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Tracing insights into human metabolism using chemical engineering approaches

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Metabolism coordinates the conversion of available nutrients toward energy, biosynthetic intermediates, and signaling molecules to mediate virtually all biological functions. Dysregulation of metabolic pathways contributes to many diseases, so a detailed understanding of human metabolism has significant therapeutic implications. Over the last decade major technological advances in the areas of analytical chemistry, computational estimation of intracellular fluxes, and biological engineering have improved our ability to observe and engineer metabolic pathways. These approaches are reminiscent of the design, operation, and control of industrial chemical plants. Immune cells have emerged as an intriguing system in which metabolism influences diverse biological functions. Application of metabolic flux analysis and related approaches to macrophages and T cells offers great therapeutic opportunities to biochemical engineers.

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Introduction

Metabolism comprises the set of coordinated biochemical reactions that are executed by cells. Thus, metabolic processes represent a crucial link between a cell's genetic program (which encodes mRNA and proteins/enzymes) and the surrounding chemical microenvironment, where substrates are converted to energy and the biosynthetic intermediates required for cell division. The metabolic state of a cell or organism is therefore tightly linked to its health, and such information is of particular use in the field of biomedicine. Many, but not all, of the most active metabolic pathways in mammalian cells have been documented and characterized over the last century. For example, new pathways associated with the tricarboxylic acid (TCA) cycle [1,2] and pentose phosphate pathway [3,4] have recently been characterized in mammals, providing new potential targets for controlling inflammation (Immune-responsive gene 1/*cis*-aconitate decarboxylase; IRG1/CAD) and cancer cell growth (transketolase-like 1; TKTL1), respectively. However, beyond such basic pathway discoveries we also have much to learn about the functional regulation of many biochemical pathways in human cells. Engineers have solved analogous problems in designing mechanical, electrical, and chemical systems, offering lessons for biomedicine which can enhance our understanding of disease pathogenesis. Neither engineering or biology alone can succeed in this task. Crucial insights into the phenotype of metabolic disorders and diseases in general will come from the clinic [5]. Detailed information on the behavior of such interconnected metabolic networks will come from systems-based analytics [6]. In the coming years these approaches will become increasingly integrated to advance our understanding of human physiology in the coming years [7].

In this review we highlight the utility of engineering concepts in studying cellular metabolism. We relate cellular functions and human metabolic physiology to that of an industrial chemical plant, highlighting the utility of real-time process parameters in operation of the latter while pointing out the need for analogous data in human metabolism. We highlight recent advances in the areas of analytical chemistry, computational analysis of metabolomics datasets, and biological engineering that are now facilitating the acquisition of human biochemical process conditions. Finally, we discuss recent studies that have explored the role of metabolism in regulating the immune system, an area of intense interest within the biomedical community that holds great therapeutic potential. The convergence of engineering and biomedical science on these problems is likely to catalyze many discoveries in the coming years.

Chemical process plant as an engineered model system

The regulation of cellular biochemical processes has grown more complex throughout evolution, with functional specialization increasing from prokaryotes to eukaryotes and, in turn, to multicellular organisms. In a simplified form, human cell and tissue metabolism can be viewed as a set of interacting chemical reaction sequences. Conceptually, a chemical plant functions similarly to the human body in that both use interconnected chemical processes

to execute specialized functions. Chemical engineers design, troubleshoot, and optimize such systems by breaking them down into smaller unit operations, as such plants consist of various units that execute specific steps of the overall chemical process. Piping of transfer fluids from one unit to the next connects each unit operation, and products from one reactor are substrates in downstream chemical reactions and/or separations processes. Since malfunction in a single unit operation can affect the entire process, chemical plants are highly controlled and regulated. Detailed study of individual unit operations as well as systems-level process control analysis are therefore crucial for designing robust and productive chemical processes.

