ANNUAL REVIEWS

Annual Review of Immunology

Systems Immunology Approaches to Metabolism

Denis A. Mogilenko,[1,2,*] Alexey Sergushichev,[1,3,*] and Maxim N. Artyomov[1]

[1]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri, USA; email: alsergbox@gmail.com, martyomov@wustl.edu

[2]Current affiliation: Department of Medicine, Department of Pathology, Microbiology, and Immunology, and Vanderbilt Center for Immunobiology, Vanderbilt University Medical Center, Nashville, Tennessee, USA; email: denis.mogilenko@vumc.org

[3]Computer Technologies Laboratory, ITMO University, Saint Petersburg, Russia

Annu. Rev. Immunol. 2023. 41:317–42

The *Annual Review of Immunology* is online at immunol.annualreviews.org

https://doi.org/10.1146/annurev-immunol-101220-031513

*These authors contributed equally to this article

Keywords

systems immunology, immunometabolism, computational biology, transcriptomics, metabolomics, metabolic imaging

Abstract

Over the last decade, immunometabolism has emerged as a novel interdisciplinary field of research and yielded significant fundamental insights into the regulation of immune responses. Multiple classical approaches to interrogate immunometabolism, including bulk metabolic profiling and analysis of metabolic regulator expression, paved the way to appreciating the physiological complexity of immunometabolic regulation in vivo. Studying immunometabolism at the systems level raised the need to transition towards the next-generation technology for metabolic profiling and analysis. Spatially resolved metabolic imaging and computational algorithms for multi-modal data integration are new approaches to connecting metabolism and immunity. In this review, we discuss recent studies that highlight the complex physiological interplay between immune responses and metabolism and give an overview of technological developments that bear the promise of capturing this complexity most directly and comprehensively.

1. INTRODUCTION

Immunometabolism is an interdisciplinary field of research that connects metabolism, at intracellular and whole-body levels, to immunology. The immune system requires substantial energy input to function: even mild immune responses initiated by respiratory infections increase resting metabolic rate, which accounts for the total number of calories burned in rest, to around 10% (1). This increased metabolic rate reflects the metabolic cost of the immune response, at least partially due to direct metabolic activation of immune cells. Immune cells can fine-tune their energy demands and preferred metabolic substrates based on specific triggers. Indeed, pathogen-associated molecular patterns (PAMPs), such as Toll-like receptor (TLR) agonists, in macrophages and dendritic cells, and TCR (T cell receptor) and BCR (B cell receptor) signaling in T and B cells rapidly reprogram these cells to switch to differential metabolic substrates and metabolic pathways (2–4). Initial immune activation is typically linked to preferential glucose oxidation and the generation of ATP in the cytoplasm (3) but also profoundly affects the utilization of other metabolic substrates such as amino acids and lipids (5, 6). Multiple classical approaches to interrogate immunometabolism include analyzing extracellular fluxes, intracellular metabolite composition, and metabolic pathways using isotope tracing strategies. Recent reviews have extensively explained how these techniques are implemented in immunometabolism research (7–9).

Metabolism not only supports the energy demands of immune cells but also guides their activation, differentiation, and function in a context-dependent manner in homeostasis and disease (10). Moreover, metabolic control at the levels of cells, tissues, and the whole body optimizes the allocation of limited supplies by integrating inflammatory and other physiological signals (11). The systems immunology approach to immunometabolism aims to understand how complex interactions between cell types, signaling molecules, and metabolic pathways coordinate immune responses at the levels of individual cells, organs, and the whole organism (12–14). Recent technological and computational advances expanded a toolbox of techniques for studying immunometabolism and enhanced our understanding of the functions of metabolites in immune regulation. In this review, we focus on systems-level methods of immunometabolism that connect immune (and nonimmune) cells in tissues via metabolic communications and reveal cellular and functional heterogeneity of immunometabolic circuits.

2. APPROACHES TO STUDYING IMMUNOMETABOLISM

Approaches to studying immunometabolism can be broadly defined at two conceptual levels (**Figure 1**). On the one hand, immunometabolism describes the relationship between intracellular metabolic pathways in immune cells and their functions, including activation, differentiation, and migration (15). This approach to immunometabolism is focused on cell-intrinsic metabolic processes in specific subsets of immune cells and, in the furthermost case, attempts to understand metabolic processes in individual immune cells (9). On the other hand, immunometabolism studies cross talk between immune cells and their metabolic and tissue environments (e.g., in the liver, adipose tissue, and other tissues) and whole-body metabolic homeostasis (16–18). This type of research focuses on cell-extrinsic metabolic processes, including immune functions modified in response to extracellular metabolic cues and the direct effect of immune cells on metabolic processes in nonimmune tissues. These two approaches are not mutually exclusive and should be considered together to understand the rules of metabolic regulation of immunity. Excellent recent reviews elucidated these concepts and discussed discoveries in immunometabolism (19–23). In parallel, technical advances in single-cell and computational biology and metabolic profiling made it possible to study immunometabolism at a more integrative and comprehensive level, bringing systems-based approaches to immunometabolism (8). This new and growing field of

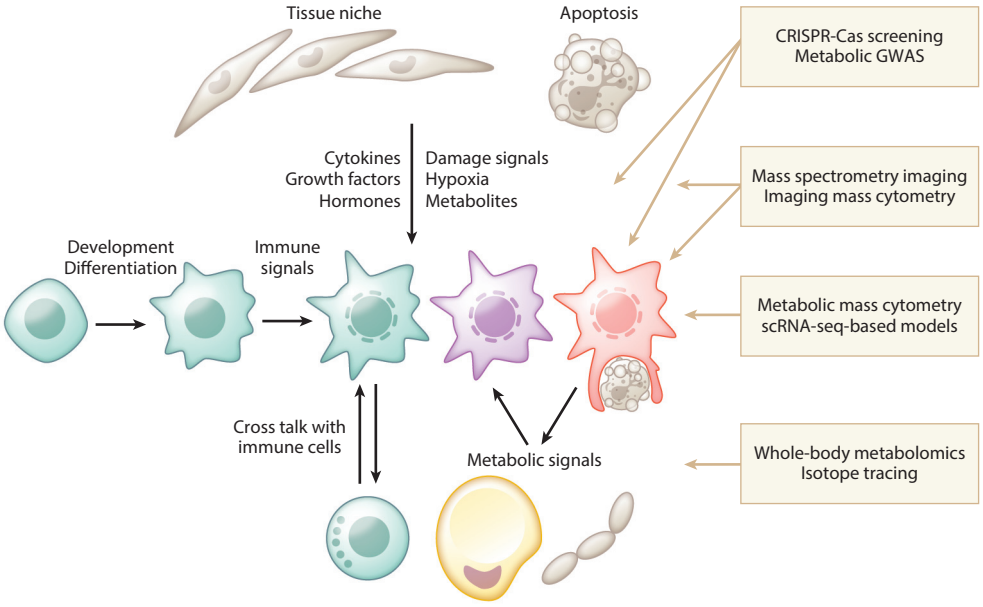

Figure 1

Experimental approaches to studying systems-level immunometabolism. (*Left*) Biological processes that result in metabolic and functional heterogeneity of immune cells. The heterogeneity of metabolic phenotypes of immune cells is determined by their developmental origin, differentiation trajectory, and a plethora of immune signals that the cells experience in tissues. During immune responses, immune cells undergo metabolic reprogramming (that is, dynamic reorganization of metabolic pathways and bioenergetics) to adapt to their function. Metabolic reprogramming of immune cells is affected by cross talk with surrounding immune and nonimmune cells, distant metabolic tissues, and the microbiota. This cross talk is mediated by cytokines, growth factors, hormones, and metabolites in tissue niches. Moreover, the metabolic response of immune cells is sensitive to damage signals in tissue niches, such as hypoxia, damage-related metabolites, and apoptotic cells. Immune cells rely on metabolic adaptation to the microenvironment to produce signaling molecules and metabolites to control tissue homeostasis. (*Right*) Methods and approaches that capture metabolic heterogeneity of immune responses. Various methods of genetics, transcriptomics, metabolomics, and cytometry can be used to decipher metabolic processes in multiple modes to reconstruct immune and metabolic functions at systems-level resolution. Abbreviations: GWAS, genome-wide association study; scRNA-seq, single-cell RNA sequencing.

systems immunometabolism integrates techniques and knowledge from several disciplines and is a blossoming source of new insights and discoveries.

2.1. Cell-Centric Immunometabolism

The cell-centric approach to immunometabolism is focused on intracellular metabolic pathways and how they interact with signaling pathways inside the immune cells to understand immune homeostasis and disease (9). For example, aerobic glycolysis (that is, the conversion of glucose to lactate in conditions of sufficient oxygen in the microenvironment) is essential for the functional activation of various immune cell types, including macrophages (24–26), dendritic cells (27), T cells (28), B cells (29), and natural killer (NK) cells (30). Early studies that identified metabolic demands of activated immune cells often relied upon in vitro models and a simplified view of metabolic cues from the microenvironment. Although these studies generated robust in vitro results and established the basics of immunometabolism, it has been recognized that variations in developmental origin, differentiation and activation states, tissue residence of immune cells, and their microenvironment have a profound impact on the interaction between metabolism and cell function (31, 32). For example, in vitro proinflammatory activation of macrophages is associated

with high glycolytic activity. Still, the anti-inflammatory polarization of macrophages with enhanced tissue repair potential does not require increased glycolysis for their activation. Instead, it relies upon oxidative phosphorylation (OXPHOS) into mitochondria and glutamine fuel into the tricarboxylic acid (TCA) cycle to produce ATP (33). To complicate the picture further, the metabolic reprogramming can also depend on cell ontogeny and a tissue niche, thus differentially affecting the embryonically derived resident alveolar macrophages and the hematopoietically derived interstitial macrophages in the lungs (34, 35). Finally, the tissue environment directly affects the metabolic identity of the immune cells. For example, the lung niche regulates the metabolic activity of alveolar macrophages, including glycolytic activity, through locally produced cytokine signals (36). These examples show that our understanding of the immunometabolism of immune cells is not complete without a systems approach that provides information about immune cell heterogeneity, microenvironment, and cross talk between different types of cells.

