REVIEW | Article in Press

Navigating metabolomics in the era of immunometabolism

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Received: 21 July 2026 / Accepted: 11 September 2026
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Abstract

Over the past decade, immunology has undergone a profound metabolic awakening. Long considered a hallmark of tumour cells, altered metabolic states are now recognised as a defining feature of immune cells, whose functions are intricately regulated by their bioenergetic and biosynthetic states. This evolving appreciation of immunometabolism has driven demand for analytical tools that can capture metabolic states in immune cells under diverse physiological and pathological conditions. Metabolomics, the comprehensive profiling of metabolites, provides a powerful means to interrogate immune cell metabolism and can offer real-time insights into pathway activity and cellular bioenergetics. However, for many immunologists, metabolomics remains a technically challenging and conceptually opaque area. In this review, we provide a simple guide for designing initial metabolomics experiments, outlining key methodological approaches and addressing common challenges along with potential solutions. Importantly, we present a step-by-step roadmap for interpreting nutrient tracing data, enabling readers to make sense of complex isotopologue datasets and draw meaningful conclusions. Not only will Immunologists be better able to interpret metabolomic data from others, but by lowering the barrier to entry, this review aims to empower them to confidently incorporate metabolomics into their research, ultimately advancing our understanding of immune cell function and its metabolic regulation.

Abbreviations

TCA	Tricarboxylic cycle
Oxphos	Oxidative phosphorylation
MS	Mass spectrometry
ATP	Adenosine triphosphate
PPP	Pentose phosphate pathway
NMR	Nuclear magnetic resonance
GC	Gas chromatography
LC	Liquid chromatography

The field of immunometabolism has evolved into a dominant area of research. There are now numerous excellent publications which clearly illustrate how immune cells adopt particular metabolic configurations to support and

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https://doi.org/10.1186/s12967-026-08998-y

Published online: 20 October 2026

regulate immune functions [1–7]. Forced adaption of a metabolic configuration, for example due to hypoxia or a local microenvironment change, may confer a particular phenotype or function [8–10]. These discoveries have established metabolism as both a driver and a constraint of immunity, creating an urgent need for approaches capable of interrogating metabolism accurately. Although genetic and transcriptional studies have provided important insights into immunometabolism, they cannot fully resolve how nutrients are utilised by immune cells. Metabolomics, particularly in combination with nutrient tracing, is required to study the flow of carbons into different fates, for example into the tricarboxylic acid cycle (TCA) cycle versus lipid synthesis, or indeed preferential use of a particular fuel in specific settings.

Many immunologists today are familiar with the basics of glucose metabolic pathways of glycolysis, TCA and oxidative phosphorylation (Oxphos) to generate adenosine triphosphate (ATP) within immune cells [1, 11] (Fig. 1). This review is written for those immunologists who are planning to undertake a deeper analysis of cellular metabolism to explain why their cell type of interest behaves as it does under certain conditions. The goals of this review are two-fold and will be discussed in Part 1 and Part 2 respectively: (1) to provide a simple guide to planning and designing initial metabolomics experiments, and (2) to provide a roadmap for interpreting and understanding metabolomic tracing data. Given the vast potential of metabolomics for many applications [12], we are specifically focussing on those nutrients most commonly employed in immunometabolism research,

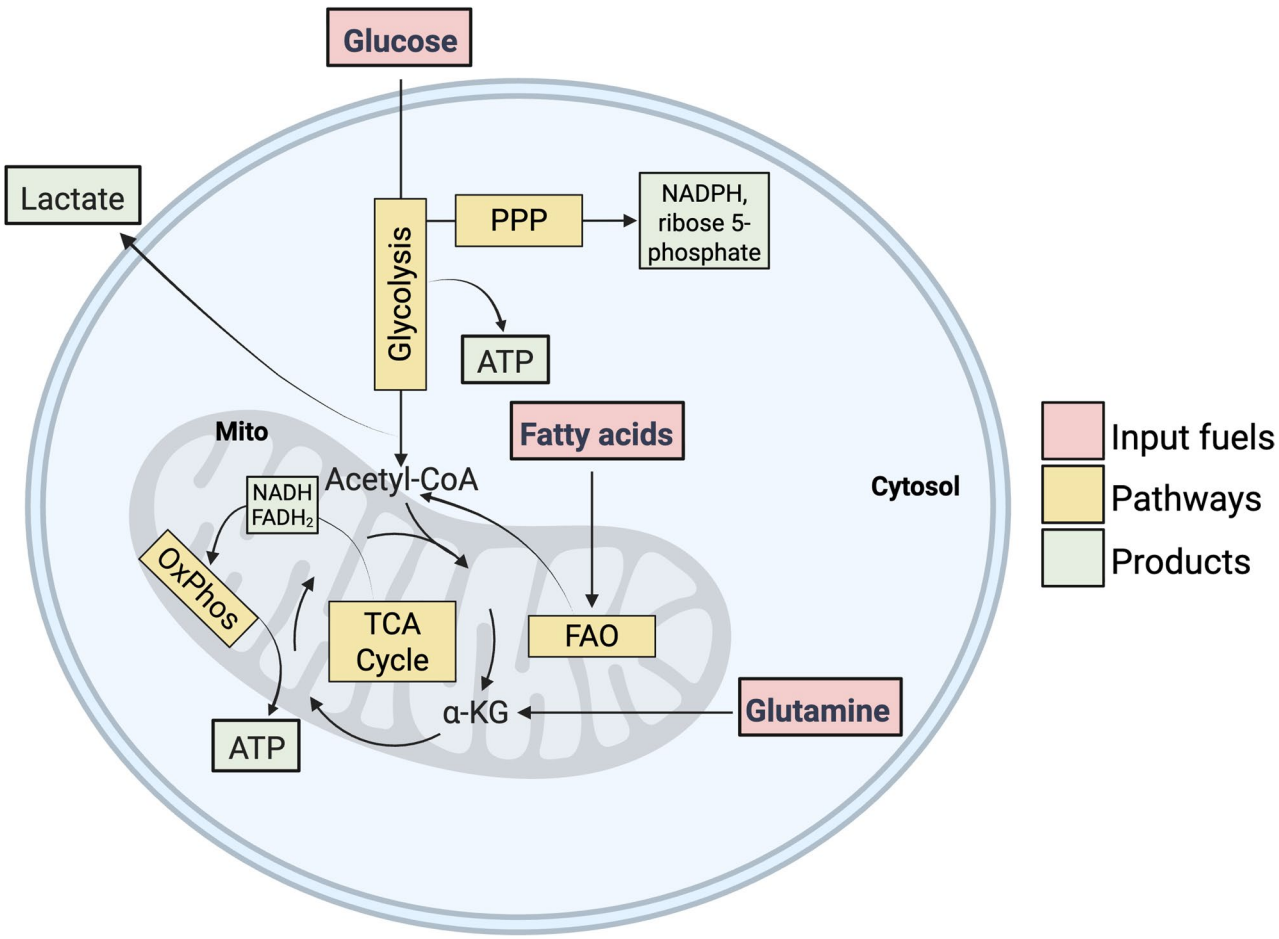


Fig. 1 The key pathways involved in glucose, fatty acid and glutamine catabolism in immune cells. Glucose is metabolised through glycolysis and/or the PPP in the cytosol, with key products including ATP, NADPH, acetyl CoA and lactate. Acetyl CoA feeds the TCA cycle in the mitochondria, producing the reducing equivalents NADH and FADH2, which are used by the electron transport chain during Oxphos for ATP production. Glutamine also fuels the TCA cycle through anaplerosis. Fatty acids are oxidised in the mitochondria, producing acetyl CoA which can enter the TCA cycle

specifically glucose and glutamine, although some more specialist applications are mentioned throughout. Additional information is provided in boxes throughout which can be referred to as required.

Metabolic analysis, often with the Agilent Seahorse platform (which couples specific metabolic inhibitors with correlates of glycolysis - pH change, or Oxphos - O_2 consumption), or more recently with flow cytometry based SCENITH (which uses *de novo* protein synthesis rates as a proxy for ATP production) [13, 14], facilitated progress in defining key pathways used by immune cells in given circumstances [15]. While this was a major advance, the intrinsic limitations of various technologies have researchers now turning to metabolomics for better answers. For example, 2-DG which inhibits the first step of glycolysis, is regularly used in Seahorse experiments where calculations associate small changes in the pH of the media with the cells' glycolytic capacity. However, 2-DG also inhibits the PPP so the relative contribution of the PPP potentially feeding back to glycolysis versus direct glycolysis to lactate production is not clear. Furthermore, 2-DG has historically been employed by biochemists to rapidly deplete intracellular stores of ATP [16] and the impact of this on interpretation of Seahorse data, particularly as glucose also fuels the TCA cycle and thus Oxphos, has not been clearly addressed. To answer specific questions about how fuels are used within immune cells, metabolomics can be used in a variety of ways, some of which are outlined below. Most immunologists will not carry out metabolomics in-house but subscribe to specialist service providers who can provide both technical and analytic support. Accordingly, before considering how to interpret metabolomic data, it is important to understand the key decisions that underpin a well-designed metabolomics experiment.

Part 1: A guide to planning metabolomics experiments

Experimental design

Careful experimental design is essential for generating metabolomics data that is both biologically meaningful and technically robust. Unlike some other molecular approaches, metabolomics is highly sensitive to factors such as sample collection, cell number, culture conditions, timing, and analytical methodology. Decisions made before an experiment begins can therefore have a major impact on data quality and interpretation. While metabolomic specialists can provide valuable technical guidance, investigators should first consider the biological questions they wish to address and the practical constraints of their system. The following sections outline key considerations when designing metabolomics and stable isotope tracing experiments.

What is your research question?

While it may sound trivial, spending time thinking about what you want out of the experimental dataset is the most important step in planning a metabolomics experiment. For example, whether you are testing a specific hypothesis or undertaking a more holistic/hypothesis generating project will impact which type of analysis is most suitable. Knowing what metabolites you want to measure will ensure the appropriate extraction and analysis method can be used and the specialist can provide additional advice to optimise your experiment.

How many cells are required?

Obtaining sufficient material for metabolomic analysis can be a major challenge in immunology, particularly when studying rare immune cell populations. The number of cells required depends largely on the metabolites of interest, their expected abundance, and the analytical platform being used. Considerations must also be made during stable isotope tracer analysis (Box 1) because any labelled metabolite signal become distributed across multiple isotopologues and progressively diluted as metabolites are processed through metabolic pathways. For analysis of immune cells, this is important given the requirement for a highly pure starting population of cells

Box 1 Stable isotope labelling

Stable isotope labelling involves using non-radioactive, stable isotopes as metabolic tracers [17–19]. Isotopes are versions of a chemical element such as carbon or nitrogen, with the same number of protons but a unique number of neutrons in the nucleus resulting in a unique atomic mass. This unique mass allows for detection of specific isotopes using MS or NMR. For example, the most abundant carbon isotope contains 6 protons and 6 neutrons, resulting in an atomic weight of 12, and it is therefore denoted carbon-12 (^{12}C). While the ^{12}C isotope accounts for ~99% of the carbon on Earth, carbon-13 (^{13}C), which contains 6 protons and 7 neutrons forms the remaining 1% [20]. In immunometabolism research, nutrients such as glucose containing ^{13}C in place of ^{12}C can be purchased commercially and added to cell cultures so the fate

of these ^{13}-carbons can be mapped throughout downstream metabolic pathways [21]. As ^{13}C is heavier than ^{12}C, stable isotope labelling is also referred to as 'heavy labelling'. Other available isotopes include ^{5}N (useful in amino acid and nucleotide tracing), ^{2}H (redox reactions), ^{18}O (phosphates), and ^{34}S (cysteine, methionine and other sulphur- containing compounds) [22]. The technique is specifically called 'stable' isotope labelling as many elements also have unstable isotopes, however these are not used in metabolomic tracing analysis.

and the technical limitations associated of purifying enough of a rare cell type. For many LC-MS analyses of lymphocytes, 1–2 million cells are often sufficient, although the optimal cell number should be determined in consultation with the analytical facility and may vary according to the experimental design and metabolites under investigation. An early pilot experiment will ensure there is sufficient signal for detecting the metabolites of interest. It is important to note that immune activation is often accompanied by increased cell size, which can result in higher absolute metabolite abundance independent of changes in metabolic activity. Therefore, when comparing metabolite levels between cells of different sizes or activation states, appropriate normalisation (e.g. to protein content) may be required to accurately interpret metabolic differences.

How many samples should be included?

It is generally preferable to prioritise biological replicates over technical replicates in metabolomics to increase statistical power. Strategic thinking about the correct experimental comparisons and the number of biological repeats for each experiment will help estimate the number and size of samples possible/required. Given the low sensitivity of mass spectrometry (MS) and the technical process associated with it, there is the potential for occasional problems and extra replicates are advised. It is also worth considering that subtle changes in metabolite levels can be highly biologically relevant as compared to fold changes in other RNA or DNA 'omics' approaches that benefit from amplification. Therefore, the more powered the study, the more likely the investment will pay off. Once sample numbers have been considered, the next decision is whether the experiment aims to measure metabolite abundance alone or to investigate metabolic pathway activity using a labelled tracer nutrient.

Labelled or unlabelled analysis?

The simplest and cheapest approach involves unlabelled metabolomics, so called as it does not involve any isotope labelling. With guidance regarding sample harvesting from the MS facility, experiments can be carried out as usual. Metabolites are measured, and relative amounts can be compared under different experimental conditions. Care and consideration must be taken to keep samples cold when processing to reduce variability associated with extractions. Steady-state analysis (no isotope tracer) can be useful to determine how the amount of metabolite(s) changes in your experimental conditions e.g. an increase in abundance of many of the TCA intermediates might suggest that the TCA cycle is upregulated in your experiment. This type of analysis can give a metabolic 'snapshot' but does not indicate traffic or use of a particular pathway. The key limitation is that unlabelled analysis limits specific conclusions e.g. changes in the amount of α-ketoglutarate are just that, and do not identify a source for that change.

If the goal of the research is to understand how a particular starting fuel such as glucose is used within cells, labelled metabolomics, also known as stable isotope tracer analysis, can be very informative (Box 1). For glucose

tracing, a heavy-labelled $^{13}C_6$-glucose is purchased and added to glucose-free cell culture medium. ^{13}C-labelling provides a specific fingerprint for the carbons provided through $^{13}C_6$-glucose as MS can readily distinguish ^{12}C and ^{13}C. Any ^{13}C signal in metabolites can only come from the glucose provided at the start of the experiment e.g. if there is a ^{13}C labelled specific TCA intermediate e.g. citrate, this proves that citrate derives from glucose under those conditions. Comparing the amount of labelled and unlabelled amounts of a given metabolite provides information on the relative contribution of the provided fuel source. For example, if the pool of citrate after glucose tracing is 90% ^{13}-carbon labelled, then glucose is a key source of the citrate pool under those experimental conditions. For most nutrient tracing experiments, dialysed FCS should be used in place of normal FCS to minimise the introduction of unwanted sources of unlabelled nutrients.

For many experiments, a universally (U) labelled fuel source is used. For glucose which contains six carbons, this means that each of these ^{12}C are replaced with ^{13}C i.e. the glucose tracer contains six ^{13}C molecules; this is often abbreviated to U-^{13}C-glucose. Similarly, glutamine which has five carbons can have each of those five carbons replaced with the equivalent ^{13}C versions to give U-^{13}C-glutamine (gln). In fact, a whole range of substrate sources including palmitate and amino acids [4, 23] are now being investigated using carbon-based tracing experiments. Evidence is mounting regarding the use of alternative fuels such as acetate or ketone bodies by immune cells, which can also be used for metabolic tracing experiments. This is generally experienced when cells are stressed e.g. use of acetate or lactate rather than glucose in a tumour microenvironment when glucose is limiting, or limited access to conventional fuel sources e.g. in the bone marrow niche [24, 25]. Tracing of alternative metabolites such as heavy labelled serine can also be used to demonstrate the importance of different metabolic pathways such as the serine-glycine-one-carbon (SGOC) for T cell expansion [26].

In tracing experiments, there are usually very specific and predictable labelling patterns expected based on classical biochemistry [17], some of which are detailed below in Part 2. However, sometimes a more uniquely labelled substrate can be used to glean important information. For example, universally labelled ^{13}C-glucose does not help distinguish glycolysis and the PPP, in part due to the crosstalk and overlap between the two pathways. However, using 1,2^{13}C-glucose (in which only the first two carbons of glucose are labelled) allows pathways to be distinguished based on a biochemical understanding of how glucose is differentially processed by either glycolysis of the PPP enzymatic reactions (Fig. 3).

Although carbon tracers are the most widely used in immunometabolism, other isotope-labelled elements can provide complementary information. For example, introduction of ^{15}N-labelled substrates (usually ^{14}N predominates) enables tracking of amino-group transfer through transaminase reactions and *de novo* nucleotide synthesis, particularly from glutamine. These approaches complement conventional ^{13}C tracing by directly resolving the movement of nitrogen atoms into amino acids, nucleotides, and other nitrogen-containing metabolites, providing insights that cannot be obtained from carbon tracing alone [22, 27, 28]. Deuterium (^{2}H) labelled compounds are also frequently used to trace hydrogen transfer and biosynthetic activity. However, caution should be taken with interpretation as the deuterium–carbon bond is stronger (and heavier) than the hydrogen-carbon bond and this can impact on the reaction kinetics and thus metabolism.

As these stable isotopes occur in nature, albeit at a low frequency, a control sample containing unlabelled substrate should always be run in parallel to ensure that heavy labelling is true and to facilitate correction for natural isotope abundance during data analysis. Indeed, different elemental isotopes (e.g. C, N, H) vary in natural abundance, for example, as ^{13}C accounts for 1% of C atoms and this must be corrected for in analysis [17].

What timepoints should be chosen?

Regardless of whether a certain tracer is used, experimental timing is one of the most important determinants of the biological information obtained. Metabolites that play roles in multiple pathways may be siphoned away to meet other needs of a cell. Furthermore, metabolic pathways are characterised by flux, i.e. rate of turnover of metabolites through a pathway, and different pathways have different characteristic rates of flux. Glycolysis has a much higher rate of flux compared to TCA and Oxphos [29, 30] so shorter times may be more relevant

for analysis of glycolytic intermediates. As a general guide, labelled metabolic outputs from glycolysis and PPP can often be detected within minutes to a few hours, TCA cycle and glutaminolysis outputs from approximately 1–24 h while lipid metabolic outputs from approximately 6–48 h [12]. However, this will vary depending on cell type, cell source and experimental conditions [31, 32]. At longer times (when isotopic steady state is reached), the percent of labelled substrate will be driven mainly by substrate selection without influence from rate of turnover. Importantly, a single timepoint provides only a snapshot of metabolite abundance at that moment and cannot directly quantify metabolic flux. Analysis of multiple timepoints is therefore strongly recommended when investigating pathway activity and nutrient utilisation.

For tracing experiments, where following specific heavy-labelled carbon atoms is a goal, timing is key for successful interpretation. Following predicted labelling patterns of metabolites generally works very well for linear metabolic pathways with a labelled source material, e.g. $^{13}C_6$-glucose for glycolytic pathway utilisation. However, cyclical pathways or where there is crosstalk between pathways can make tracing analysis become complicated quite quickly. While following glucose into the TCA generates predictable metabolites for the first turn through of the TCA, a second cycle now has (i) labelled carbons coming from the first turn of the TCA cycle which is joined by (ii) labelled carbons that enter for the first time (discussed in more detail in Part 2). There is the further complication of anapleurotic supplementation of the TCA from other pathways such as glutaminolysis [4], which can dilute and disrupt predicted labelled carbon cycling. Picking a time point that captures 1–2 metabolic cycles of label is often optimal [17].

The value of supernatant analysis

In immunometabolism, metabolic pathways of interest generally occur inside cells and most measurements related to metabolites and pathways are based on cell extracts. However, cell supernatants can also provide important information on the exchange of metabolites between cells and their environment. For example, lactate, the end product of glycolysis is secreted from cells. Therefore, measurement of lactate is most relevant in the supernatant rather than in the cell extract and thus the supernatant is more relevant for defining PPP versus glycolysis using 1,2^{13}C-glucose tracing [33] (Fig. 3). When considering immune cell interaction with other cells in e.g. tumour microenvironment, analysis of the extracellular metabolites may provide insight into nutrient competition and metabolic crosstalk between cell populations. It is also worth noting that while cellular metabolites turn over as part of a pathway, secreted metabolites accumulate over the experiment. For supernatant analysis, it is important to include any unspent sample (i.e. cell-free media) that has been treated in the same manner as your test samples to measure background levels of metabolites. Comparing metabolite levels between unspent and test sample supernatants can shed light on net metabolite uptake and secretion by the cells.

What technology to use: NMR vs. MS

There are many advanced technologies for analysis of metabolites of which MS and nuclear magnetic resonance (NMR) are most used. The MS specialist will help decide on which technology is best suited for the project and they will consider experimental parameters such as the concentration range of expected metabolites (fM to mM), their intrinsic chemistry (polar versus non-polar) and ability to separate metabolites e.g. neutral lipids from polar metabolites. NMR offers some advantages including identification of a large range of metabolites, position of stable isotope incorporation (Box 1) and absolute quantification, all without sample destruction [34]. The drawback is its low sensitivity, requiring more concentrated samples, and the high cost of instrumentation and robotics. Furthermore, while NMR can be used to identify many polar metabolites, this can get complicated quickly requiring advanced NMR techniques and analysis. As an immunologist, it is not required to understand the technical details e.g. derivatization of samples (explained in Box 2) but it is useful to understand the terminology and if your sample will survive (MS will destroy samples, NMR will not). Currently, metabolomics facilities tend to be equipped with both high-resolution mass spectrometers (e.g. quadrupole time of flight, orbitrap) and lower,

Box 2 Sample derivatization

Sample derivatization is a chemical process used to modify certain metabolites in a sample before analysis, which helps to make them easier to detect [35, 36]. Sample derivatization is often necessary before MS-based metabolomic analysis, for reasons including:

Enhancing Volatility: Many metabolites, especially polar compounds like sugars, amino acids, and organic acids, are non-volatile and cannot be analysed directly using gas chromatography-mass spectrometry (GC-MS). Derivatization improves their volatility, making them suitable for analysis.

Improving Thermal Stability: Some metabolites degrade at high temperatures. Derivatization can stabilise them, preventing decomposition during GC-MS analysis.

Increasing Ionization Efficiency: In LC-MS or GC-MS, certain metabolites ionize poorly. Derivatization introduces functional groups that enhance ionization, improving sensitivity and detection limits.

Enhancing Chromatographic Separation: Metabolites with similar structures may co-elute during chromatography, e.g. glucose 6-phosphate and fructose 6-phosphate, however derivatization can allow for their specific detection. Fatty acids are commonly converted to fatty acid methyl esters (FAMEs) prior to GC-MS analysis, which improves volatility and chromatographic separation [35].

Table 1 The strengths and weaknesses of metabolomics analysis platforms

Platform	Major strengths	Typical isotope-tracing applications	Limitations
LC-MS	High sensitivity, broad metabolome coverage, minimal derivatisation, excellent for polar metabolites and labile pathway intermediates. Widely used for ^{13}C and ^{15}N tracing.	Glycolysis, TCA cycle intermediates, amino acid metabolism, nucleotide biosynthesis, acyl-carnitines, coenzymes and lipidomics.	Some sugar phosphates and structural isomers may be challenging to resolve.
GC-MS	Excellent chromatographic resolution, highly reproducible electron-ionisation fragmentation patterns, extensive spectral libraries, strong performance for central carbon metabolism.	^{13}C tracing of sugars, organic acids, amino acids and fatty acids following derivatisation.	Requires derivatisation and is less suitable for many thermally labile metabolites.
NMR	Quantitative without external standards, non-destructive, provides positional information and direct observation of isotope incorporation. Particularly valuable for flux analysis and validation.	Positional ^{13}C enrichment, metabolic flux analysis, verification of isotopomer structures.	Lower sensitivity than MS-based methods, requiring larger sample amounts and often detecting fewer metabolites.

unit resolution mass spectrometers (e.g. single ortriple quadrupole, ion traps). See Table 1 for a summary of the major strengths, limitations and typical applications. Generally, high-resolution systems are used for hypothesis generating or untargeted experiments (metabolomics or lipidomics) as well as stable isotope tracer analysis while low resolution instruments are used for hypothesis driven or targeted metabolite profiling, and less frequently for absolute quantification.

What MS-based metabolomics is right for your experiment

LC-MS vs. GC-MS

The key consideration here is deciding which metabolites are most important for your research question as this will likely determine the method of analysis. For example, GC-MS has better resolution of phospho-hexoses, for example it can distinguish glucose-6-phosphate and fructose-6-phosphate. It can also be used for the lower half of glycolysis (3-carbon compounds), TCA cycle and amino acids with excellent reproducibility, but it requires derivatization of metabolites (Box 2) to improve volatility and stability [35]. LC-MS is usually a more direct procedure which can give data on glycolytic intermediates, PPP and TCA intermediates, nucleotides, amino acids and many more including polar compounds such as lipids. The GC-MS and LC-MS extraction procedures differ

https://doi.org/10.1186/s12967-026-08998-y

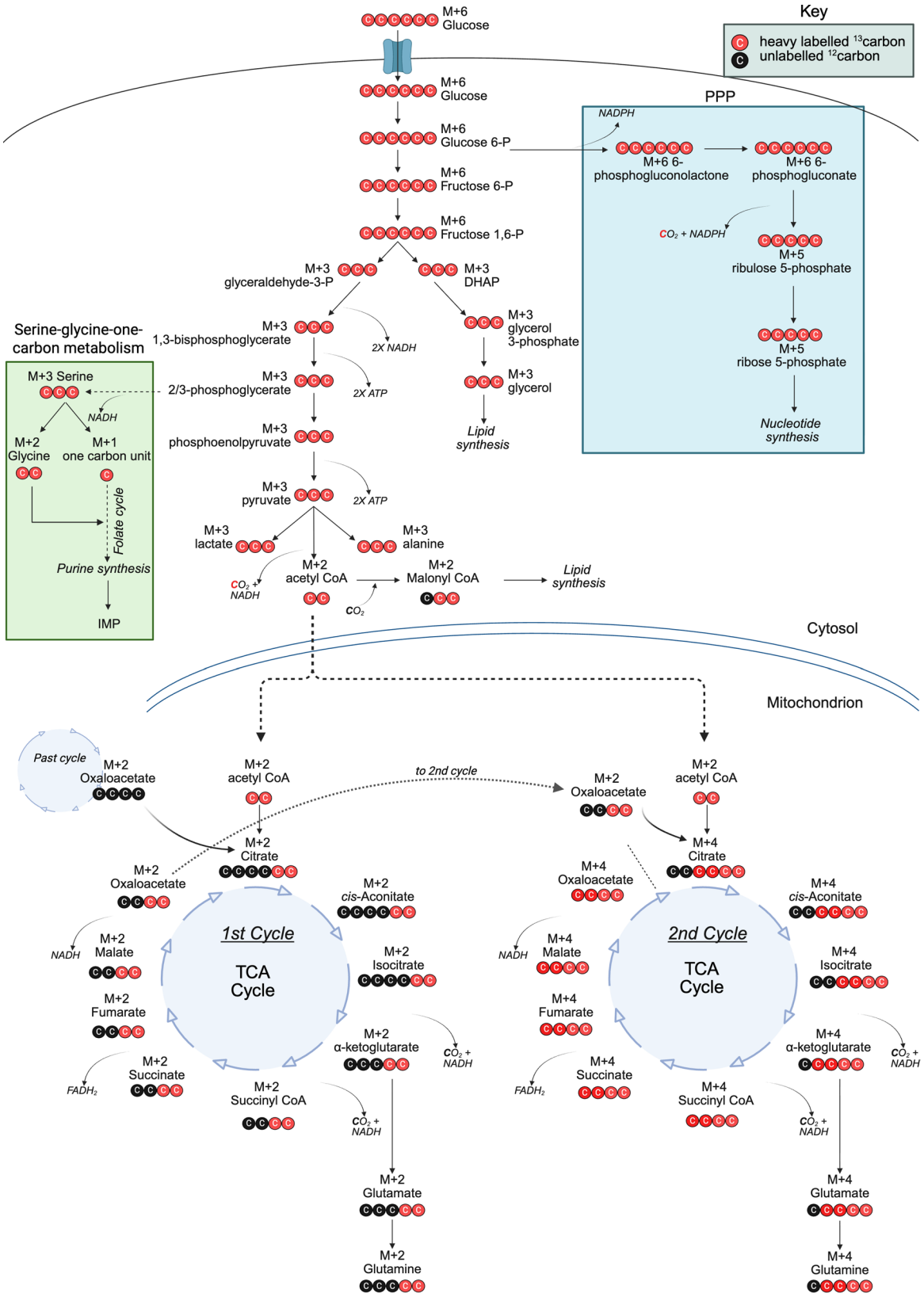


Fig. 2 Predicted labelling patterns in U-^{13}C glucose tracing. The flow of heavy labelled carbons (red) is indicated by the arrows. U-^{13}C-glucose breakdown through glycolysis is signified by M+6 upper glycolytic intermediates and M+3 lower glycolytic intermediates. Metabolism through the pentose phosphate pathway yields M+5 ribulose 5-phosphate and M+5 ribose 5-phosphate. When M+2 acetyl CoA, derived from glycolysis, enters the TCA cycle and condenses with unlabelled oxaloacetate, this yields M+2 TCA cycle intermediates. If M+2 acetyl CoA condenses with M+2 oxaloacetate, this yields M+4 TCA cycle intermediates. Note: the first entry of glucose-derived acetyl CoA into the TCA uses unlabelled oxaloacetate from a previous TCA cycle to generate citrate. P=phosphate, PPP=pentose phosphate pathway

and have been optimised accordingly. Ask for extraction and shipping methods appropriate for your samples (tissue, cells, plasma, other biofluid) and metabolites of interest.

Targeted vs. untargeted metabolomics

Most immunologists starting out are concerned with understanding what metabolic pathways are used in their cells, and what fuels are important for them e.g. while glucose is required for glycolysis, the TCA cycle can have input from many sources including glucose, glutamine, other amino acids and/or lipids (Fig. 1). Therefore, targeted metabolomics which has a pre-defined set of metabolites to measure is usually more useful. For those who want to engage in more 'discovery' type of analysis, then untargeted metabolomics may be a better option. Without a priori decisions regarding what metabolites to measure, the potential for discovering unexpected metabolites in unexpected places remains. However, proper validation of differential metabolites (for example with authentic standards or 'spike-in' experiments) should be done, and for lipidomics, this is expensive and requires highly specialist analysis.

Important caveats and limitations to consider

Unknown subcellular location

MS is very powerful and gives detailed and quantitative information on metabolites. However, it does not give any information as to where in the cell each metabolite is found. A key principle of biochemistry is the sequestration of metabolic pathways e.g. glycolysis occurs in the cytosol while the TCA cycle occurs in the mitochondria. However, many metabolic intermediates are used by numerous pathways depending on the cellular requirements at a given time. Pyruvate generated by glycolysis can pass into the mitochondria and enter the TCA cycle as acetyl-CoA or remain in the cytosol where it can either be used to make alanine or oxidised for secretion as lactate as the last step of glycolysis [37]. Therefore, a pyruvate signature from metabolomics in isolation does not provide information as to where it is or what the cell is using it for. Similarly, a strong glutamate signature does not inform as to whether this is in the mitochondria (where it can replenish the TCA) or in the cytosol (e.g. for amino acid, pyrimidine or polyamine synthesis).

Difficulty in separating structurally similar metabolites

MS struggles to distinguish isomers of some molecules (compounds that have the same molecular formula but are structurally different) which can be problematic depending on the research question. For example, hexose sugars (6 carbon sugars) can be detected but unless separated before detection (Box 2), MS does not distinguish between glucose-6-phosphate and fructose-6-phosphate. When compounds like this co-elute, they are frequently reported together. For most purposes, measuring glycolysis pathway utilisation is more important than identifying each of individual phospho-sugar species. Therefore, MS reporting of glycolytic intermediates often has hexose sugars, or indeed 2/3-phosphoglycerate reported together. Measuring branch point metabolites such as 3-phosphoglycerate (coming from PPP or glycolysis) which can then go towards the generation of other metabolites (towards purines, pyrimidines or lipid production, for example) is important (Fig. 2, green box).

Instability of certain metabolites

Metabolites differ in stability and half-life. While choosing the best MS approach can help, low abundance of a particular metabolite may be due to a variety of factors. For example, stability (e.g. oxaloacetate is unstable and rarely detected), a high rate of use or a technical issue with detection such as surviving the high temperatures of GC-MS or ionizing efficiency in electrospray ionization LC-MS. Therefore, pathways should be considered more holistically. Interesting and unexpected findings should be validated with a variety of approaches.

Impact of unlabelled nutrients

The composition of tracing media requires careful consideration, as unlabelled nutrients can substantially dilute tracer incorporation into downstream metabolites [38]. For example, the presence of unlabelled pyruvate may reduce the enrichment of glucose-derived carbon entering the TCA cycle, complicating interpretation of glucose-tracing experiments. Where the objective is to quantify glucose contribution to mitochondrial metabolism, pyruvate is therefore often omitted from tracing media. Similarly, for fatty acid tracing experiments such as using labelled palmitate, using lipid-free serum and conjugating the tracer to fatty-acid-free albumin will minimise background fatty acid contamination and improve accurate interpretation of isotopic enrichment.

The considerations above will help generate robust metabolomic datasets. We next discuss how these data are interpreted, with a particular focus on stable-isotope tracing experiments.

Part 2: Understanding and interpreting MS-based metabolomics data

The following section is for those who have either conducted their experiments and collected their data, or who wish to understand the metabolomics data that is increasingly found in the literature. It is a guide for interpreting metabolomic tracing data, particularly for glucose and glutamine tracing, which is arguably the most critical and challenging aspect of conducting metabolomic tracing analysis. With MS-based metabolomics, detailed data files are usually provided as part of your dataset. These include quality control information, e.g. good/weak/ambiguous peak for each metabolite detected in your samples. Depending on the type of MS analysis performed, different levels of detail are provided which are explained below. These include (i) total ion abundance (ii) % labelled metabolite and (iii) isotopologue analysis. In brief, total ion abundance gives the relative amount of a metabolite detected in a sample (unless internal standards, calibration curves and corrections for ion suppression are made). Where stable isotope tracing has been performed, the relative amount of total labelled and unlabelled metabolite (% labelled metabolite) can be calculated informing the proportion of an individual metabolite derived from the provided labelled precursor molecule. Finally, isotopologue analysis provides a deeper level of analysis by identifying how many ^{13}C-labelled carbons are in each metabolite measured (as a fraction of possible heavy labelled species). This will vary depending on the labelled compound given to cells and the activity of metabolic pathways that use the labelled substrate. Thus, if a labelled fuel is metabolised by two pathways, the relative use of each might be identified by differing isotopologue patterns (see PPP example below).

Glucose tracing

As glucose is the most commonly used tracer in immunometabolism, we first consider how U-^{13}C-glucose-derived carbons move through central carbon metabolism.

Glycolysis

U-^{13}C-glucose tracing is used to study the catabolism of glucose into various intermediates of central carbon metabolism pathways [4, 39, 40]. The best way to interpret this data is to understand how carbons flow through these pathways; where carbons are lost and where there are opportunities for entry of unlabelled carbons from other sources, or from already-labelled metabolites undergoing a second round of metabolism [12]. The flow of carbons through glycolysis is straight forward, and we recommend the reader to follow Fig. 2 while reading this section. The six carbon, U-^{13}C-glucose (isotopologue signature of M+6) is phosphorylated to yield the six carbon glucose-6-phosphate, which is then isomerised to the six carbon fructose-6-phosphate intermediate. Although it is sometimes possible to distinguish between them when samples are derivatised in a particular manner (Box 2), they are often difficult to resolve by chromatography and are therefore reported together as M+6 hexose 6-phosphate. Following this, the six carbon sugar is cleaved, producing two three carbon sugar phosphates; dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate, each having an M+3 signature. Glyceraldehyde 3-phosphate is oxidised to produce M+3 1,3-bisphosphoglycerate, yielding 2 NADH molecules. 1,3-bisphosphoglycerate is phosphorylated to produce 3-phosphorglyerate, yielding 2 ATP molecules, which is subsequently isomerised to 2-phosphorglyerate. Again, if the chromatography used cannot resolve these isomers (they are generally resolved by GC-MS), then they are interpreted together as M+3 2/3-phosphoglycerate. 2-phosphoglyerate is converted to M+3 phosphoenolpyruvate, and finally, phosphoenolpyruvate is dephosphorylated to M+3 pyruvate, yielding 2 more ATP molecules. Overall, U-^{13}C glucose is traced through glycolysis by monitoring M+6 hexose-6-phosphate and M+3 for all downstream intermediates. The presence of heavy labelled carbons in any metabolite means that, after corrections for natural abundance, it derives from the provided U-^{13}C glucose.

One potential source of unlabelled glucose carbons entering this pathway is from glycogen stores-derived glucose which could enter as M+0 glucose-6-phosphate and therefore dilute labelling by glucose [41]. An interesting branching off point along this pathway is at phosphoglycerate, wherein M+3 3-phosphoglyerate can be converted to M+3 serine. Serine is subsequently converted to M+2 glycine, generating one-carbon units that enter the folate cycle, linking glycolysis to the serine-glycine-one-carbon metabolism pathway. Together, glycine-derived carbons and folate-bound one-carbon units contribute to *de novo* purine synthesis, resulting in labelled purine intermediates such as inosine monophosphate (IMP) [42, 43] (Fig. 2, green box).

PPP

The PPP branches out from glycolysis at the point of glucose-6-phosphate. 1,2-^{13} C-glucose tracing is preferable when investigating the PPP as it produces distinct labelling patterns in certain metabolites such as phosphofructokinase or 3-phosphoglycerate which can be used to distinguish glycolytic versus PPP-mediated carbon breakdown, as they have a characteristic M+2 signature from glycolysis compared to an M+1 signature from the PPP (although crosstalk can complicate this) [18, 33, 34]. In 1,2-^{13} C-glucose tracing, secreted lactate may be more useful to measure as it has an M+2 signature when derived from glycolysis but an M+1 signature when derived from the PPP pathway (Fig. 3).

Nonetheless, it is still possible to gain some insight into glucose-fuelled PPP when using U-^{13}C glucose. M+6 glucose 6-phosphate is converted to the six carbon 6-phosphogluconolactone yielding 1 molecule of NADPH, which is then converted to 6-phosphogluconate (Fig. 2, blue box). 6-phosphogluconate is decarboxylated to ribulose 5-phosphate, yielding another molecule of NADPH and crucially, releasing 1 molecule of CO_2. This loss of 1 carbon changes the isotopologue signature from M+6 6-phosphogluconate to M+5 ribulose 5-phosphate. Ribulose 5-phosphate is then isomerised to ribose 5-phosphate, which is important in nucleotide synthesis. In general, identification of M+5 ribulose/ribose 5-phosphate signals that glucose is being used to fuel the PPP.

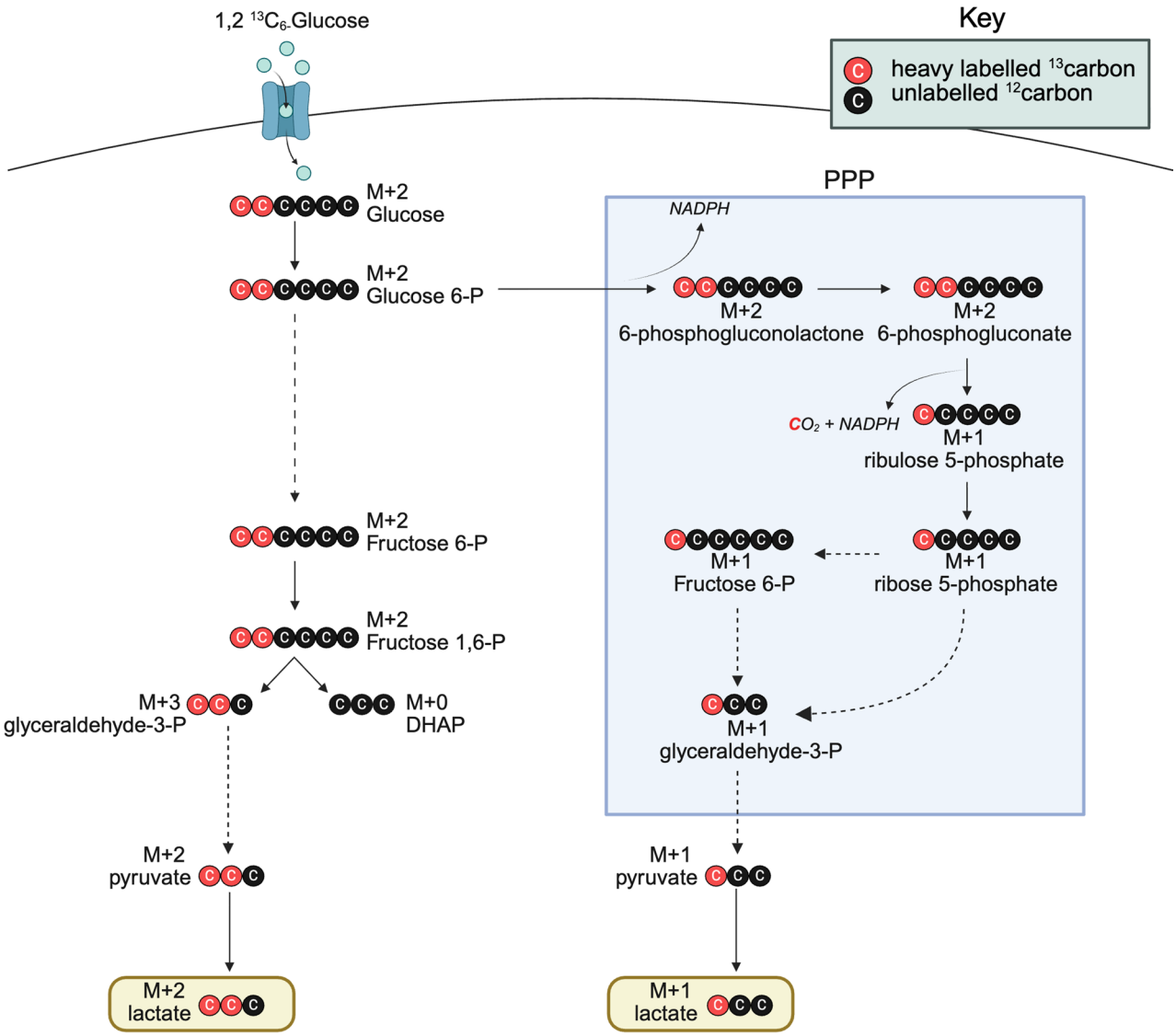


Fig. 3 1,2 ^{13}C glucose tracing distinguishes between glucose catabolism through glycolysis or the PPP. The flow of heavy labelled carbons (red) is indicated by the arrows. 1,2-^{13} C-glucose tracing leads to M+2 glucose 6-phosphate which enters the PPP to form M+2 6-phosphogluconolatone and M+2 6-phosphogluconate. M+2 6-phosphogluconate is decarboxylated to M+1 ribulose 5-phosphate which is converted to M+1 ribose 5-phosphate. Further metabolism yields M+1 fructose 6-phosphate and M+1 glyceralderhyde 3-phosphate. M+1 glyceralderhyde 3-phosphate is converted to M+1 pyruvate, which is further converted to M+1 lactate. P=phosphate, PPP=pentose phosphate pathway

TCA cycle and beyond

While the PPP diverts glucose-derived carbon toward NADPH production and nucleotide synthesis, most of the glucose-derived pyruvate continues towards mitochondrial metabolism and the TCA cycle. One common fate of pyruvate is decarboxylation to acetyl-CoA for entry into the TCA cycle [44] (Fig. 2). In this reaction, one carbon is lost through CO_2, resulting in a change in the isotopologue signature from M+3 pyruvate to M+2 acetyl CoA. This two carbon acetyl CoA molecule enters the TCA cycle in the mitochondria. Firstly, acetyl CoA is condensed with oxaloacetate (rarely measured) and H_2O to form a six carbon M+2 citrate molecule, where the 2 carbons from U-^{13}C glucose derived from acetyl CoA are labelled, but the 4 carbons from oxaloacetate are unlabelled. This citrate is converted to M+2 *cis*-aconitate and then to M+2 isocitrate. Isocitrate is decarboxylated to

α-ketoglutarate, yielding one molecule of NADH and releasing one molecule of unlabelled CO_2 (from original unlabelled oxaloacetate), resulting in a five carbon M+2 α-ketoglutarate. Similarly, α-ketoglutarate is decarboxylated to succinyl CoA, yielding one molecule of NADH and releasing one molecule of unlabelled CO_2, resulting in a four carbon M+2 succinyl CoA. No more carbons are lost in the remaining steps of the TCA cycle, wherein M+2 succinyl CoA is converted to M+2 succinate, then M+2 fumarate, then M+2 malate and finally M+2 oxaloacetate (Fig. 2).

The examples above describe the first turn of the TCA cycle, which is generally the simplest to interpret, however it gets more complicated during later cycles. As carbon atoms are rearranged during successive TCA cycle reactions, identical isotopologues can arise from different metabolic reactions. One scenario for the second cycle involves M+2 acetyl CoA condensing with M+2 oxaloacetate from the end of the first cycle, to form M+4 citrate. This then goes through the cycle of chemical reactions to yield M+4 TCA cycle intermediates (Fig. 2). Another scenario involves M+0 acetyl CoA (e.g. from FAO) condensing with M+2 oxaloacetate from the end of the first cycle, to form M+2 TCA cycle intermediates. During a third cycle, there are many possible combinations depending on the labelling pattern of acetyl CoA and oxaloacetate, and for M+2 oxaloacetate, this includes whether the first two carbons or the last two carbons contain the label (see [45] for further explanation). This progressive redistribution of label positions, often termed isotope scrambling, can make it difficult to uniquely infer pathway activity from mass isotopologue data alone and may limit the conclusions that can be drawn [17].

It is important to be aware that other nutrients commonly feed into the TCA cycle through anaplerosis, most commonly glutamine (Fig. 1) [4, 46], and this can dilute the labelling signal observed. Pyruvate can also feed directly into the TCA cycle through anaplerosis [47], wherein M+3 pyruvate is carboxylated, gaining one unlabelled carbon and forming M+3 oxaloacetate (rarely detected for technical reasons), which can be converted to M+3 malate and then M+3 fumarate (Fig. 4) [48]. Should this M+3 oxaloacetate continue in the TCA cycle and condense with M+2 acetyl CoA, this will result in M+5 citrate, cis-aconitate and isocitrate. Overall, while M+2 and M+4 labelling of TCA cycle intermediates suggest glucose fuelling of the first and second round of the TCA cycle respectively, it is important to be aware of the other ways carbon can enter and leave the TCA cycle, and the impact that experimental timing can have on different labelling patterns.

The malate-aspartate shuttle

After the first round of the TCA cycle, an alternative fate for M+2 oxaloacetate is entry to the malate-aspartate shuttle, which is important in transporting redox equivalents from the cytosol into the mitochondria [49, 50]. As NADH cannot cross the mitochondrial membrane, this shuttle is used to move its reducing power inside. M+2 oxaloacetate is converted M+2 aspartate (through the transamination of oxaloacetate and glutamate) which is exported to the cytosol (Fig. 5). Cytosolic M+2 aspartate is converted to M+2 oxaloacetate. NADH donates electrons to M+2 oxaloacetate, converting it into M+2 malate, which is then transported back into the mitochondria. M+2 malate now re-enters the TCA cycle and donates its electrons to NAD^+, regenerating NADH (which enters the electron transport chain to make ATP) and producing M+2 oxaloacetate.

Beyond glycolysis, the PPP and the TCA cycle, U-^{13}C-glucose tracing can provide insight into other, less familiar pathways. Glycerol, important in the synthesis of the certain lipids, can be produced from glucose via glycolysis. The three carbon M+3 DHAP is converted to M+3 glycerol 3-phosphate and then to M+3 glycerol (Fig. 2). Acetyl CoA can also contribute to lipid synthesis through a carboxylation reaction, converting the two carbon M+2 acetyl CoA to the three carbon M+2 malonyl CoA. The converse of this is also true, whereby lipids can contribute to the pool of M+0 acetyl CoA through fatty acid oxidation, and this has been demonstrated in NK cells using U-^{13}C-palmitate tracing [23]. Different amino acids can be synthesised at multiple points along these pathways, for example M+2 α-ketoglutarate, generated in the first round of the TCA cycle, can be converted to M+2 glutamate and then to M+2 glutamine (Fig. 2). Overall, U-^{13}C-glucose tracing is a powerful technique that provides mechanistic insights into how glucose is catabolised through central carbon metabolism pathways and

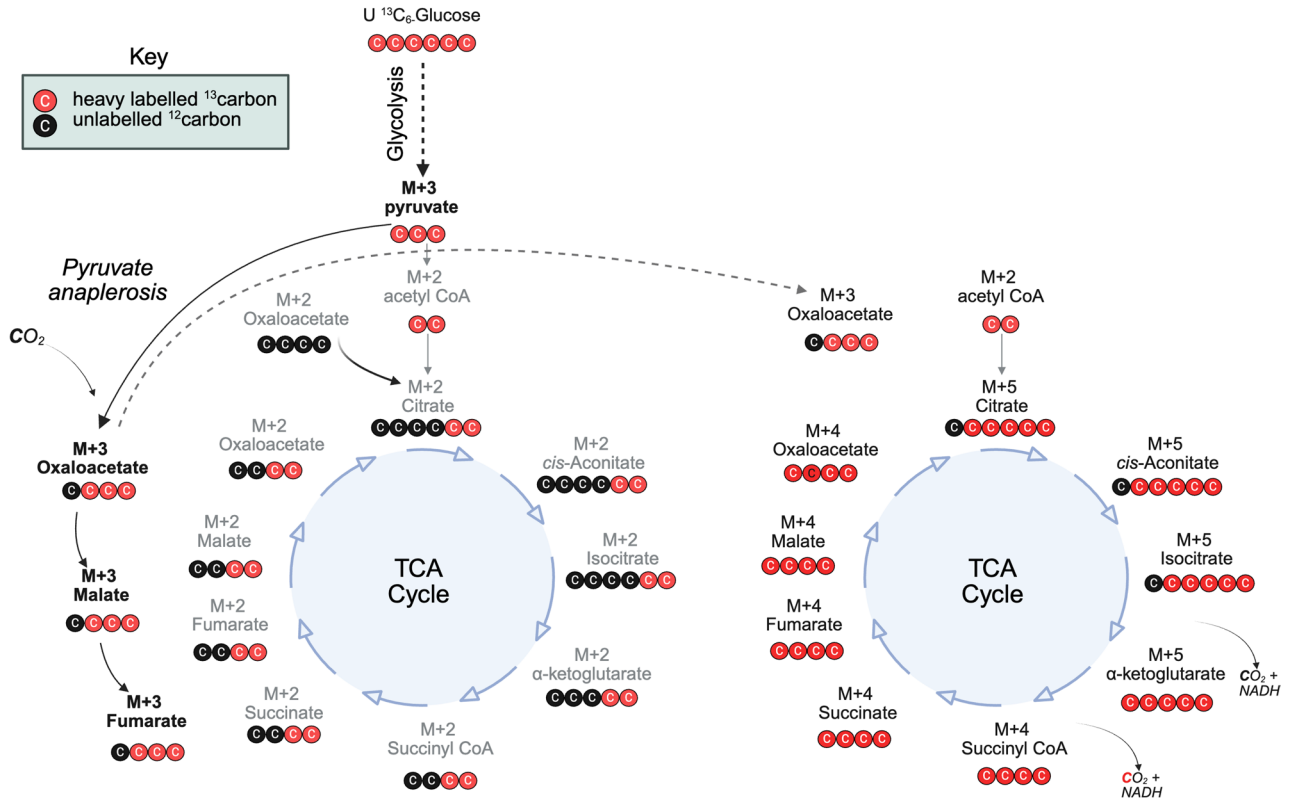


Fig. 4 Pyruvate anaplerosis feeds carbons into the TCA cycle and results in M+3 malate and fumarate in U-^{13}C glucose tracing experiments. The flow of heavy labelled carbons (red) is indicated by the arrows. M+4 pyruvate can enter the TCA cycle through pyruvate anaplerosis through carboxylation to M+3 oxaloacetate. M+3 oxaloacetate can then be converted into M+3 malate and M+3 fumarate. Should M+3 oxaloacetate condense with M+2 acetyl CoA, this will yield M+5 and M+4 TCA cycle intermediates

beyond. By understanding the flow of carbons through these pathways and their key outputs, we can appreciate the biochemical role that glucose metabolism plays in supporting the functions of the cells.

U-^{13}C-glutamine tracing

The gold standard for studying glutamine metabolism in immune cells is using U-^{13}C-glutamine tracing [2, 4, 51, 52]. Like U-^{13}C -glucose tracing, these data are best interpreted by understanding how labelled carbons derived from U-^{13}C-glutamine can enter and leave various metabolic pathways. For glutamine, this is often focused on anaplerosis of the TCA cycle in the mitochondria, although it is important to remember that the subcellular location of measured metabolites is unknown. Indeed, there are a wide variety of possible fates for U-^{13}C-glutamine-derived carbons and using isotopologue analysis to understand the movement of these carbons through relevant metabolic pathways we can gain insight into the molecular role that glutamine plays in fuelling the immune system.

Fully labelled, M+5 glutamine enters the TCA cycle by conversion to M+5 glutamate and then M+5 α-ketoglutarate (Fig. 6, main start point is indicated in pink box). During the TCA cycle, M+5 α-ketoglutarate is decarboxylated yielding 1 molecule of NADH and releasing 1 molecule of labelled CO_2, resulting in M+4 succinyl CoA. This M+4 isotopologue signature is maintained during the next steps TCA cycle (Fig. 6A), producing M+4 succinate, fumarate, malate, oxaloacetate, citrate, *cis*-aconitate and isocitrate [18]. Should the M+4 isocitrate continue cycling without entry of more labelled carbons, this will either yield the same labelling pattern, or else M+3 α-ketoglutarate and M+2 succinyl CoA, succinate, fumarate, malate and oxaloacetate, depending on

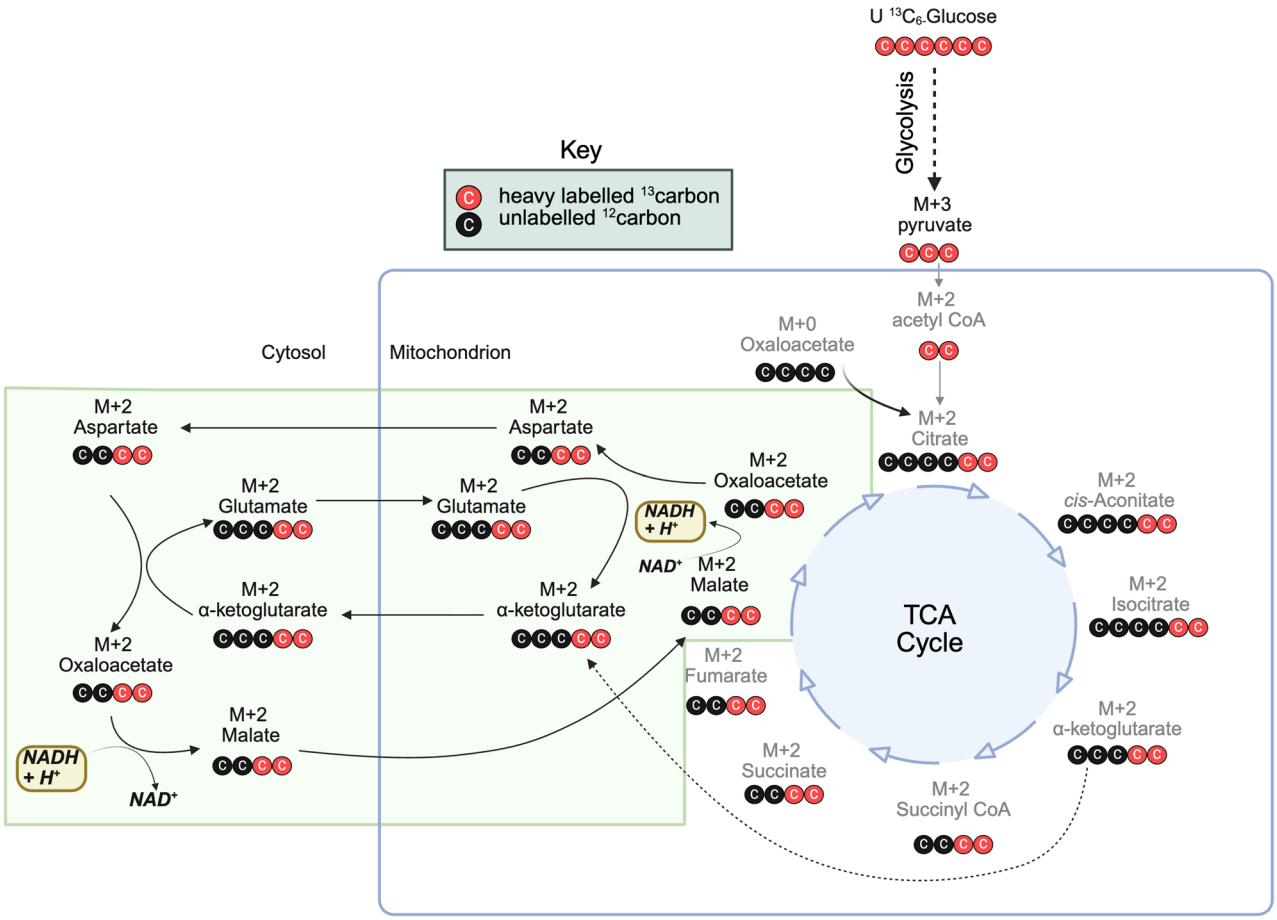


Fig. 5 The malate-aspartate shuttle transfers reducing equivalents from the cytosol into the mitochondria for oxidation. The flow of heavy labelled carbons (red) is indicated by the arrows. M+2 oxaloacetate is converted to M+2 aspartate, which is transported to the cytosol and converted into M+2 oxaloacetate. NADH donates electrons to M+2 oxaloacetate, converting it into M+2 malate, which is then transported back into the mitochondria. M+2 malate re-enters the TCA cycle and donates its electrons to NAD^+, regenerating NADH and producing M+2 oxaloacetate

which carbon is removed from the M+4 isocitrate derived from the first cycle. Of note, M+4 succinate can also derive from the GABA shunt, wherein M+5 glutamine is decarboxylated to M+4 GABA, and M+4 GABA is converted to M+4 succinate (Fig. 6). While common in neurons, this shunt has also been described in murine macrophages [53].

U-13 C-glutamine-derived M+5 α-ketoglutarate can undergo reductive carboxylation, gaining one unlabelled carbon from CO_2 to form M+5 isocitrate, M+5 *cis*-aconitate and M+5 citrate (Fig. 6, blue box). This process is catalysed by isocitrate dehydrogenase in the mitochondria (IDH2) or in the cytosol (IDH1) [54]. This citrate can then be exported to the cytosol, where it is cleaved to form one molecule of M+2 acetyl CoA and one molecule of M+3 oxaloacetate (Fig. 6, green box). The resulting acetyl CoA can either be carboxylated to form M+2 malonyl CoA, a key precursor for lipid synthesis, or participate in epigenetic acetylation reactions. M+3 oxaloacetate can be converted to M+3 malate which can re-enter the mitochondria and rejoin the TCA cycle. This loop from mitochondrial citrate to cytosolic oxaloacetate and then to mitochondrial malate is known as the citrate malate shuttle [40, 50]. TCA cycle-derived M+4 citrate can similarly enter the citrate malate shuttle, producing M+2 acetyl-CoA, M+2 oxaloacetate, and M+2 malate (Fig. 6, green box).

There are many other opportunities for U-^{13}C-glutamine-derived carbons to exit the TCA cycle [55], sometimes referred to as cataplerosis. M+4 malate can be transported to the cytosol and decarboxylated to M+3 pyruvate,

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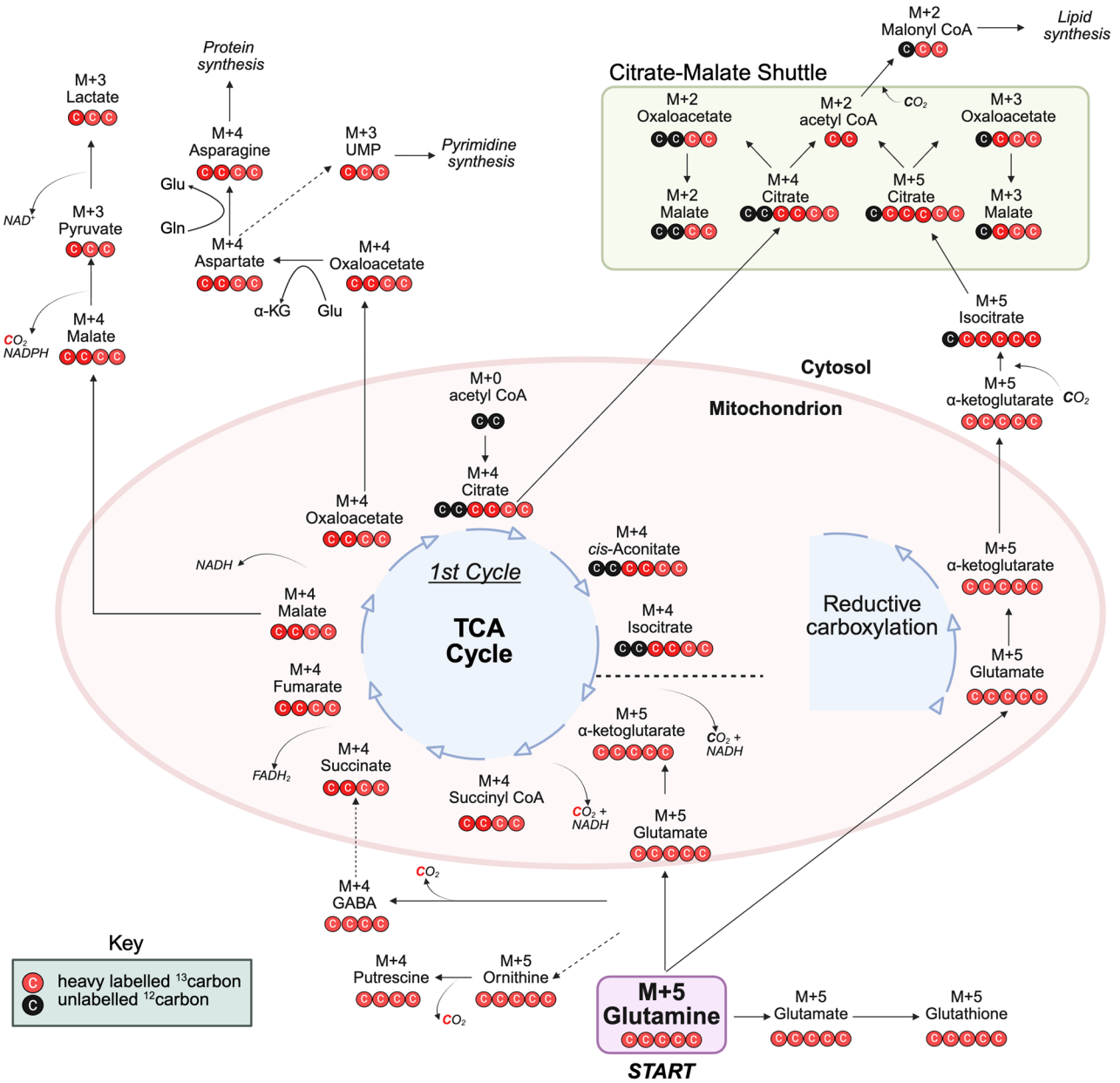


Fig. 6 Predicted labelling patterns in U-^{13}C glutamine tracing experiments. The flow of heavy labelled carbons (red) is indicated by the arrows, and suggested start points are indicated. U-^{13}C-glutamine enters the TCA cycle through anaplerosis, where it is converted to M+5 glutamate and M+5 α-ketoglutarate, which then cycles through the TCA cycle to yield M+4 TCA cycle intermediates. M+4 malate can be transferred to the cytosol to form M+3 pyruvate and M+3 lactate. M+4 oxaloacetate can be transferred to the cytosol to yield M+4 aspartate and asparagine, and M+3 UMP. In the cytosol, U-^{13}C-glutamine can be decarboxylated to M+5 GABA, which can enter the TCA cycle. It can fuel polyamine synthesis, through production of M+5 ornithine and M+4 putrescine, or be converted to M+5 glutamate and then M+5 glutathione. Glutamine can undergo reductive carboxylation, wherein M+5 α-ketoglutarate is converted to M+5 isocitrate, *cis*-aconitate and citrate. M+5 citrate is metabolised to yield M+2 acetyl CoA and M+3 oxaloacetate as part of the citrate malate shuttle. Glu = glutamate, Gln = glutamine, αKG = α-ketoglutarate

releasing one molecule of CO_2 and yielding 1 molecule of NADPH [56]. This pyruvate can be converted to M+3 lactate yielding one molecule of NAD^+ or can be decarboxylated to form M+2 acetyl CoA. Should this M+2 acetyl CoA condense with M+4 oxaloacetate in the mitochondria, it could re-enter the TCA cycle to yield M+6 *cis*-aconitate and M+6 isocitrate.

Oxaloacetate can be transported to the cytosol where it is converted to the amino acid aspartate by the transfer of an amino group from glutamate, yielding a molecule of α-ketoglutarate [57] (Fig. 6). M+4 aspartate can then either be converted to the amino acid asparagine or undergo a series of chemical reaction to form M+3 uridine monophosphate (UMP) which is essential in pyrimidine synthesis (Fig. 6A) [43, 58]. Cytosolic M+5 glutamate can combine with cysteine and glycine to form M+5 glutathione, important in redox homeostasis. M+5 glutamate can also undergo a series of chemical reactions involved in polyamine synthesis, yielding M+5 ornithine, which is decarboxylated to M+4 putrescine.

Emerging technologies and evolving approaches

The technical capabilities of metabolomics and isotope tracing are improving all the time. Improvements in analytical sensitivity now allow for metabolomic profiling from substantially smaller cell numbers, making analysis of rare immune populations possible for the first time. While reports of single cell metabolomics are beginning to emerge [59–61], ongoing developments in instrumentation, sample preparation, and data analysis suggest that these approaches will become increasingly accessible and informative over the coming years. An established approach is single-cell metabolic regulome profiling by mass cytometry, which couples phenotypic and metabolic protein measurements to resolve metabolic states and heterogeneity across cell populations at single-cell resolution [62]. Moreover, mass spectrometry imaging (MSI) has emerged as a powerful platform for spatially resolved metabolomics. MSI enables direct metabolomic analysis of tissue sections and has advanced to the point where three-dimensional reconstructions of metabolic niches within tumours, lymph nodes, and other tissues are becoming possible [63, 64]. It can now be combined with stable-isotope tracing to visualize the spatial distribution of labelled metabolites within intact tissues, linking nutrient utilization to cellular location and tissue architecture [65]. Already demonstrated with spatial proteomics [66], metabolic adaptation of specific immune cells in different tumour compartments has been reported. Thus, combined with conventional histological analysis and more advanced image techniques, MSI can map the spatial co-localisation of immune cells, tumour cells, and metabolites, providing unprecedented insight into the organisation of tissue metabolism.

In parallel with advances in analytical technologies, experimental models are also becoming more physiologically relevant. Recognition of the limitations of traditional in vitro culture systems for studying cellular metabolism has prompted efforts to develop more physiologically relevant models [4]. Because many metabolomic approaches still require relatively large numbers of purified cells, in vitro studies are likely to remain dominant in the near term. However, the use of physiologically relevant culture media is becoming increasingly common. Commercially available formulations such as Human Plasma-Like Medium (HPLM) and Plasmax™ more closely replicate the nutrient composition of human plasma and have highlighted metabolic artefacts introduced by conventional media such as RPMI [67–69]. In addition, specialised culture systems tailored to specific tissue environments have been developed, such as Tumour Interstitial Fluid Medium (TIFM), designed by Saab et al. to mimic the nutrient make-up of the tumour microenvironment [70]. Other important physiological variables, including oxygen tension, remain largely unexplored in immunometabolism.

Beyond improvements in culture systems, stable-isotope tracing is increasingly being applied directly in vivo. Advances in tracer administration (including continuous infusion and bolus labelling), rapid tissue processing, cell isolation, flow cytometric sorting, and high-resolution LC-MS enable quantification of nutrient utilization by T cells, macrophages, and other immune populations within their native physiological microenvironment rather than relying solely on in vitro culture systems [38, 71, 72].

Indeed, studies have shown that in vivo metabolic behaviour of immune cells can differ substantially from observations made in culture. For example, ^{13}C-tracing approaches revealed distinct fuel preferences of CD8^{+} T cells in vivo, including significant use of glutamine and acetate [4], while tracer analyses have also been used to define metabolic transitions during T-cell activation, differentiation, and exhaustion [73]. These findings highlight the importance of studying metabolism within its physiological context, where nutrient availability, tissue architecture, and intercellular interactions collectively shape metabolic behaviour.

However, in vivo labelling brings new challenges (see reviews [28, 38]) in particular label recirculation, whereby tracer-derived carbons are released into the circulation as downstream metabolites, where they can be subsequently taken up by other tissues and cells [38]. One study demonstrated that circulating lactate served as a major vehicle for redistribution of glucose-derived carbon between organs, often contributing to tissue (e.g. liver) TCA-cycle labelling more strongly than direct glucose uptake [31]. Label recirculation is likely to complicate immune cell analysis in vivo and interpretation of isotopic enrichment in specific cells needs to consider both intracellular carbon rearrangements and whole-body metabolite exchange to avoid over-attributing labelling to a specific pathway or nutrient source.

The field of in vivo tracing for cancer has provided a roadmap for these approaches. Stable-isotope infusions in patients have shown that glucose, lactate, glutamine and other metabolites contribute to tumour metabolism in ways that vary between tumour types, anatomical sites and even regions within the same tumour [28, 74]. Single cell isotope tracing is now one of the most rapidly advancing frontiers in cancer metabolomics [75]. When combined with emerging technologies such as single-cell nutrient uptake assays [76], cellular timestamping approaches for temporal analysis [77], and spatial isotope tracing by MSI, these approaches have the potential to transform our understanding of how local environmental cues shape immune cell function and disease progression. Together, these advances promise a more comprehensive and physiologically relevant view of metabolism across tissues, cell types, and disease states.

Summary and conclusions

Metabolomics and isotopologue tracing have transformed our understanding of the role that metabolism plays in driving and sustaining the immune response. Beyond its core role in ATP production, metabolism is now recognised as a key contributor to cellular differentiation, epigenetics, cell signalling, trained immunity, pathogen sensing, and much more. Importantly, metabolomics has shed light on how these fundamental processes become perturbed in disease, revealing exciting opportunities for immunometabolism-based therapeutics. This review has endeavoured to provide information and tools for immunometabolism researchers to harness metabolomics, and in particular nutrient tracing in their studies. Increasing accessibility to this specialist technology will further our understanding of the role that metabolism plays in supporting the immune system and may guide the development of next generation metabolic immunotherapies.

Glossary

Catabolism	The breakdown of complex molecules and nutrients into smaller units.
Liquid chromatography	The separation of compounds dissolved in a liquid mobile phase by passing the liquid over a solid stationary phase.
Gas chromatography	The separation of molecules based on their boiling points and their interaction with the stationary phase.
Derivatization	The chemical modification of certain metabolites in a sample before mass spectrometry analysis, which helps to make them easier to detect.

Ion mobility	The mobility of ions as they travel through a region filled with gas under the influence of electric fields.
Fragmentation patterns	The way molecules break apart when they are ionized, which helps in determining the structure of a molecule.
Isotopes	Versions of a chemical element with the same number of neutrons but different numbers of protons in the nucleus, resulting in unique atomic masses.
Isotopologue analysis	Analysis of the isotope composition of metabolites, often used in the tracing of stable isotopes in nutrients through metabolic pathways.
Anaplerosis	The entry of carbons into the TCA cycle to replenish TCA cycle intermediates.
Cataplerosis	The loss of carbons from the TCA cycle into other metabolic pathways.
Carboxylation	Addition of a carboxylic acid group i.e. increase of one carbon.
Decarboxylation	Removal of a carboxylic acid group i.e. loss of one carbon.
Quadrupole	A quadrupole is a device made of four metal rods that create an electric field to guide and filter ions based on their mass-to-charge ratio.
Orbitrap	An Orbitrap is a type of mass analyzer where ions move in an orbit around a central spindle. Their motion generates an electrical signal, which is then processed to determine the mass of the ions with high accuracy.

Author contributions Conceptualization, K.S. and C.M.G.; Writing – Original Draft, K.S. and C.M.G.; Writing – Review & Editing, K.S., C.M.G., G.M.O. and D.Z.A.; Visualization, K.S.

Funding C.M.G. is funded by the Irish Health Research Board and the EU.

Data availability Not applicable.

Declarations

Ethics and consent to participate Not applicable.

Competing interests No authors have any competing interests to declare.

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