A number of parallels between chemical plants and the human body emerge with respect to their function and analysis (Figure 1a). Organs such as the pancreas, liver, and kidney regulate nutrients and metabolic waste products to ensure that adequate energy and chemical building blocks are supplied to other tissues, such as the heart and brain. The vascular system serves to physiologically link each of these operation centers. At the subcellular level, distinct metabolic pathways are catalyzed by enzymes often localized in specific organelles. For example, mitochondria are the site of numerous biosynthetic and bioenergetic reactions, the lysosome and peroxisome are sites of recycling and detoxification, and the nucleus is the center of genetic control. Process control analysis is a fundamental tool of chemical engineers used to design and optimize industrial plants. Metabolic control analysis (MCA) has been analogously applied to characterize the regulation of individual enzymes and metabolic pathways [8]. However, key differences between chemical processes and cells or tissues highlight the challenges facing the biomedical community but also provide insights into approaches that can improve our understanding of human metabolism and disease pathology.

Since the design of a chemical plant is based on *a priori* knowledge, the flow of substrates, products, waste, and energy is well known. Furthermore, gauges present throughout the system provide engineers with real-time data on current process conditions (e.g. temperature, pressure) and deviations from targeted values. By contrast, human metabolic processes and their regulation are not fully characterized, and many unknowns remain to be discovered. Furthermore, given the challenges of clinical work it is difficult if not impossible to know the time-dependent concentrations and sources of metabolites within human tissues, cells, and subcellular compartments. As such, a major limiting factor in advancing our understanding of how metabolism contributes to human disease is the acquisition and analysis of biochemical information in cells and tissues. In particular the most valuable information lies in metabolic fluxes, which are the ultimate metric describing an enzyme's function. Dysregulation of fluxes (e.g. limited oxygen transport into tissues during ischemia, phosphorylation of glucose in cancer cells) is a key factor in virtually all diseases that in some cases can be used as a diagnostic biomarker (i.e. FDG-PET in cancer) [9]. Therefore, the acquisition of *quantitative* data on metabolic fluxes is needed to understand the mechanisms through which metabolism impacts or drives disease. Indeed, Lazebnik first related the function of apoptotic signaling pathways to electrical engineering concepts applicable to the circuitry of a transistor radio [10]. Rather than approach the integrated circuitry of such pathways by knocking out components one-by-one, a more systematic method was proposed using quantitative information on pathway function. In terms of metabolism the situation is similar, as fluxes cannot be effectively characterized as 'ON' or 'OFF.' Instead, the molar rates of reactions (in some cases relative to other pathways) are most informative. Fortunately, technological advances have now greatly improved our ability to estimate metabolic fluxes in complex biological systems.