2.2. Immunometabolism of Cell Communications in Metabolically Active Tissues

Communication between cells in tissues is a mechanism of adaptation to environmental changes (37). Multiple signaling pathways allow immune cells to regulate metabolism at the levels of tissues and the whole body. Immune cells resident in adipose tissue are among the best-characterized immunometabolic players. One of the early discoveries showed that macrophages resident in adipose tissue could directly inhibit the response of adipocytes to insulin (insulin sensitivity) through inflammatory signals dependent on the activity of JNK kinase (38). Subsequent studies identified multiple roles of adipose macrophages in developing obesity-related metabolic disorders and highlighted the cellular heterogeneity that programs differential functional roles of macrophage subsets (39).

Metabolic regulation in adipose tissue involves a complex interplay between various immune cell types. For instance, cytotoxic $CD8^+$ T cells accumulate in fat depots during obesity to initiate macrophage infiltration and promote insulin resistance (40). In contrast, $CD4^+$ regulatory T cells (Tregs) can suppress inflammation in adipose tissue and restore glucose metabolism (41). Intriguingly, adipose Treg phenotype is profoundly controlled by sex hormones via cross talk with IL-33-producing stromal cells specific to male adipose tissue, ultimately leading to pronounced sexual dimorphism in adipose tissue inflammation and metabolism (42). It remains unclear how obesity reprograms fat-resident immune cells through metabolic cues and signals. Systems-level tools in immunometabolism are needed to understand metabolic communication between adipocytes and immune cells.

The liver is another example of an immune and metabolically active organ whose functions are directly controlled by hepatic immune cells (43). Obesity and diets enriched in fat and sugar lead to the accumulation of lipids in the liver (known as hepatic steatosis) and can result in hepatic inflammation and the disease state of nonalcoholic steatohepatitis (NASH) (44). Almost all subsets of innate and adaptive immune cells reside in the liver and contribute to metabolic inflammation and NASH development, involving a network of metabolic signals and spatial-temporal dynamics of cross talk between hepatocytes and immune cells (18, 45). These examples show that immune cells can integrate signals at multiple levels to establish immune and metabolic coregulation in tissues. Still, the molecular mechanisms of these processes are far from being fully understood.

2.3. Metabolic Cross Talk in the Tumor Microenvironment

Metabolic alterations in the tissue microenvironment can dramatically change the functions of the immune cells. The tumor microenvironment (TME) is a specific tissue niche with unique immune

and metabolic characteristics that reprograms cell-cell communications and alters antitumor immune responses (46). For example, competition between cancer cells and intratumor immune cells for limited nutrients can dampen tumor immunosurveillance and interfere with therapeutic interventions (47). The metabolic landscape inside the tumor is not random and is characterized by distinct areas. A recent study used positron emission tomography (PET) tracers to understand how the metabolism of immune cells is dysregulated in the TME in the MC38 tumor model. This analysis of access to metabolites inside the TME showed that cancer cells actively take up glutamine, whereas myeloid cells preferentially take up glucose (48). The metabolic partitioning in the TME is programmed through the mechanistic target of rapamycin complex 1 (mTORC1) activity and involves inhibiting glucose uptake by active glutamine metabolism (48). It remains to be understood how other signaling and metabolic pathways are implicated in establishing the metabolic partitioning in TME. The interconnected immunometabolism in the TME involves complex and hierarchical regulation of nutrient partitioning and metabolic crosstalk between cancer and immune cells as a critical regulator of antitumor immunity.

Distinct immune cell subsets inside the tumor can vary in metabolic demands and phenotypes. For example, macrophages are metabolically heterogeneous within the TME, and metabolites abundant in the TME, such as lactate, differentially regulate the transcriptome and function of MHC-II^{hi} and MHC-II^{lo} tumor-associated macrophages (49). T cells inside tumors have a broad spectrum of functional and metabolic states. Intratumoral CD4$^+$ Tregs adapt to the TME and upregulate pathways of lactate metabolism, including lactate uptake through monocarboxylate transporter 1 (MCT1), which results in increased immune suppression and tumor growth (50). Exhausted CD8$^+$ T cells characterized by progressive loss of effector functions and metabolic dysregulation are a distinct cell lineage abundant in cancers (51). Reversal of the metabolic dysfunction in exhausted CD8$^+$ T cells, for example by promoting OXPHOS through IL-10-Tc fusion peptide, enhances their expansion and effector function, leading to increased antitumor immunity in mice (52). Moreover, dysfunctional effector T cells characterized by high glycolysis but disturbed lipid metabolism develop in the TME in response to signals from cancer cells and Tregs. Antitumor responses can be enhanced by inhibiting group IVA phospholipase A2 and reprogramming lipid metabolism in effector T cells in the TME (53). Future studies will expand our understanding of metabolic regulators of tumor-infiltrating immune cells in responses to anticancer therapies.

Approaches that can capture the metabolic heterogeneity of the TME in an unbiased manner will be instrumental in studying tumor immunometabolism. Thus, recent advances in single-cell transcriptomics revealed connections between whole-body metabolism and tumor metabolism and intratumor immune cells. For example, integrated analysis of single-cell RNA sequencing (scRNA-seq) and metabolomics data revealed that obesity increases the competition between tumor cells and tumor-infiltrating CD8$^+$ T cells for extracellular fatty acid, which results in decreased utilization of fatty acid by CD8$^+$ T cells and immune dysfunction, contributing to accelerated tumor growth in obese mice (54). Naturally, disturbed fatty acid metabolism in obesity is not limited by T cells and has a broader effect on immune responses. For example, metabolic reprogramming of NK cells by obesity depends on fatty acid–mediated activation of peroxisome proliferator-activated receptors (PPARs) α and δ and inhibits glycolysis-dependent trafficking of cytotoxic mediators to the immune synapse between NK cell and tumor cell (55). Thus, obesity paralyzes the cellular metabolism of NK cells and impairs tumor surveillance. Moreover, in the context of obesity and skin inflammation, fatty acids reprogram the metabolism of activated dendritic cells, triggering metabolically driven unfolded protein response (UPR), which transcriptionally reprograms cytokine responses of dendritic cells via the UPR transcription factor XBP1 in obesity (56). Whether metabolic reprogramming of dendritic cells by obesity alters tumor

antigen presentation and disturbs antitumor immune responses of dendritic cells remains to be studied.

2.4. Immunometabolism in Tissue Damage and Homeostasis

Metabolic cues play an important role in the reprogramming of immune cells in a state of perturbed tissue homeostasis (57). For example, tissue hypoxia induces generation of 2-hydroxyglutarate (2-HG), which can act as an epigenetic regulator (58, 59). Recent studies show that 2-HG inhibits the anti-inflammatory functions of Tregs (60) and alters the differentiation of $CD8^+$ T cells (61). In addition to hypoxia, tissue injury induces the massive death of cells by apoptosis, followed by engulfment and elimination of apoptotic cells by tissue macrophages in the process of efferocytosis to prevent inflammation (62). Critically, efferocytosis overloads the macrophages with metabolites that boost fatty acid oxidation in mitochondria and reprogram IL-10 and TGF-β secretion. This metabolic circuit connecting efferocytosis and immune responses has been shown to regulate cardiac repair after myocardial infarction (63). In addition, efferocytosis induces enhanced glucose uptake in macrophages, which is mediated by the SLC2A1 transporter and leads to increased production and release of lactate to promote an anti-inflammatory tissue microenvironment (64). In dendritic cells, the activity of amino acid transporters, such as SCL7A11 (the subunit of the cysteine-glutamate antiporter system xc−) exchanging intracellular glutamate for extracellular cysteine, inhibits efferocytosis and suppresses skin wound healing in part through the action of GDF15 (65). This illustrates that immune cells can engage specific metabolic regulators to coordinate immune function with cues from the tissue microenvironment.

A coordinated response to the microenvironmental cues in tissues goes beyond metabolic reprogramming of immune cells and involves metabolic communications with nonimmune cells. One of the recent findings connecting immune cells to the stromal cells through the metabolic axis is the effect of glutamine derived from tissue macrophages on muscle regeneration (66). Although glutamine is among the most abundant amino acids in circulation (67), in some circumstances, such as muscle injury and aging, the abundance of glutamine in tissues is limited. Importantly, tissue macrophages can synthesize glutamine via the enhanced activity of glutamine synthetase. This macrophage-derived glutamine is transported inside muscle satellite cells through the SLC1A5 transporter to improve muscle regeneration after acute injury, ischemia, and aging (66). Glutamine metabolism controls stem cell fate in multiple cellular niches, such as bones (68) and the hair follicle (69), suggesting that it can mediate the effects of immune cells on tissue homeostasis in inflammation and aging.

Changes in inflammatory and metabolic signaling can rewire metabolism in immune cells and alter their homeostatic functions. Peritoneal macrophages isolated from old mice show suppressed glycolysis and mitochondrial respiration accompanied by increased production of inflammatory cytokines and decreased phagocytosis of bacteria compared to macrophages from young mice (70). Intriguingly, this age-associated metabolic and immune dysfunction of the macrophages was induced by prostaglandin E_2 (PGE_2), a lipid mediator of inflammation (71) whose levels are increased in aged mice and humans (70, 72). Moreover, blockade of PGE2 receptor EP2 in myeloid cells restored metabolic fitness of macrophages and improved brain inflammatory states and cognitive function in old mice (70). T cells can also connect immune cell–intrinsic metabolism to whole-body homeostasis. For example, T cells deficient in mitochondrial transcription factor A (TFAM) that have defective mitochondrial respiration induce multiple physiological dysfunctions in middle-aged mice, such as chronic inflammation, cardiac atrophy, and signs of neurological disability—the features of accelerated aging (73). In the future,

better understanding of how metabolic pathways intertangle immune and nonimmune cells will be critical to decode immunometabolism at the systems level.

2.5. Cross Talk Between Microbiota and Immunometabolism

In addition to regulating tissues, immune cells rely upon metabolic signaling to coordinate their functions with the microbiota (74). For example, gut microorganisms produce short-chain fatty acids (SCFAs) that inhibit histone deacetylases and activate G protein–coupled receptors in hematopoietic and nonhematopoietic cell lineages, thus regulating immune and metabolic homeostasis in the host (75). SCFAs have anti-inflammatory effects in macrophages (76) and dendritic cells (77) and support the differentiation of Tregs (78–80). The immune system coevolved with several classes of microbial metabolites to promote immune homeostasis. One of such classes of metabolites extensively modified by microbiota is bile acids. Bile acids are cholesterol-derived metabolites produced in the liver and secreted into the intestine and are critical for lipid digestion and glucose metabolism (81). The primary bile acids pool is converted into secondary bile acids by intestinal bacteria, and both primary and secondary bile acids act as ligands for the transcription factor farnesoid X receptor (FXR) (also known as bile acid receptor or nuclear receptor subfamily 1 group H member 4) (82, 83). Acting as signaling molecules, these metabolic products regulate innate and adaptive immunity, connecting hepatic metabolism and microbiota-controlled metabolism with immune homeostasis (84). Screening of the major species of bile acid metabolites identified the secondary bile acid 3β-hydroxydeoxycholic acid (isoDCA) and two distinct derivatives of lithocholic acid (LCA), 3-oxoLCA and isoalloLCA, as T cell regulators (85, 86). Similar to SCFAs, these classes of bile acids control Treg differentiation and function (85, 86). Interestingly, isoDCA mediates its regulatory functions in Tregs through the FXR signaling axis in dendritic cells (85), giving an example of a multilevel metabolic regulatory circuit that connects the liver and microbial metabolism with different classes of communicating immune cells. Emerging evidence from multi-omics and systems biology shows that microbiome-mediated metabolism affects human immunity in response to environmental cues (87) and alters the response to antitumor radiation therapy in mice and humans (88).

3. SYSTEMS-LEVEL TECHNIQUES TO STUDY IMMUNOMETABOLISM

To better understand the complexity of metabolic regulation of immunity, its components should be studied in the context of the tissue microenvironment, genetic and physiological background, and spatial and temporal dimensions of immune responses. Individual immune cells vary in their differentiation states and responses to the tissue microenvironment. Recently, single-cell genomics of human immune cells demonstrated a high degree of plasticity and adaptability of immune cells to organs of their residence (89). Likewise, immune cells can show intercellular metabolic variability and adapt metabolically to tissue niches and environmental cues (90). However, the metabolic composition of immune cells is sensitive to cell isolation conditions. Classical approaches to immune cell isolation, such as fluorescence-activated cell sorting (FACS), alter concentrations of many metabolites in cells (91). One of two major directions can overcome this limitation: either proxy techniques that measure metabolically relevant proteins or transcripts or direct measurements of metabolites via mass spectrometry imaging (MSI). Here we discuss these approaches to defining the systems-level state of metabolism in the tissues.

3.1. Mass Cytometry as a Proxy for Single-Cell Metabolic Profiling

Bulk cell metabolic analyses help us understand changes at the level of immune cell subsets, but they do not capture information about the metabolic heterogeneity of individual cells. Although

direct measurement of metabolites in individual cells is possible, the optimal sensitivity of this method has not yet been reached, and its effective throughput is low (92). As an alternative to single-cell metabolomics, single-cell-level measurement of rate-limiting metabolic enzymes can provide essential information about the metabolic state of the cell, serving as a proxy to metabolic pathways. This approach is based on labeling of immune cells with antibodies that recognize metabolic enzymes or regulators. Conventional flow cytometry can identify 10–20 features using fluorescently labeled antibodies as a readout. In contrast, mass cytometry (a fusion of flow cytometry and mass spectrometry using antibodies labeled with heavy metal ion tags) or spectral flow cytometry (93) provides approximately 40 cellular parameters at single-cell resolution, enabling high-dimensional and in-depth analysis of cellular and metabolic composition (94, 95). To capture essential metabolic information, antibodies included in these panels can recognize the main metabolic enzymes, such as hexokinase, phosphofructokinase 2, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which regulate glycolysis; citrate synthase and succinate dehydrogenase, which control TCA cycle activity; membrane metabolite transporters such as GLUT1 (for glucose) and CD36 (for fatty acids); and other transporters and rate-limiting metabolic enzymes (9).

Several recent studies applied mass spectrometry to understand the functional roles of metabolic pathways in immune cells in mice and humans. One of these studies used flow cytometry of immune cell populations in human blood combined with the measurement of 10 critical metabolic proteins, such as rate-limiting enzymes (e.g., hexokinase 1, CPT1A, and IDH2) and transporters (e.g., GLUT1 and SLC20A1), in individual cells (96). The authors found that T cell activation increases protein expression of glucose transporter GLUT1 and glycolytic and OXPHOS enzymes. Moreover, this method revealed differences in metabolic phenotypes between naive, effector memory, and central memory $CD4^+$ T cells, including elevated expression of fatty acid oxidation regulator CPT1A in effector memory and central memory subsets (96). Mass spectrometry analysis with approximately 20 critical metabolic enzymes, regulators, and transporters has been performed to study antigen-specific mouse $CD8^+$ T cells during activation and differentiation in response to *Listeria monocytogenes* infection in vivo (97). This analysis showed that transient, early-activated $CD8^+$ T cells profoundly increase the expression of proteins controlling glycolysis (e.g., GLUT1, GAPDH, and HIF1α), OXPHOS (e.g., citrate synthase and ATP5A), and fatty acid oxidation (e.g., CPT1A), in line with peaking glycolytic activity and increasing mitochondrial activity (97). Thus, this unbiased analysis revealed a metabolically specialized transient state of activated $CD8^+$ T cells before differentiation to effector or memory states.

3.2. Imaging Mass Cytometry

Antibodies suitable for mass spectrometry can also be repurposed to label their targets on histological tissue slides, adding histological context to cell identification and metabolic profiling (95). For example, this approach has been used to characterize the phenotype and metabolic regulome of single human $CD8^+$ T cells using a mass spectrometry panel evaluating multiple metabolic proteins (98). Integration of 48 parameters revealed heterogeneity of $CD8^+$ T cell metabolic remodeling during TCR activation, including distinct temporal phases of glycolysis and amino acid metabolism followed by decreased metabolic activity. Moreover, the authors adapted the mass spectrometry detection of the metabolic markers to a high-dimensional imaging platform (MIBI-TOF). They analyzed human tumor and nonmalignant samples with a resolution of approximately 400 nm. This spatial analysis demonstrated that cells expressing enzymes and transporters with the same metabolic specificity are enriched in the same tissue niches, suggesting the existence of metabolic microenvironmental drivers that coordinate the metabolic identity of immune cells

in human tissues (98). Combining classical mass spectrometry with high-dimensional imaging techniques is a promising approach to revealing metabolic regulators in individual cells and their relation to the tissue environment.

3.3. Transcriptomics Approaches to Immunometabolism

RNA sequencing is another technology that can be used as a proxy for studying immunometabolism (**Figure 2**). RNA sequencing is highly scalable and has been used to profile cohorts of hundreds and thousands of bulk samples (99) and millions of individual blood cells (100). In the context of metabolism, transcriptomics provides information about mRNA expression levels for all enzymes and transporters. Network analysis approaches to bulk RNA sequencing and scRNA-seq datasets have been used to characterize base-level metabolism in tissue-specific immune populations (32, 101). Modeling of cellular metabolic states applied to scRNA-seq data revealed the metabolic specificity of T helper type 17 (Th17) cell subpopulations and linked Th17 cell pathogenicity to polyamine metabolism (102). Integration of blood metabolomics with scRNA-seq data of patients with multiple sclerosis revealed altered regulation of aromatic amino acid metabolism in monocyte populations associated with the disease (103). A comprehensive

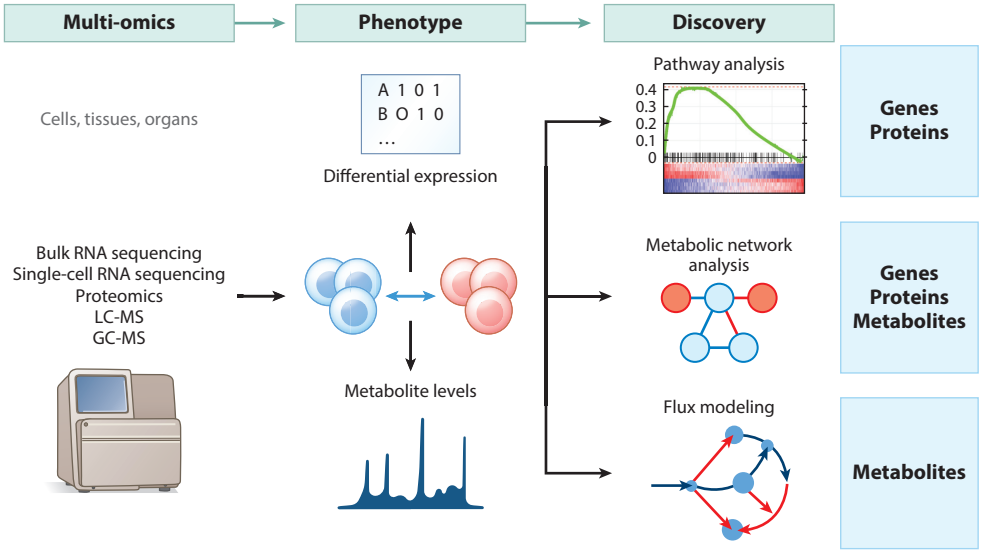

Figure 2

Multi-omics approaches to studying immunometabolism. (*Left*) Various techniques can be used to generate omics data from purified cells or entire organs or tissues, including gene expression (transcriptomics), protein expression and modifications (proteomics), and metabolite levels [metabolomics by liquid chromatography–mass spectrometry (LC-MS) or gas chromatography–mass spectrometry (GC-MS)]. (*Center*) These data are used to calculate the differential expression of genes and proteins or the differential abundance of metabolites and their statistical significance by comparing groups defined by phenotypes or experimental conditions. (*Right*) The information generated in this data analysis step can be used as input for methods predicting pathways and networks with significant biological phenotypes. Gene set enrichment analysis approaches reveal biological pathways enriched in overexpressed genes and proteins. Metabolic pathway analysis uses information about gene, protein, and metabolite levels to identify metabolic subnetworks, whose activity is differentially regulated depending on experimental conditions. Finally, flux modeling can identify balance between fluxes of various metabolites in possible reactions and reveal shifts in intracellular metabolism. Figure adapted from images created with BioRender.com.

example of using scRNA-seq and blood metabolomics combined multiple computational tools, including network analysis and flux modeling, to systematically characterize the immunometabolic landscape of individuals with COVID-19 and correlate it with disease severity (104). Transcriptomics approaches are an essential part of immunometabolism research, and they will provide valuable insights into the metabolism of immune responses and cell-cell communications.

3.4. Metabolic Imaging Techniques

MSI is currently the only technique that allows robust direct, systematic quantification of metabolism in intact cell populations (**Table 1**). As in any mass spectrometry approach, it includes two key steps. First, compounds of interest are ionized (105–107) using a focused laser or a primary ion beam whose location relative to the sample can be automatically controlled, thus achieving spatially resolved mass spectra. Second, the generated ions are quantified with a

Table 1 Characteristics of metabolic imaging techniques

Ionization method	Sample	Resolution	Number of features	*m/z* range	Reference
MALDI	dHepaRG hepatocyte culture	50 μm grid spacing, 30 μm beam diameter	740 metabolites	200–1,100	109
SIMS	Tissue sections: mouse liver, stomach, pancreas, kidney, lung, and small intestine; nontumor tissue regions from patients with liver cancer	1.5 μm per pixel	200–300 selected ion species	50–500	115
SIMS	Frozen hydrated HeLa cells	1 μm × 1 μm × 400 nm voxels	NA (continuous spectra, focused analysis)	90–900	118
MALDI	Mouse brain tissue section	1.4 μm spot diameter, 2 μm step size	89 phospholipid ion signals	700–900	119
MALDI	Mouse kidney and brain tissues	*x–y* raster width was set to 20–50 μm	200 canonical metabolites were identified	70–300	113
SIMS	Human tonsil tissue	580 nm × 580 nm, 138 subsequent slices of 5-μm-thick tissue samples	189 metabolites	NA	117
MALDI	Mouse heart tissue sections	30 μm per pixel	NA	300–2,000	122
MALDI	Human dermal fibroblasts	5–7 μm	296 lipids	400–1,600	124
MALDI	Cortex tissue sections of patients with glioblastoma	50 μm	NA	75–1,000	125
MALDI	Kidney tissue sections from mice with *Staphylococcus aureus* infection; heart tissue sections from a patient with infective endocarditis	15-μm spacing with 10-μm beam diameter for mouse tissues; 40-μm spacing, 30-μm beam diameter for human tissues	NA	200–2,000	194
MALDI	Lung sections from rabbits infected with *Mycobacterium tuberculosis*	50-μm spacing	NA	300–500	195

Abbreviations: MALDI, matrix-assisted laser desorption/ionization; NA, not available; SIMS, secondary ion mass spectrometry.

mass-to-charge ratio (m/z) detector. Depending on the application, a balance between improving spatial resolution and increasing the complexity of systematic molecular identification must be achieved (105). This guides the choice of a specific ionization approach, with two main alternatives: matrix-assisted laser desorption/ionization (MALDI) and secondary ion mass spectrometry (SIMS).

MALDI is the most common approach in spatial metabolomics (107). In MALDI, a sample is coated with a matrix—a crystalline structure that absorbs laser energy (108). When the sample is irradiated with a laser, the matrix absorbs the laser energy and acts as a proton donor, thus ionizing the sample. MALDI is a soft ionization method where the main mode of analyte compounds is protonated without fragmentation, which significantly simplifies the identification of molecules in measured spectra. A MALDI-based approach aimed to democratize spatial metabolomics integrates commercially available MALDI imaging and bright-light microscopy, avoiding the requirement for a custom setup (109). There are several significant steps in this approach. First, a bright-field microscopy image of the sample is obtained. This pre-MALDI image is used to identify cells and additional morphological and fluorescence-based features. Second, the sample goes through MALDI-based imaging. Finally, a post-MALDI microscopy image is obtained. The post-MALDI image is needed to align MALDI spectral images and pre-MALDI images based on pen marks (connecting pre-MALDI and post-MALDI images) and laser ablation marks (connecting MALDI and post-MALDI images). While bright-field microscopy has a submicrometer resolution, the MALDI image corresponds to a grid with a spacing of approximately 50 μm, allowing distinct analysis only for groups of cells or sufficiently large cells. Still, the authors have been able to identify a specific subpopulation of steatotic hepatocytes in the lipid-stimulated cell culture (109). Another study combined high-resolution MALDI-based MSI with confocal microscopic imaging of human pancreas tumor samples and found an accumulation of long-chain fatty acids in intratumoral $CD8^+$ T cells that impaired their mitochondrial function (110).

Additionally, not only can MALDI be run in a standard grid pattern, but the acquisition spatial pattern can be controlled programmatically (111, 112). One example of this approach has been used to analyze the metabolome of extracellular dense-core vesicles in secretory cells of the sea slug *Aplysia* (112). First, a bright-field microscopy image was used to detect the positions of micrometer-scale vesicles. Next, a distance filter was applied to remove vesicles closer than 200 μm to each other. Finally, a MALDI image was obtained, and while the laser had a 100-μm spot size, the previously applied filter ensured that only a single micrometer-scale vesicle was sampled per MALDI pixel. This approach can analyze the metabolic composition of separate organelles, such as microvesicles, in immune cells. Lastly, MALDI-based metabolic imaging is compatible with isotope labeling experiments. For example, ^{13}C- and ^{15}N-labeled nutrient infusion coupled with MALDI-based metabolic imaging revealed metabolic spatial organization of the kidney and brain, including spatial gradation in carbon input impacted by a ketogenic diet (113). The authors showed the presence of glycolytic and gluconeogenic regions in the kidney and that glucose is uniformly used as a primary carbon source in the brain under a standard diet (113).

SIMS ionization methods provide different technological balances—optimizing the spatial resolution at the expense of molecular identification (105). In SIMS, the sample is radiated by a primary ion beam, leading to analyte ionization (114). In contrast to MALDI, SIMS does not require a matrix coating, as the sample is directly radiated, simplifying the sample preparation procedure. SIMS-based technologies offer submicrometer resolution. However, the trade-off is higher metabolite fragmentation. In one of the recent examples of SIMS-based methods, a resolution of approximately 1.5 μm per pixel was reported, which is sufficient not only for single-cell-level

analysis but also to resolve subcellular structures (e.g., nuclei) (115). This approach identified four metabolically distinct subpopulations of hepatocytes in human liver tissue slides. However, SIMS-based methods produce highly fragmented spectra, which complicate the identification of the precise molecular composition of each pixel. As such, the analysis is typically limited to identifying overall metabolic patterns rather than individual metabolites across the samples. To connect the metabolic patterns to biological functions, the authors combined the SIMS-based MSI analysis with spatial transcriptomics using serial sections, which allowed them to highlight a metabolic signature of hepatocytes connected to the fibrosis (115).

Multi-layer imaging and 3D reconstruction of the sample are essential advantages of SIMS-based technologies (116, 117). In this setup, two ion beams are used in the same experiment: one for analyte ionization and another for removal of the sample layer after the round of scanning. Pareek et al. (118) used 3D metabolic scanning to resolve 1 μm $\times$ 1 μm $\times$ 400 nm for each voxel. Due to fragmentation, interpretation of the obtained spectra is challenging, and the authors validated experimental results, including pure standards for metabolites and experiments, with isotope labeling. This approach revealed the presence of voxels highly enriched in 5-aminoimidazole-4-carboxamide (AICAR), an intermediate of the de novo purine biosynthesis pathway, and indicated functional colocalization of the de novo purine biosynthesis pathway enzymes (118).

A promising MSI approach combining high resolution and low fragmentation is atmospheric pressure MALDI (AP-MALDI) MSI (119). Kompauer et al. (119) optimized a laser setup and matrix application procedure to achieve 1.4-μm resolution. The approach is also compatible with tandem mass spectrometry, which can be used for accurate metabolite identification. The same AP-MALDI approach was further improved for integrating fluorescence in situ hybridization (FISH) (120). In this work, the authors modified the AP-MALDI setup to decrease tissue destruction while retaining micrometer lateral resolution, and they further additionally optimized the post-MALDI fixation procedure and FISH probes. Together, this allowed them to discern host-microbe interaction in deep-sea mussel symbiosis with sulfur-oxidizing and methane-oxidizing bacteria. First, the authors obtained an AP-MALDI image of the cryosection with a pixel size of 3 μm, and then the same tissue section was analyzed with fluorescence microscopy with FISH probes specific to 16S rRNA of sulfur-oxidizing and methane-oxidizing bacteria and an additional DAPI staining for both host and bacterial DNA. This technique allowed the authors to link the metabolome either to individual eukaryotic cells or to patches of 50–100 bacterial cells and showed the spatial partitioning of the metabolome into submetabolomes depending on the presence of the symbiotic bacteria (120).

Integrative multimodal MSI approaches provide an exciting avenue for a multifaceted and complementary understanding of metabolic regulation in tissues (121). Other examples of multimodal measurement in the same tissue sample include a combination of MALDI lipidomics and proteomics (122) and MALDI and SIMS metabolomics (123), which have allowed MALDI-like spectra with SIMS-like resolution to be achieved computationally. Further, even the metabolomics data combined with other data types in partially independent samples have generated essential insights (115, 124, 125). For example, Capolupo et al. (124) showed how nonspatial single-cell transcriptomics can connect transcriptional programs with lipid heterogeneity in human fibroblasts. Another example is the simultaneous integration of spatial transcriptomics, metabolomics, and proteomics that deciphered the spatial architecture of glioblastoma and identified immunosuppressive interactions between immune and tumor cells in segregated niches (125). MSI, combined with multi-omics in the same or parallel tissue section, is a powerful tool to identify metabolic regulators of immune cells and understand metabolic communication between cells in tissue niches.

4. GENETIC APPROACHES IN IMMUNOMETABOLISM

Systems-level approaches, such as metabolic imaging and transcriptomics-based predictions, can reveal metabolites associated with distinct immune cell subsets or connected to specific cell differentiation states. Because cellular metabolism is an interconnected system that depends on enzymatic and regulatory inputs from many pathways, it is essential to validate the roles of the metabolic regulators in immune responses functionally. In mice, genome-editing and -screening techniques targeting metabolically relevant genes can provide a functional link to immunometabolism. In humans, analysis of the associations between natural variations in genomes (e.g., single-nucleotide polymorphisms) and immune and metabolic phenotypes can provide essential information about immunometabolic regulators and their functions.

4.1. Genome-Editing Tools

Genome-editing technologies characterize the functional roles of multiple genes in one screening experiment (126). Prokaryote-derived CRISPR-Cas genome-editing systems are essential tools in modern biological research (127). Due to their scalability and flexibility, CRISPR-Cas techniques are ideal for simultaneously interrogating multiple genes in pooled libraries and are actively used both in vitro and in vivo. In a recent study, to understand how metabolic signaling affects $CD8^+$ T cell fate, the authors designed an in vivo CRISPR-Cas9 screening system that included a library of 3,017 metabolism-associated genes introduced into mouse $CD8^+$ T cells that express P14-transgenic TCRs recognizing the $gp33_{33–41}$ epitope of lymphocytic choriomeningitis virus (LCMV) (128). Acute LCMV infection in these mice leads to rapid specification of terminal effector and memory precursor subsets of virus-specific $CD8^+$ T cells that can be distinguished based on the expression of KLRG1 and CD127 ($KLRG1^+CD127^-$ terminal effector and $KLRG1^-CD127^+$ memory precursor). The CRISPR-Cas9 screening system allowed the authors to identify approximately 300 genes from the metabolic library whose frequencies were altered in terminal effector versus memory precursor cells around seven days after infection. Among these genes, the authors validated the effects of downregulating *Acaca* on decreasing memory precursor cell formation and the opposite effect in the case of deletion of *Pten*. This approach also showed that loss of amino acid transporters *Sls7a1* and *Slc38a2* profoundly diminished memory precursor cell formation, whereas deletion of glycosyltransferase *Pofut1* increased proportions of effector T cells and cytokine expression in effector T cells (128).

Similar to $CD8^+$ T cells, $CD4^+$ T cells expressing transgenic TCRs specific to the cognate antigen are suitable for massive genetic screening of metabolic regulators in vivo. A recent study used an in vivo CRISPR-Cas9 screening system to identify components of one-carbon metabolism that regulate the differentiation of mouse $CD4^+$ T cells (129). The authors designed the CRISPR-Cas9 library using ovalbumin-specific OT-II $CD4^+$ T cells and transferred the cells into $Rag1^{-/-}$ mice subjected to ovalbumin-induced lung inflammation. This approach revealed several one-carbon metabolism genes depleted from expanded OT-II $CD4^+$ T cells in the lungs. The authors confirmed that the metabolism enzyme methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) is required for maximal $CD4^+$ T cell activation, proliferation, and cytokine production. Interestingly, MTHFD2 was essential to suppress Treg differentiation in mice and humans, and MTHFD2 inhibition suppressed inflammation in mouse models of T cell–dependent delayed-type hypersensitivity, experimental autoimmune encephalomyelitis, and allergic airway inflammation (129).

$CD4^+$ T follicular helper (Tfh) cells regulate the germinal center reaction to initiate and maintain long-term antibody responses; however, unbiased understanding of Tfh cell metabolic regulation is limited. A recent in vivo CRISPR-Cas9 screening model containing approximately

400 single guide RNAs aimed to identify Tfh cell metabolic regulators in physiological settings (130). The authors used this library in the acute LCMV infection model and identified targeted genes that discriminate Tfh from Th1 cell development in mice, including the hypoxia-inducible factor 1 gene *Hif1a*. Genetic validation of the results confirmed that deletion of Hif1a from CD4-expressing cells increased the expansion of germinal center Tfh cells compared to that of wild-type phenotype mice infected with LCMV (130). These examples show that CRISPR-Cas systems are a powerful tool to decipher immunometabolic regulators of $CD4^+$ T cells in vivo. Future applications of CRISPR-Cas genome-editing systems to immunometabolism research will foster approaches to study other actively proliferating and differentiating immune cell subsets, such as B cells, monocytes, and dendritic cells.

4.2. Metabolic Genome-Wide Association Studies

Natural variations in human genomes are a valuable tool to decipher how metabolism is connected to immunity in humans. Genetic regulation of metabolism in humans can be inferred using metabolic genome-wide association studies (131). For example, a correlation between genotypes and plasma metabolite levels can be studied (132), as accruing blood samples from a sufficiently large cohort of individuals is more feasible than collecting metabolic profiles from other tissues. However, linking such studies to immune function requires additional steps, such as adding blood cell transcriptomics on top of metabolomics to serve as a proxy for immune processes (133). Another approach is to start from a pronounced immunometabolic trait and conduct a small-scale genomic study. This approach revealed a gain-of-function mutation in the *SDHA* gene (encoding succinate dehydrogenase subunit A) as a genetic factor contributing to polyclonal B cell lymphocytosis development (134).

5. COMPUTATIONAL CHALLENGES IN DATA ANALYSIS

The generation of massive and complex datasets using techniques such as mass spectrometry presents new challenges in data analysis (**Figure 3**). The first of these challenges is that the data must be simplified and structured for visualization and initial exploratory analysis. This is typically achieved with dimensionality reduction and clustering methods that can be applied to raw intensity data. Biological interpretation of the data requires metabolite identification and pathway and network analysis. This section briefly considers critical computational steps in the system-level analysis.

5.1. Dimensionality Reduction and Unsupervised Learning

Systems-level metabolic data are inherently high-dimensional. Data of this type represent many objects, such as individual cells or tissue regions with complex 2D and 3D geometry, and are quantified with many modalities (metabolomics, proteomics, transcriptomics), each consisting of hundreds or thousands of features. Thus, even the simplest tasks of data visualization and exploration require unsupervised machine learning methods to elucidate the internal structure of the data. Here we give a brief overview of the existing approaches and refer the reader to a recent comprehensive review for more details (135).

The first class of methods is dimensionality reduction. It is a classical computational problem whose goal is to represent the points originally defined in high-dimensional space in a lower-dimensional space while preserving some of the higher-dimensional structure. As an example, for analysis of MSI data, the points can correspond to points on an image grid, and initial high-dimensional space would correspond to measured ion intensities. Typically, one wants to make a

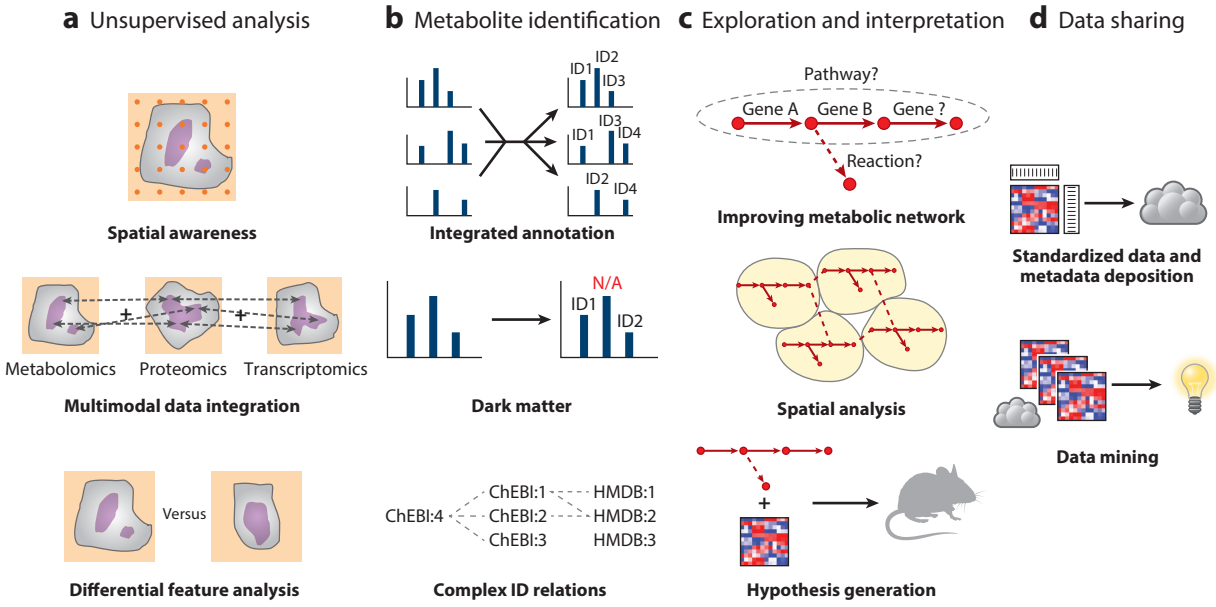

Figure 3

Computational challenges in systems immunology. (*a*) Unsupervised analysis of systems immunology data heavily relies on more general data analysis and machine learning methods. However, it is important to account for field-specific aspects of the data. The profiled tissue slides have a certain spatial organization that shapes the measurement. Multi-omics profiling has the potential to give a fuller picture, but different types of data should be appropriately aligned and correlated before the analysis. Due to the complex sample structure, the problem of differential feature analysis should be properly formulated and addressed with the development of computational methods. (*b*) Knowing what metabolites have been measured is critical for the understanding of the mass spectrometry data. Integration of many mass spectra measurements can give a better understanding of the processes influencing the measurements, such as fragmentation or ionization, and help to improve annotation methods. Expanding the known universe of metabolites is essential as well. Another problem lies in the inherent complexity of biochemical species hierarchy, which leads to complex relations between different systems of identifiers. (*c*) Interpreting the systems metabolism data requires studying not just individual entities (e.g., metabolites or genes) but also their complex interactions in metabolic processes. The first challenge lies in extending our knowledge of the potential interactions, including discovering new reactions and new enzymatic functions and defining new metabolic pathways. The spatial nature of the data makes many of the classical analysis methods inapplicable, as they were designed to compare two relatively uniform sets of samples in a steady-state condition. Going from exploratory analysis to the generation of experimentally testable hypotheses also remains a challenge. (*d*) Systematic sharing of the generated data increases the impact of the research. However, to be useful for reanalysis, the data and metadata deposition should happen in a standardized fashion. Finally, systematically reanalyzing and searching the data can benefit the whole immunometabolism field.

2D plot illustrating the metabolic diversity of the sample. This is generally achieved with nonlinear projection methods, such as *t*-distributed stochastic neighbor embedding (*t*-SNE) and uniform manifold approximation and projection (UMAP), which focus on preserving different aspects of the high-dimensional structure (136, 137). An alternative approach has been used to automatically assign colors to the MSI grid points, where the lower-dimensional space corresponds to the 3D red-green-blue (RGB) color space (115). Dimensionality reduction methods are also used to obtain intermediate data representation, simplifying further computational tasks, for example clustering or spatial pattern detection (138).

Clustering is another classical data analysis method commonly used in the context of metabolomics. The goal of clustering is to group inside one cluster objects that are more similar to each other than the objects in other clusters. Clustering can be applied to spectra and grid points or cells for the imaging data. In the first case, ions with similar spatial patterns are grouped

(139). In the second case, the cells with similar metabolic profiles are grouped (115). Clustering procedures depend on the definition of similarity; notably, spatial similarity measurement, that is colocalization, must be carefully selected (140).

The problem of detection of spatial patterns can be further considered for interpretation and follow-up experiments. For this task, Alexandrov and Bartels (141) introduced a spatial chaos metric that distinguishes structured patterns from unstructured ones. Dimensionality-reduction methods explicitly or implicitly detect patterns in the data and can also be used. Classical methods, like factor analysis (142, 143), or more modern ones, like autoencoders (138), can detect patterns in mass spectrometry datasets. Finally, if the initial sample contains labeled segments (e.g., tumor and normal tissue), statistical discriminative analysis methods can be applied to mass spectrometry data analysis (144).

Cross analysis of multimodal data requires its own methods (145). Classical statistical methods, such as canonical correlation analysis or partial least squares regression, are successfully applied to find correlated structures between two data modalities (123, 146). Spatial integrated analysis of cellular composition and metabolic niches provides additional challenges to data analysis. For example, integrating multiplex immunofluorescent imaging and MSI can reveal connections between immune cell heterogeneity and its metabolic context in complex tissues (147). More specialized multi-omics integration methods (148) and methods focused on spatial data (149) have also been developed and can be used to analyze multimodal datasets connecting immunology and metabolism.

5.2. Metabolite Identification

A natural task of metabolomics is identifying and annotating metabolites in measured spectra. This step is challenging even for the bulk metabolomics (150), and it is increasingly difficult in the case of MSI. The classical approach to metabolite assignment of measured mass-to-charge ratio consists of the preliminary matching of ions with the metabolites based on their masses and potential adducts. The next step is filtering obtained metabolites based on additional data, such as chromatography column retention time or secondary spectra from tandem mass spectrometry (151). Advanced machine learning methods, for example based on deep neural network architectures, are emerging as potential systematic solutions for metabolite identification tasks (152, 153).

Typically, only the direct mass spectrometry spectra are used for annotation in MALDI-based MSI technologies. One of the early efforts in that direction was made by Palmer et al. (154), who presented a computational framework to annotate image-based mass spectrometry data. In this approach, only some chemical formulas are assigned to mass spectrometry signals, and isomers are not resolved. The method uses three measures to match mass spectrometry signals with metabolites: (*a*) spatial informativeness of the principal peak, (*b*) spectral similarity between theoretical isotopic patterns and measured intensities, and (*c*) spatial colocalization between isotopic patterns. However, this approach cannot resolve isotopic patterns. To combat false positive assignment, the authors suggested a false discovery rate (FDR) estimation procedure based on decoy metabolites with implausible adducts (154). This approach later became a foundation for the METASPACE platform, allowing researchers to annotate MSI data (155). Still, this approach enables annotating only approximately 1% of MSI data (e.g., 10^2 molecules out of a dataset with 10^4 m/z channels) (155). The rest of the measured features remain unannotated, the dark matter of metabolomics (156), underscoring the need for more advanced metabolite annotation methods.

Network-based metabolite identification methods represent a promising direction for metabolite annotation and discovery (120, 157, 158). These methods are based on the idea that the organism's metabolome is connected: No metabolite can be obtained from nowhere. Thus, a network can be constructed where nodes correspond to metabolites and their derivatives, and

edges represent the gain or loss of specific chemical moieties. For example, one of such methods classifies the edges as metabolic (due to biochemical reactions) and abiotic (due to adducts, isotope composition, and fragmentation) (158). After the network is constructed, it can be used for metabolite identification based on the assumption that the neighbors of measured metabolites are more likely to be also present in the sample. The exact procedure varies, from a heuristic greedy search (157) to a more advanced optimization approach (158).

Particular attention is required to expand the universe of known biological compounds and physiological reactions. For example, the chemical database ChEBI (159) contains 60,000 annotated compounds, but the reaction database Rhea (160) contains only 15,000 reactions involving 13,000 ChEBI compounds. While there are more biochemical databases (160–164), quality reactions are significantly underrepresented. A recent study attempted to bring more compounds to reactions (165). First, the authors gathered a large metadatabase (bioDB) of 1.5 million unique biological or bioactive compounds and 56,000 unique biochemical reactions. This metadatabase was used to form reaction rules and determine active sites, which allowed the authors to predict 5.2 million hypothetical reactions (165).

5.3. Pathways, Networks, and Fluxes

There are three major approaches to moving from metabolite- or reaction-level analysis to metabolic processes: canonical pathway analysis, metabolic network analysis, and flux modeling (9) (**Figure 2**).

Pathway analysis methods work with a collection of predefined pathways and identify pathways that show some level of regulation in the experiment. Pathways for the analysis can be obtained from metabolically focused databases like KEGG (161) or Reactome (164), as well as from more general pathway databases like Gene Ontology (166) and MSigDB (167). Pathways defined in terms of enzymes and the corresponding genes can be used to characterize metabolic states of an individual cell or MSI pixels based on transcriptional data (168). Metabolite-based pathway analysis is more nuanced, as the mapping from mass spectrometry peaks to metabolites is not entirely accurate and specific. However, specialized algorithms have been developed to implement metabolite-based pathway analysis (169–172).

Distinct from pathway analysis, network-based methods do not depend on predefined pathways and instead consider a global network of interconnected reactions (173). In a simple case, the network nodes correspond to metabolites and connections to biochemical reactions. Such networks can infer pathways in a data-independent manner based on connectivity (174). When the omics data are available and can be summarized into numeric values characterizing metabolite or reaction importance, connected subnetworks with high combined importance can be found (175, 176). These approaches have proven helpful for multiple data types, including single-cell-level transcriptional data (101, 177). The natural next step is to extend these approaches toward MSI data in the future.

Flux modeling is a more structured alternative to network analysis. In such a case, reaction connections are considered, and the steady-state flux structure is enforced. The foundational framework for such approaches is the flux balance analysis (178). There, metabolic models are considered with a defined set of possible reactions. Then, under the assumption of a steady state, a space of potential reaction fluxes is determined, such that mass balance constraints (equal rates of metabolite production and utilization) are satisfied. Flux balance analysis can be integrated with gene expression data to investigate metabolic regulation, both in the bulk transcriptomics (179, 180) and scRNA-seq (102, 181, 182). Aside from gene expression, flux modeling methods can also be integrated with isotope labeling data (183). However, we are not aware of the application of these methods to MSI isotope labeling data.

5.4. Data Sharing and Reanalysis

Public data sharing proved to be a valuable instrument for accelerating biomedical research (184). Facilitated by journal policies, deposition of high-throughput gene expression data and experiment metadata to databases such as Gene Expression Omnibus (185), ArrayExpress (186), and Sequence Read Archive (187) became standard in the field of transcriptomics. This led to many datasets that are publicly available for reanalysis by researchers: For example, the Gene Expression Omnibus database now contains more than 175,000 datasets.

Presently, data deposition policies are not as strict for the metabolomics field, but metabolomics databases are also available (155, 188, 189). The Metabolomics Workbench database is focused on bulk metabolomics and contains fewer than 2,000 studies (188). METASPACE is a more recent database focused on spatial metabolomics, and it already features more than 6,000 datasets (155). A distinctive advantage of METASPACE is that it allows data sharing and can be used for research on metabolite annotation. Developing data-sharing standards in immunometabolism research, encompassing both metabolomics and immunological data, is the next important step for the community.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The field of immunometabolism has recently generated multiple novel insights into fundamental mechanisms of immune responses. The focus for bridging metabolism and immunity has been on the intracellular metabolic processes. The next major conceptual advance in this field will connect cell-level metabolism and macroscopic physiological metabolism during immune responses. This goal will require different experimental and analytical approaches compared to classical metabolomic profiling techniques currently used to decipher the metabolism of immune cell populations. Suitably, the field is on the cusp of a major technological transition directed toward spatially resolved metabolic profiling of the unperturbed (or minimally perturbed) tissues (190). While spatial metabolic imaging technologies are rapidly evolving and maturing, the approaches should become more standardized and affordable to enable full-scale immunological research. Taking further lessons from high-throughput sequencing, data sharing, and the ability to reanalyze the data and perform meta-analysis are essential components of the infrastructure for systems-level research. Metabolite identification is one of the critical bottlenecks in the current data-processing pipelines (191). Systematic accumulation of large amounts of standardized data will enable an even more robust application of machine learning approaches for metabolite identification tasks.

To further advance systems-level immunometabolism research, one can envision developing experimental systems that connect in vitro discoveries and in vivo imaging in a scalable and controlled manner. Integrative analysis of multimodal in vitro and in vivo datasets will enable mechanistic investigation of the immunometabolic regulatory processes in the context of whole-body physiological processes and tissue microenvironments. For instance, regulatory phenotypes, including immune and metabolic components, can be guessed from unbiased tissue metabolic imaging and further validated in more controlled systems in vitro. For example, immunometabolic rules might be studied by coculturing relevant cell types (such as macrophages and fibroblasts) that can produce stable multicomponent cell systems capturing critical regulatory rules of complex physiological systems (192). Moreover, rapidly developing organoid culture systems research might also serve as a powerful 3D platform to decipher immune and metabolic processes inferred from complex tissue niches in vivo (193). Lastly, the next technological frontier of immunometabolism is the ability to perform spatial metabolic profiling at a subcellular resolution. This will allow us to dissect metabolic specialties of distinct organelles that play essential roles in immune responses, such as

mitochondria, lysosomes, and endosomes. Together, these technological and computational tools will move forward systems-level and holistic research in immunometabolism.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

LITERATURE CITED

1. Muehlenbein MP, Hirschtick JL, Bonner JZ, Swartz AM. 2010. Toward quantifying the usage costs of human immunity: Altered metabolic rates and hormone levels during acute immune activation in men. *Am. J. Hum. Biol.* 22(4):546–56
2. Menk AV, Scharping NE, Moreci RS, Zeng X, Guy C, et al. 2018. Early TCR signaling induces rapid aerobic glycolysis enabling distinct acute T cell effector functions. *Cell Rep.* 22(6):1509–21
3. Kelly B, O'Neill LAJ. 2015. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res.* 25(7):771–84
4. Waters LR, Ahsan FM, Wolf DM, Shirihai O, Teitell MA. 2018. Initial B cell activation induces metabolic reprogramming and mitochondrial remodeling. *iScience* 5:99–109
5. Murray PJ. 2016. Amino acid auxotrophy as a system of immunological control nodes. *Nat. Immunol.* 17(2):132–39
6. O'Neill LAJ, Pearce EJ. 2015. Immunometabolism governs dendritic cell and macrophage function. *J. Exp. Med.* 213(1):15–23
7. Voss K, Hong HS, Bader JE, Sugiura A, Lyssiotis CA, Rathmell JC. 2021. A guide to interrogating immunometabolism. *Nat. Rev. Immunol.* 21(10):637–52
8. Purohit V, Wagner A, Yosef N, Kuchroo VK. 2022. Systems-based approaches to study immunometabolism. *Cell Mol. Immunol.* 19(3):409–20
9. Artyomov MN, Van den Bossche J. 2020. Immunometabolism in the single-cell era. *Cell Metab.* 32(5):710–25
10. Jung J, Zeng H, Horng T. 2019. Metabolism as a guiding force for immunity. *Nat. Cell Biol.* 21(1):85–93
11. Ye J, Medzhitov R. 2019. Control strategies in systemic metabolism. *Nat. Metab.* 1(10):947–57
12. Davis MM, Tato CM, Furman D. 2017. Systems immunology: just getting started. *Nat. Immunol.* 18(7):725–32
13. Poon MML, Farber DL. 2020. The whole body as the system in systems immunology. *iScience* 23(9):101509
14. Villani A-C, Sarkizova S, Hacohen N. 2018. Systems immunology: learning the rules of the immune system. *Annu. Rev. Immunol.* 36:813–42
15. O'Neill LAJ, Kishton RJ, Rathmell J. 2016. A guide to immunometabolism for immunologists. *Nat. Rev. Immunol.* 16(9):553–65
16. Schipper HS, Prakken B, Kalkhoven E, Boes M. 2012. Adipose tissue-resident immune cells: key players in immunometabolism. *Trends Endocrinol. Metab.* 23(8):407–15
17. Trim WV, Lynch L. 2022. Immune and non-immune functions of adipose tissue leukocytes. *Nat. Rev. Immunol.* 22(6):371–86
18. Huby T, Gautier EL. 2022. Immune cell-mediated features of non-alcoholic steatohepatitis. *Nat. Rev. Immunol.* 22(7):429–43
19. Lercher A, Baazim H, Bergthaler A. 2020. Systemic immunometabolism: challenges and opportunities. *Immunity* 53(3):496–509
20. Kaymak I, Williams KS, Cantor JR, Jones RG. 2021. Immunometabolic interplay in the tumor microenvironment. *Cancer Cell* 39(1):28–37
21. Wang A, Luan HH, Medzhitov R. 2019. An evolutionary perspective on immunometabolism. *Science* 363(6423):eaar3932
22. Zhao S, Peralta RM, Avina-Ochoa N, Delgoffe GM, Kaech SM. 2021. Metabolic regulation of T cells in the tumor microenvironment by nutrient availability and diet. *Semin. Immunol.* 52:101485

23. Corrado M, Pearce EL. 2022. Targeting memory T cell metabolism to improve immunity. *J. Clin. Investig.* 132(1):e148546
24. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, et al. 2013. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* 496(7444):238–42
25. Jha AK, Huang SC-C, Sergushichev A, Lampropoulou V, Ivanova Y, et al. 2015. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity* 42(3):419–30
26. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, et al. 2016. Succinate dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. *Cell* 167(2):457–70.e13
27. Everts B, Amiel E, Huang SC-C, Smith AM, Chang C-H, et al. 2014. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. *Nat. Immunol.* 15(4):323–32
28. Gubser PM, Bantug GR, Razik L, Fischer M, Dimeloe S, et al. 2013. Rapid effector function of memory $CD8^+$ T cells requires an immediate-early glycolytic switch. *Nat. Immunol.* 14(10):1064–72
29. Raza IGA, Clarke AJ. 2021. B cell metabolism and autophagy in autoimmunity. *Front. Immunol.* 12:681105
30. Sheppard S, Santosa EK, Lau CM, Violante S, Giovanelli P, et al. 2021. Lactate dehydrogenase A-dependent aerobic glycolysis promotes natural killer cell anti-viral and anti-tumor function. *Cell Rep.* 35(9):109210
31. Makowski L, Chaib M, Rathmell JC. 2020. Immunometabolism: from basic mechanisms to translation. *Immunol. Rev.* 295(1):5–14
32. Artyomov MN, Sergushichev A, Schilling JD. 2016. Integrating immunometabolism and macrophage diversity. *Semin. Immunol.* 28(5):417–24
33. Wang F, Zhang S, Vuckovic I, Jeon R, Lerman A, et al. 2018. Glycolytic stimulation is not a requirement for M2 macrophage differentiation. *Cell Metab.* 28(3):463–75.e4
34. Russell DG, Huang L, VanderVen BC. 2019. Immunometabolism at the interface between macrophages and pathogens. *Nat. Rev. Immunol.* 19(5):291–304
35. Ogger PP, Byrne AJ. 2021. Macrophage metabolic reprogramming during chronic lung disease. *Mucosal Immunol.* 14(2):282–95
36. Svedberg FR, Brown SL, Krauss MZ, Campbell L, Sharpe C, et al. 2019. The lung environment controls alveolar macrophage metabolism and responsiveness in type 2 inflammation. *Nat. Immunol.* 20(5):571–80
37. Elmentaite R, Teichmann SA, Madissoon E. 2019. Studying immune to non-immune cell cross-talk using single-cell technologies. *Curr. Opin. Syst. Biol.* 18:87–94
38. Han MS, Jung DY, Morel C, Lakhani SA, Kim JK, et al. 2013. JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. *Science* 339(6116):218–22
39. Chakarov S, Blériot C, Ginhoux F. 2022. Role of adipose tissue macrophages in obesity-related disorders. *J. Exp. Med.* 219(7):e20211948
40. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, et al. 2009. $CD8^+$ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat. Med.* 15(8):914–20
41. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, et al. 2009. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* 15(8):930–39
42. Vasanthakumar A, Chisanga D, Blume J, Gloury R, Britt K, et al. 2020. Sex-specific adipose tissue imprinting of regulatory T cells. *Nature* 579(7800):581–85
43. Kubes P, Jenne C. 2018. Immune responses in the liver. *Annu. Rev. Immunol.* 36(1):247–77
44. Haas JT, Francque S, Staels B. 2016. Pathophysiology and mechanisms of nonalcoholic fatty liver disease. *Annu. Rev. Physiol.* 78:181–205
45. Sutti S, Albano E. 2020. Adaptive immunity: an emerging player in the progression of NAFLD. *Nat. Rev. Gastroenterol. Hepatol.* 17(2):81–92
46. Elia I, Haigis MC. 2021. Metabolites and the tumour microenvironment: from cellular mechanisms to systemic metabolism. *Nat. Metab.* 3(1):21–32
47. Li F, Simon MC. 2020. Cancer cells don't live alone: metabolic communication within tumor microenvironments. *Dev. Cell* 54(2):183–95

48. Reinfeld BI, Madden MZ, Wolf MM, Chytil A, Bader JE, et al. 2021. Cell-programmed nutrient partitioning in the tumour microenvironment. *Nature* 593(7858):282–88
49. Geeraerts X, Fernández-Garcia J, Hartmann FJ, de Goede KE, Martens L, et al. 2021. Macrophages are metabolically heterogeneous within the tumor microenvironment. *Cell Rep*. 37(13):110171
50. Watson MJ, Vignali PDA, Mullett SJ, Overacre-Delgoffe AE, Peralta RM, et al. 2021. Metabolic support of tumour-infiltrating regulatory T cells by lactic acid. *Nature* 591(7851):645–51
51. McLane LM, Abdel-Hakeem MS, Wherry EJ. 2019. CD8 T cell exhaustion during chronic viral infection and cancer. *Annu. Rev. Immunol.* 37:457–95
52. Guo Y, Xie Y-Q, Gao M, Zhao Y, Franco F, et al. 2021. Metabolic reprogramming of terminally exhausted $CD8^+$ T cells by IL-10 enhances anti-tumor immunity. *Nat. Immunol.* 22(6):746–56
53. Liu X, Hartman CL, Li L, Albert CJ, Si F, et al. 2021. Reprogramming lipid metabolism prevents effector T cell senescence and enhances tumor immunotherapy. *Sci. Transl. Med.* 13(587):eaaz6314
54. Ringel AE, Drijvers JM, Baker GJ, Catozzi A, García-Cañaveras JC, et al. 2020. Obesity shapes metabolism in the tumor microenvironment to suppress anti-tumor immunity. *Cell* 183(7):1848–66.e26
55. Michelet X, Dyck L, Hogan A, Loftus RM, Duquette D, et al. 2018. Metabolic reprogramming of natural killer cells in obesity limits antitumor responses. *Nat. Immunol.* 19(12):1330–40
56. Mogilenko DA, Haas JT, L'homme L, Fleury S, Quemener S, et al. 2019. Metabolic and innate immune cues merge into a specific inflammatory response via the UPR. *Cell* 177(5):1201–16.e19
57. Singer BD, Chandel NS. 2019. Immunometabolism of pro-repair cells. *J. Clin. Investig.* 129(7):2597–607
58. Intlekofer AM, Dematteo RG, Venneti S, Finley LWS, Lu C, et al. 2015. Hypoxia induces production of L-2-hydroxyglutarate. *Cell Metab*. 22(2):304–11
59. Oldham WM, Clish CB, Yang Y, Loscalzo J. 2015. Hypoxia-mediated increases in L-2-hydroxyglutarate coordinate the metabolic response to reductive stress. *Cell Metab*. 22(2):291–303
60. Xu T, Stewart KM, Wang X, Liu K, Xie M, et al. 2017. Metabolic control of T_H17 and induced T_{reg} cell balance by an epigenetic mechanism. *Nature* 548(7666):228–33
61. Tyrakis PA, Palazon A, Macias D, Lee KL, Phan AT, et al. 2016. *S*-2-Hydroxyglutarate regulates $CD8^+$ T-lymphocyte fate. *Nature* 540(7632):236–41
62. Doran AC, Yurdagul A, Tabas I. 2020. Efferocytosis in health and disease. *Nat. Rev. Immunol.* 20(4):254–67
63. Zhang S, Weinberg S, DeBerge M, Gainullina A, Schipma M, et al. 2019. Efferocytosis fuels requirements of fatty acid oxidation and the electron transport chain to polarize macrophages for tissue repair. *Cell Metab*. 29(2):443–56.e5
64. Morioka S, Perry JSA, Raymond MH, Medina CB, Zhu Y, et al. 2018. Efferocytosis induces a novel SLC program to promote glucose uptake and lactate release. *Nature* 563(7733):714–18
65. Maschalidi S, Mehrotra P, Keçeli BN, De Cleene HKL, Lecomte K, et al. 2022. Targeting SLC7A11 improves efferocytosis by dendritic cells and wound healing in diabetes. *Nature* 606(7915):776–84
66. Shang M, Cappellesso F, Amorim R, Serneels J, Virga F, et al. 2020. Macrophage-derived glutamine boosts satellite cells and muscle regeneration. *Nature* 587(7835):626–31
67. Stein WH, Moore S. 1954. The free amino acids of human blood plasma. *J. Biol. Chem.* 211(2):915–26
68. Yu Y, Newman H, Shen L, Sharma D, Hu G, et al. 2019. Glutamine metabolism regulates proliferation and lineage allocation in skeletal stem cells. *Cell Metab*. 29(4):966–78.e4
69. Kim CS, Ding X, Allmeroth K, Biggs LC, Kolenc OI, et al. 2020. Glutamine metabolism controls stem cell fate reversibility and long-term maintenance in the hair follicle. *Cell Metab*. 32(4):629–42.e8
70. Minhas PS, Latif-Hernandez A, McReynolds MR, Durairaj AS, Wang Q, et al. 2021. Restoring metabolism of myeloid cells reverses cognitive decline in ageing. *Nature* 590(7844):122–28
71. Kawahara K, Hohjoh H, Inazumi T, Tsuchiya S, Sugimoto Y. 2015. Prostaglandin E_2-induced inflammation: relevance of prostaglandin E receptors. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1851(4):414–21
72. Hayek MG, Meydani SN, Meydani M, Blumberg JB. 1994. Age differences in eicosanoid production of mouse splenocytes: effects on mitogen-induced T-cell proliferation. *J. Gerontol.* 49(5):B197–207
73. Desdín-Micó G, Soto-Heredero G, Aranda JF, Oller J, Carrasco E, et al. 2020. T cells with dysfunctional mitochondria induce multimorbidity and premature senescence. *Science* 368(6497):1371–76

74. Rooks MG, Garrett WS. 2016. Gut microbiota, metabolites and host immunity. *Nat. Rev. Immunol.* 16(6):341–52
75. Fan Y, Pedersen O. 2021. Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* 19(1):55–71
76. Chang PV, Hao L, Offermanns S, Medzhitov R. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *PNAS* 111(6):2247–52
77. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, et al. 2014. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat. Med.* 20(2):159–66
78. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, et al. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504(7480):446–50
79. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, et al. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504(7480):451–55
80. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, et al. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* 341(6145):569–73
81. Kuipers F, Bloks VW, Groen AK. 2014. Beyond intestinal soap—bile acids in metabolic control. *Nat. Rev. Endocrinol.* 10(8):488–98
82. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, et al. 1999. Identification of a nuclear receptor for bile acids. *Science* 284(5418):1362–65
83. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, et al. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 284(5418):1365–68
84. Chen ML, Takeda K, Sundrud MS. 2019. Emerging roles of bile acids in mucosal immunity and inflammation. *Mucosal Immunol.* 12(4):851–61
85. Campbell C, McKenney PT, Konstantinovsky D, Isaeva OI, Schizas M, et al. 2020. Bacterial metabolism of bile acids promotes generation of peripheral regulatory T cells. *Nature* 581(7809):475–79
86. Hang S, Paik D, Yao L, Kim E, Trinath J, et al. 2019. Bile acid metabolites control T_H17 and T_{reg} cell differentiation. *Nature* 576(7785):143–48. Erratum. 2020. *Nature* 579:E7
87. Stražar M, Temba GS, Vlamakis H, Kullaya VI, Lyamuya F, et al. 2021. Gut microbiome-mediated metabolism effects on immunity in rural and urban African populations. *Nat. Commun.* 12(1):4845
88. Guo H, Chou W-C, Lai Y, Liang K, