possible from the plate edges (to minimize solvent evaporation). Always plan for 2–3 wells less than expected from your total cell counts, to compensate for pipetting inaccuracies.

29. After only a few hours, cells will be fully settled at the round bottom of the wells. In addition, the anti-CD3ε coating will lead to the cells slightly adhering to the wells, which facilitates medium removal/addition. In any case, be as careful as possible when removing/adding medium, so as to minimize the risk of losing and/or perturbing the cells. To do so, hold the multichannel pipette at a 45° angle relative to the plate, and let the pipette tips rest against the walls of the wells, right at the union with the round bottom, when removing medium. Proceed in a similar fashion for adding medium, this time letting the tips rest at the top of the wells' walls.
30. Here we replace 50% of the medium daily to prevent certain nutrients (specifically glucose and glutamine) from depleting over the course of the 72 h of activation.
31. Assess the quality of the activation by monitoring the changes in morphology experienced by the cells under a microscope. Upon full activation, cells will look roughly 2 times bigger in size and more irregularly shaped than when initially seeded as smaller, spherical naïve cells. In addition, cells will form clearly distinguishable clumps. You should also expect the medium color to shift towards yellow after the activation, as a result of lactic acid production by the activated cells.
32. You should expect your $CD8^+$ T-cell population to at least double during the 72 h activation, so that you will end up with at least 8–10 x 10^6 activated $CD8^+$ T cells starting from an 8–12 week-old C57BL/6N spleen. This should be enough to fill at least 80–100 wells at a density of 10^5 cells/well, and therefore you will need at least two 96-well plates at the beginning of the differentiation (provided you use all cells and stick to the 60 inner wells).
33. As in the case of activation, we find a seeding density of 0.5 x 10^6 cells/mL to be optimal for effector differentiation when using BLM as the differentiation medium and performing daily medium replacements. Higher densities (e.g. 10^6 cells/mL) may lead to medium over-acidification or to the cells running out of some nutrients after a 24 h time span. Lower densities in turn impose the burden of increasing the number of culture plates used for the differentiation, and thus the amount of work.
34. Expect your $CD8^+$ T-cell population to at least double during every 24 h differentiation period in standard BLM.
35. Based on our experience, a total of 72 h under 10 ng/mL IL-2 in standard BLM is sufficient to polarize activated $CD8^+$ T cells into an effector phenotype, characterized by high proliferation and high IFN-γ and TNF-α cytokine production. Here, the medium switch at 48 h differentiation (from standard BLM to the customized formulations corresponding to each condition to be further investigated) is meant to ensure

the full metabolic adaptation of the cells to the medium conditions under which their metabolism will be probed on the next step of the protocol.

36. Depending on the nutrient condition being investigated, your $CD8^+$ T-cell population may proliferate less than usual during these last 24 h of differentiation.
37. Here, we normally choose to seed the 3 mL with a 200–1000 µL pipette (3 x 1 mL pipettings), rather than using a larger (e.g. 5 mL) serological pipette, for increased accuracy. After seeding each plate, gently rock the plate back and forth and sideways over the surface of the flow, to improve homogeneity before incubating.
38. An accurate determination of the cell density both before and after the 24 h labeling experiment is key not only for a precise determination of cell proliferation rates, but also to later be able to infer metabolite uptake and secretion rates based on the metabolite levels of cell-containing and cell-free medium samples. Therefore, count twice on these steps, to help average out the technical variability due to the counting method used.
39. Similarly, the simultaneous incubation of cell-free medium for each condition investigated is required to further be able to accurately determine these metabolite uptake and secretion rates.
40. Metabolism is a rapidly adapting process. Therefore, it is critical to prepare all materials for this stage of the protocol ahead of time, and to work as fast as possible during the metabolic quenching. This will minimize perturbations to cellular metabolism, and result in more representative results. In particular, it is not necessary to work under sterile conditions in this stage of the protocol, which allows a more swift operation.
41. The use of 15 mL tubes here (rather than larger, 50 mL tubes) will lead to more compact cell pellets upon spin-down, which will further minimize the risk of accidental suction upon aspiration.
42. Label the tube caps instead of the bodies, to prevent the ink from washing out in the ethanol bath.
43. Work one well at a time. Here, we normally use a 200–1000 µL pipette set at 1 mL for the resuspension and transfer. This requires pipetting 3 times to transfer each well, but the resuspension is more gentle and the harvesting more accurate than with a larger (e.g. 5 mL) serological pipette, preventing the formation of bubbles and minimizing cell loss.
44. When resuspending the wells, tilt the plate at an angle towards you, so that the suspended cells concentrate at the bottom of the well. Then, draw liquid from the bottom of the well and pour it onto the top, paying attention to the well edges, to wash all cells down to the bottom. Repeat this 5–6 times and

then, keeping the plate tilted, transfer the 3 mL cell suspension from the bottom of the well into the 15 mL tube. Upon the last 1 mL transfer, pipet up and down a few times to homogenize the contents of the tube before sampling cells for counting.

45. Leave the tube lightly capped, to be able to easily manipulate it with one hand on the next steps. Do not insert it fully into the dry ice in the box, but rather just let it stand over it. Otherwise, the Quenching Buffer will cool down too much, which may lead to freezing of the carryover medium left in the cell-containing tubes upon pouring Quenching Buffer on them.
46. Work one 15 mL tube at a time. To speed up the process, use the 200–1000 µL pipette with the same tip for sampling the first 1 mL of medium and discarding the remaining 2 mL. Try to remove as much of the supernatant as possible, both on this step and during the subsequent two washes, to minimize medium carryover. However, give priority to working fast: there are two 10–15 mL washes ahead, so even if 100 µL of liquid remain on each wash, this will amount to at most an equivalent 0.01 µL carryover medium in the final sample.
47. Try to pour the Quenching Buffer fast and directly onto the pellet. If pouring too gently and along the tube walls, the Quenching Buffer may not mix properly with the carryover medium. This may lead to the upper layer of medium freezing, preventing dilution of the carryover medium in subsequent washes.
48. Avoid dropping the tubes into the rack from a height, and prevent any violent movements, to minimize the risk of perturbing the cell pellet.
49. Since the caps contain your labels, put them aside in the same order as the tubes are in the rack, to be able to further identify the tubes.
50. Pipet up and down several times, alternating with scratching of the bottom of the 15 mL tube with the pipette tip, in order to fully detach the quenched cell pellet. If possible, work with the bottom of the 15 mL tube standing on dry ice, to minimize metabolite degradation. Use always inert-plastic, filterless tips, to prevent the undesired contamination of the sample with plastic and/or filter residues soluble in the strong solvents used for extraction.
51. To avoid disturbing the protein layer, do not transfer the whole phase, but rather leave some 50 µL excess. Any losses will be accounted for by the internal norvaline/glutarate standard.
52. To minimize disturbance of the protein layer, release the pipette plunger slightly before immersing into the tube, and press the resulting volume of air out as bubbles while crossing the layer into the lower phase.

Again, do not transfer the whole phase, but rather leave some 50 µL excess, since any losses will be accounted for by the internal C_{17} standard. Finally, be careful to not drag part (or all) of the protein layer while removing the pipette tip from the lower phase.

53. Polar metabolite samples should be dried for approximately 8 h at 4°C, to prevent metabolite degradation. Fatty acid and protein samples may be dried for approximately 1 h at 20°C. If drying cannot be carried out immediately, store the samples at -80°C and dry within the next 48 h.

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