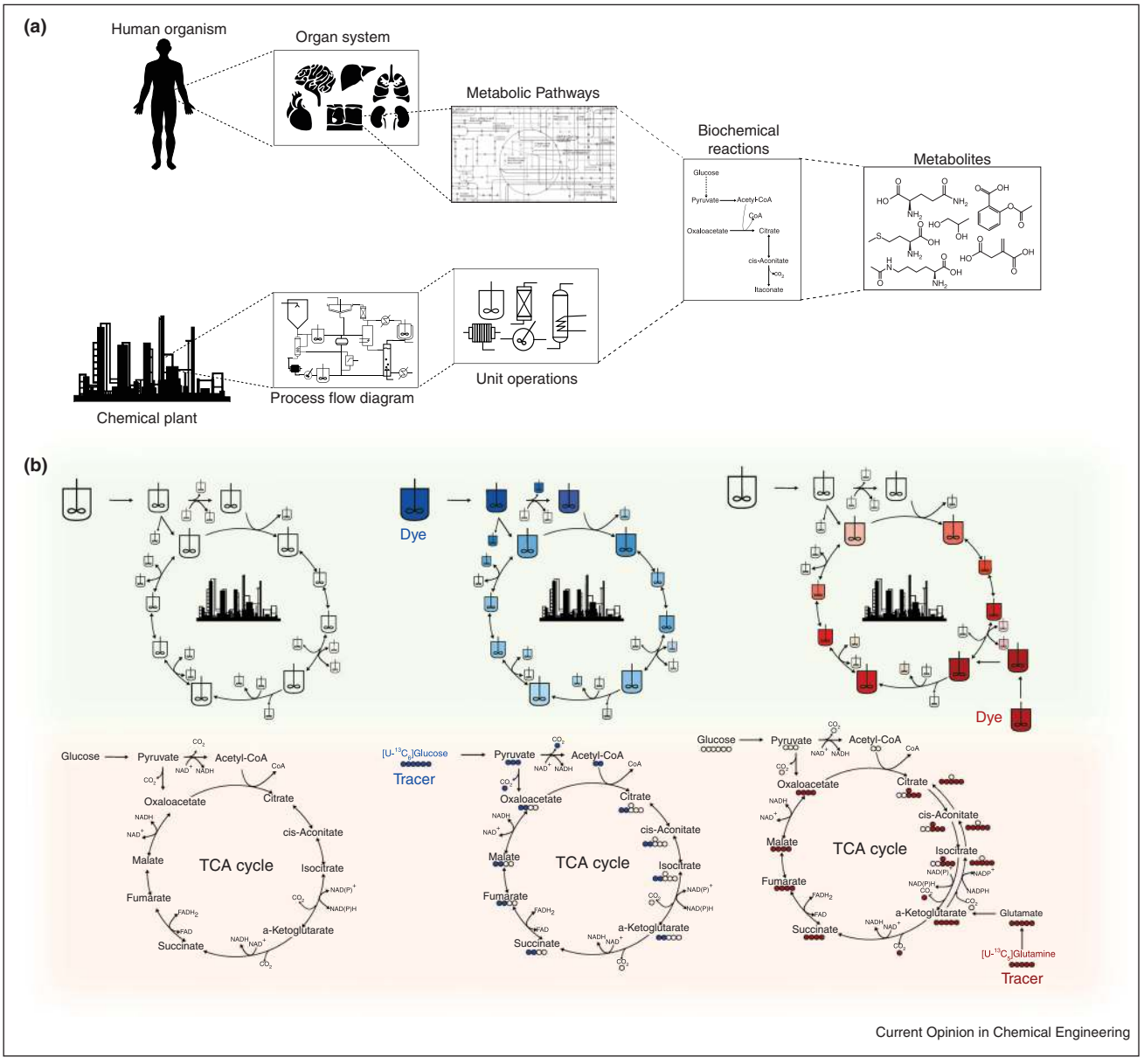
Technological advances in studying metabolism

In the last few decades, major advances in the areas of analytical chemistry, biological engineering, and computational interpretation of fluxes has greatly improved our ability to quantify metabolism in cells and organisms (Figure 2). Striking improvements in mass spectrometry and other analytical platforms is increasing the chemical information available to biomedical researchers. New software tools are allowing metabolic researchers to interpret and catalog these data and resolve pathway fluxes in unprecedented detail. Next-generation tools for genome engineering have enabled researchers to screen for crucial metabolic pathways in certain cell populations and interrogate the function of enzymes and pathways [11,12]. These advances are beginning to impact our understanding of human metabolic physiology and are reviewed in detail below. We subsequently highlight some examples where metabolic flux analysis (MFA) and related approaches have contributed to our understanding of immune cell regulation by metabolic pathways. Importantly, our knowledge of metabolic pathways and their regulation are by far not complete, and continued innovation in our ability to probe these phenomena are required to elucidate the physiological mechanisms of disease.

Analytical measurements of metabolic pathways

A wide variety of analytical platforms, especially nuclear magnetic resonance spectrometry (NMR) and mass spectrometry (MS)-based techniques, have improved the sensitivity and resolution of metabolite quantitation in biological systems. ^{1}H, ^{13}C, and ^{31}P NMR-based techniques offer crucial information to biomedical scientists given their non-invasiveness [13]. Magnetic resonance imaging (MRI) techniques have successfully been

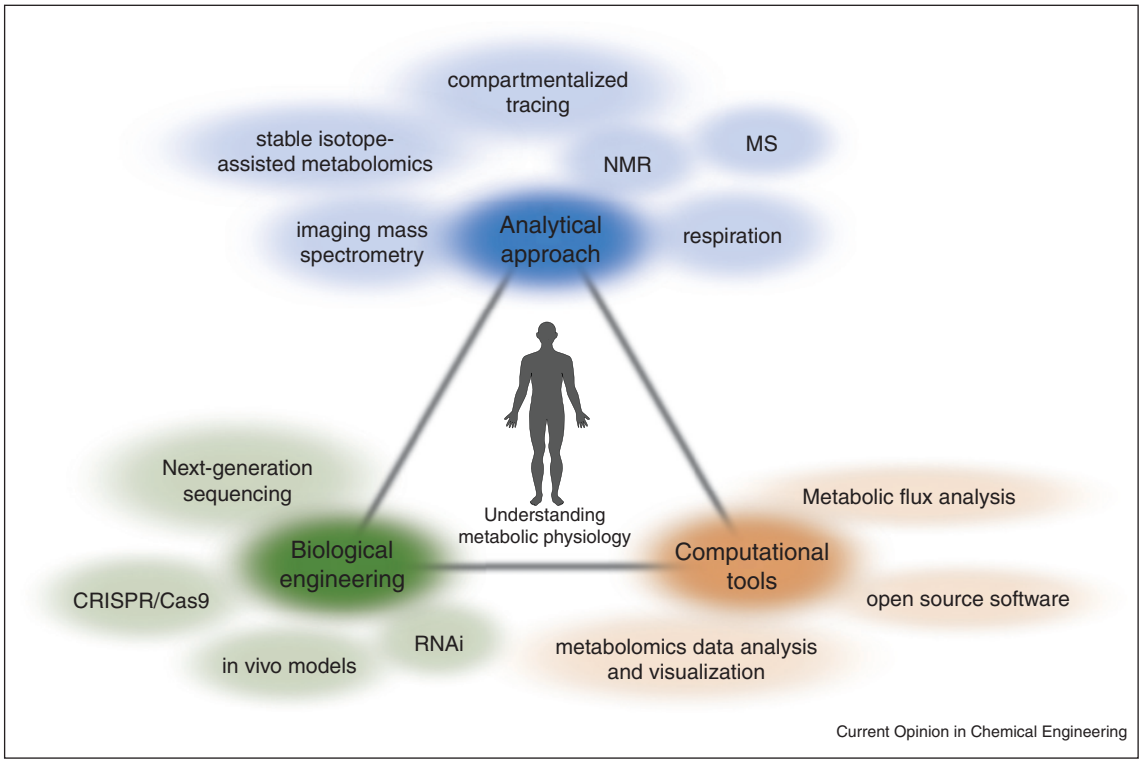
Figure 1



Human physiology viewed as an industrial chemical process. **(a)** The human body consists of numerous functional systems with interacting components that execute metabolic reactions. Conceptually, chemical plants function similarly, and both can be characterized in terms of distinct unit operations. Quantitative information on physiological states within the body are therefore required to optimize and improve human health. **(b)** Stable isotope-assisted metabolomics facilitates visualization of metabolic fluxes. Injection of dyes generates a color distribution that can be used to calculate flows across continuous stirred tank reactors (CSTRs). Isotope tracers and metabolomics allow visualization of metabolic dynamics from one metabolic pool to the next. Labeling of TCA intermediates is shown when metabolizing [U-$^{13}C_6$]glucose and [U-$^{13}C_5$]glutamine, with open circles depicting ^{12}C atoms and closed circles depicting ^{13}C isotopes.

applied to measure metabolites *in vivo*, such as 2-hydroxyglutarate (2HG) in the brains of patients with gliomas carrying isocitrate dehydrogenase (IDH) mutations [14]. Although the sensitivity of NMR is relatively low compared to MS-analytics, when used with stable isotope tracers NMR provides information on the position of labeling (isotopomers) which is particularly informative in pathways such as the TCA cycle [15]. To further enhance the sensitivity of such measurements ^{13}C substrates can be hyperpolarized before administration. In this manner, dynamic nuclear polarization (DNP) can greatly improve both *in vivo* and *ex vivo* quantitation of isotopomers and thus metabolic activity associated with pyruvate and TCA metabolism [16–18]. More detailed

Figure 2



Key technological advances that facilitate quantitation of metabolic processes. A number of innovative technologies allow for detailed metabolic analysis, including analytical chemistry approaches (blue), new methods for engineering biological systems (green), and computational tools for interpreting metabolomics data or estimating flux (red).

and comprehensive descriptions of this technology with applications in perfused tissues and cell cultures are available [19–21], as NMR continues to be an indispensable tool for biochemists.

Mass spectrometry (MS) has emerged as a versatile tool for quantifying small molecules in biological systems at varying levels of mass resolution. For greater separation, mass spectrometry is typically combined with other separation methods, including gas chromatography (GC–MS), liquid-chromatography (LC–MS) or capillary electrophoresis (CE–MS) [22,23]. Whereas GC provides high chromatographic resolution that is particularly suited for volatile analytes and molecules such as fatty acids [24], chemical derivatization is often required. LC or direct infusion into high resolution MS instruments is increasingly used in metabolomics and flux-based applications as well. Some approaches can provide spatial information on metabolites via imaging mass spectrometry, including matrix-assisted laser desorption ionization (MALDI), desorption electrospray ionization MS (DESI-MS), and secondary ion mass spectrometry (SIMS), as reviewed in detail elsewhere [25]. Given the diversity of chemistries within the metabolome, no single approach can provide a complete metabolic profile to the user. As such, the choice of technology should be considered carefully and tailored to the system and hypothesis.

Importantly, conventional metabolomics studies provide only a snapshot of metabolite levels rather than valuable, quantitative information on fluxes noted above. Therefore, stable-isotope tracing is often combined with MS or NMR analysis of isotopologue or isotopomer quantitation, respectively [23]. In principal, the interconversion of metabolites from one pool to the next is similar to the flow through the unit operations or tanks of a chemical plant. In the same way that an inputted dye can be used to determine residence times or generate color distributions that are a function of the flow from one unit to the next, isotopic labeling allows biomedical researchers to visualize the dynamics and interconnectivity of metabolic pathways (Figure 1b). The most useful stable isotopes used for observing intermediary metabolism in mammalian systems are ^{13}C, ^{15}N, and ^{2}H (deuterium), though any 'label' carried through a reaction can be employed [26]. Administration of tracer in mammalian cell culture is fairly straightforward, though the choice of labeled substrate and undefined nature of some medium components must be considered. *In vivo* applications are increasingly common and can be enhanced by infusion of tracer to

achieve steady-state enrichment in plasma [27••]. Combinations of different tracers may also be employed to increase the information available for calculating fluxes [28]. In addition to measuring free metabolites, isotopic enrichment is readily quantified into components of cellular biomass, which can be separated and analyzed to increase flux observability. Recent studies have isolated RNA, DNA, glycogen, and glycans to quantify isotope enrichment in metabolic precursors to enhance signal and provide additional information for flux calculations [29–32], drawing on related approaches that quantify acetyl-coenzyme A labeling from fatty acid measurements [33,34]. Finally, improved quantitation of metabolite labeling via tandem mass spectrometry has the potential to enhance flux resolution in MFA applications [35–37]. Broadly, these analytical approaches are increasing the data available for modeling intracellular metabolism, with the hope that more information increases the fidelity of results.

In addition to the metabolomics approaches noted above, direct measurements of fluxes also provide useful information when characterizing the metabolic state of higher cells. Measurement of uptake and secretion fluxes from cultured cells can provide valuable information on amino acid and glycolytic metabolism [38]. On the other hand, microplate-based assays have been increasingly used to monitor respiration rates to assess mitochondrial dysfunction [39]. Notably, in combination with plasma membrane permeabilization, measurements of mitochondrial respiration in permeabilized cells can allow for control of mitochondrial substrate provision, resulting in more in-depth mitochondrial studies using respirometry [40] or metabolomics [41] approaches. Increasingly, combinations of one or more analytical approaches are used to decipher the metabolism of complex systems, as each measurement may provide specific information for improved flux resolution [32,42••]. Such orthogonal measurements facilitate a more integrative view on metabolism but also require increasingly complicated software for analysis.

Computational advances

The analytical approaches described above generate highly complex data sets that can include absolute or relative metabolite levels, direct flux measurements (e.g. glucose uptake, respiration), and tracer-specific isotopologue or isotopomer abundances. As a result, researchers have developed numerous algorithms for interpreting these data, resolving fluxes, and designing experiments [43••,44]. Some of the most complex software is designed to estimate fluxes and associated sensitivities or confidence intervals for a given system. The user must input a metabolic network (often pared down to focus on intermediary metabolism) and measurements of fluxes, isotopologues, and when required metabolite abundances. The latter data must be considered when conducting kinetic or non-stationary metabolic flux analysis (NSMFA) [45]. Versatile software packages are becoming available to resolve fluxes using various tracers (e.g. ^{13}C, ^{15}N, ^{2}H), and these have been reviewed in detail elsewhere [46]. Often, the most informative data lies in flux confidence intervals obtained via parameter continuation [47], as not all fluxes will be resolvable for a given network and experimental dataset.

Tracer choice directly impacts the isotopologues and fluxes that can be determined from a particular experiment, and computational approaches have been developed to evaluate, optimize, and design tracer combinations with enhanced resolution [48–50]. For example, combinations of glucose tracers are useful for studying the pentose phosphate pathway [51], and glutamine tracers are particularly informative when studying tricarboxylic acid (TCA) metabolism in proliferating cancer cells [48]. However, post-mitotic cells such as differentiated myoblasts (i.e. myotubes) exhibit very low glutamine anaplerosis [52•], and alternate tracers should be considered depending on the metabolic state of the system to be studied.

Various software are available for extracting information from metabolomics datasets generated on different platforms. Here we focus on software used to determine isotope enrichment in analytes. Mathematical correction of natural isotope abundance using sum formula and theoretical abundance within targeted metabolomics datasets is fairly straightforward [53], and opensource software is available for this purpose [54,55]. Algorithms have recently been developed to identify labeled compounds in an untargeted manner. Non-targeted tracer fate detection (NTFD) facilitates the identification and quantitation of isotopic labeling of all detectable metabolites downstream of a given tracer in GC-MS datasets [56]. Similar software has been developed for LC-MS platforms [57]. Increasingly, researchers are incorporating tools for extracting and interpreting isotopologue data within their metabolomics software platforms, which will facilitate the use of isotope tracing and MFA in more complex biological systems [58].

These data-driven approaches are crucial tools for understanding the metabolic state of a cell, tissue, animal, or patient. Various software-based approaches that balance fluxes based on network stoichiometry and gene or protein expression are also available, allowing researchers to explore the importance of pathways *in silico*. Although challenges in the choice of objective function (i.e. how to optimize metabolism) and modeling of compartmentalized systems remain, these tools provide a unique means of generating metabolic hypotheses to be functionally tested [59•,60]. Importantly, knowledge of metabolism is a requirement for interpreting results from such models, as software algorithms alone are unlikely to provide useful data for researchers.

Engineering biological systems

Metabolism represents the biochemical phenotype of a biological system, though genetic mutations, transcription, translation, and post-translational modifications all exert significant control over these pathways. As such, it is increasingly important to study metabolism in the context of engineered biological systems to shed new light on metabolic regulation. Although the technologies described above have significantly improved our ability to study metabolism, perhaps even greater advances have been made in technologies used to engineer genes, proteins, and the microenvironment in mammalian systems. For example, numerous techniques are now available to control gene expression, including conventional RNA interference [61] or recently developed clustered regularly interspaced short palindromic repeats (CRISPRs)/Cas-system gene editing tools. The latter provides a powerful tool for modifying DNA sequences in a site-specific manner for numerous applications [62,63]. Indeed, researchers have applied CRISPR/Cas9 *in vivo* to correct mutations associated with the human metabolic disease hereditary tyrosinemia [64] and muscular dystrophy [65–67]. Furthermore, CRISPR-Cas9-based genetic screens have been applied to identify synthetic lethalities in metabolism [12]. On the other hand, engineering cells with targeted knockouts in folate-mediated one carbon metabolism has improved our ability to characterize this pathway [11]. Although further research is needed to increase efficiency and decrease off-target effects [68], this technology has already been applied to improve our understanding of metabolic pathway function in mammalian cells.

Another major challenge in deciphering the metabolism of higher cells is their compartmentation [69], as many reactions and enzymes are localized to one or more subcellular organelles. Analysis of isolated mitochondria or selective cell permeabilization applied in conjunction with metabolomics, isotopic tracing, and/or respirometry can provide some information on the function of these organelles [41,52•,70]. However, mitochondria in 'isolation' likely exhibit different phenotypes compared to those within active cells. Also, given the fast turnover rates of many metabolites, separation of organelles before MS or NMR analysis is not ideal. Subcellular compartmentalization is increasingly incorporated into MFA models [52•], often to account for labeling discrepancies in related metabolites like pyruvate, lactate, and alanine. Microbial co-cultures present analogous problems to MFA studies, as similar reactions may operate differently in adjacent cells (or organelles). Incorporation of biomass labeling into more complex models has been effective for resolving fluxes in microbial co-cultures [71], and such approaches may be effective in studying compartmentalized and/or multi-cellular mammalian tissue systems. Recently we developed a genetically encoded reporter system that works in conjunction with ^{2}H tracers (and in theory other isotopes) to provide information on compartment-specific NAD(P)H metabolism [72••]. By inducibly expressing mutant IDH1 or IDH2 in the cytosol or mitochondria, respectively, one can quantify ^{2}H-labeling on (D)2HG produced in each compartment to determine how folate-mediated one carbon metabolism contributes to compartment-specific NADPH pools. This method also provided insights into the function of reductive carboxylation in anchorage-independent cancer cells [73•].

Cell metabolism is commonly studied in 2D-cell culture models, but this microenvironment does not necessarily reflect the actual *in vivo* environment of cancer cells [27••]. Therefore, *in vivo* model organisms, in particular rodents, are valuable tools that help to advance our understanding of metabolism. The ability to engineer model organisms is increasing further as the CRISPR-Cas9 genetic toolbox enables rapid generation of new genetically engineered *in vivo* model systems [74]. In addition to *in vivo* model organisms, substantial progress has been made in engineering cellular microenvironments which more accurately reflect *in vivo* situations, such as human organs-on-chips [75] or HuMiX, a model of gastrointestinal human–microbe interface to study complex interactions between human cells and bacteria [76]. Given the complexities of metabolic systems and their regulatory requirements, it is unlikely that such engineered models will replace *in vivo* testing completely. Although, the relative simplicity of *in vitro* systems can provide a better means of elucidating molecular mechanisms, any conclusions should be viewed in the context of the system used.

Case study: immunometabolism

A large number of detailed studies employing metabolomics and/or flux analysis approaches to study disease pathogenesis have been published recently. Notably, the large number of metabolic investigations and discoveries makes it impossible to comprehensively review studies in all human tissues. In particular, analyses of the heart, liver, brain, and tumors have been described in detail. Here we focus on applications in the immune system, an area of emerging interest and tremendous therapeutic potential.

The immune system is comprised of diverse cell types present in various tissue microenvironments around the body. As such, immune cells must sense and respond to a highly complex set of physiological settings. We are now beginning to appreciate that many of these signals converge on metabolic enzymes and pathways to exert control over immune cell function. During an immune response cells of the innate and adaptive immune system become activated and reprogram metabolism to execute their diverse functions, which may involve rapid proliferation, regulatory cross-talk amongst different cell types, or

clearance of dead tissue and pathogens. Upon differentiation to their downstream lineages immune cells therefore exhibit strikingly distinct metabolic phenotypes, so there is much to learn in each situation. To date, most studies have focused on metabolic changes occurring within T cells and macrophages [77], but the many cell types present in the immune system present immunologists and biochemical engineers with a deep set of questions to be addressed in the coming years.

Although naïve T cells rely on oxidative phosphorylation and fatty acid oxidation for energy production, activated T cells must reprogram metabolism to fulfill the metabolic requirements of proliferation and cytokine production [78]. Therefore, activated T cells increase glucose uptake, glycolytic rates and glutamine catabolism. The mammalian target of rapamycin complex 1 (mTORC1), a central regulator of metabolism and cell growth, has emerged as a crucial regulator of T cell function [79]. Modulation of metabolic pathways, including glycolysis and components of the electron transport chain strongly influence T cell expansion and function [80•,81]. More unique mechanisms associated with these pathways are now coming to light. For example, aerobic glycolysis facilitates binding of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to effector cytokine mRNA to influence T cell function [82]. Furthermore, the glycolytic metabolite phosphoenolpyruvate (PEP) acts as a signal under nutrient replete conditions to mediate T cell function, and in low-glucose such as the tumor microenvironment low PEP levels can compromise the anti-tumor effects of the immune system [83]. Metabolic studies have also recently demonstrated a role for serine and folate-mediated one carbon metabolism in T cell proliferation [84]. On the other hand, a functional electron transport chain is also required for expansion of activated T cell populations [85]. Notably, changes in lipid metabolism elicit strong effects on immune cell function. In response to pathogens, induction of lipogenic pathways is an integral part of antigen-driven blastogenesis and clonal expansion in CD8+ T cells [86]. In memory T cells, regulation of mitochondrial fatty acid oxidation by cytokines is crucial for generating energy and cell survival [87]. In addition, isotopic tracer analysis has revealed co-regulation of cholesterol metabolism and the type 1 interferon (IFN) pathway which allows macrophages to coordinate antiviral responses [88•].

Macrophages are present in almost all tissues and play crucial roles in the immune response, as they facilitate clearance of invading pathogens and mediate tissue homeostasis associated with inflammation. Recently, an integrated analysis of transcriptomic and metabolomic data revealed new insights into the distinct metabolic signatures of classical (M1) and alternative (M2) polarized macrophages. Unlike alternatively polarized macrophages, M1-like macrophages have a distinct metabolic pattern characterized by high glycolytic flux and an impaired TCA cycle reminiscent of decreased IDH and succinate dehydrogenase (SDH) activities [89••]. This regulation allows for accumulation of *cis*-aconitate and succinate. The former is a precursor of the antimicrobial metabolite itaconate, which can accumulate to mM levels in stimulated macrophages and microglia [90–92]. Succinate can mediate various biological functions and is thought to enhance interleukin (IL)-1β expression via stabilization of hypoxia-inducible factor (HIF)-1α [93]. Recently, we and others applied metabolomics, stable isotope tracing, and respiratory to uncover a link between these two phenomena, where itaconate acts as an endogenous SDH inhibitor to reprogram immune metabolism and modulate succinate levels [1,2,94]. Notably, though HIF is stabilized under pro-inflammatory conditions, metabolic tracing has demonstrated that stimulated macrophages maintain pyruvate flux into the TCA cycle via pyruvate dehydrogenase (PDH) to sustain itaconate production [95]. It is therefore quite valuable to understand how these molecules are produced during immune cell stimulation, as itaconate and other molecules with immunomodulatory function may emerge as promising therapies.

Conclusions

The dynamics of immune cell populations in the body presents both challenges and opportunities to the biomedical research community. Ultimately, MFA studies using models of increasing complexity will become important for elucidating the regulation and function of metabolic pathways within the immune system. Application to cell models and *in vivo* systems will be required, with an eye to deciphering mechanisms and interesting phenotypes. Given the importance of immune cell function in combatting infections, clearing tumor cells, and autoimmune diseases, immunometabolism will remain an active area of study in the foreseeable future. Engineers and immunologists will need to work together to understand and control these systems effectively.

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- • of special interest
- •• of outstanding interest

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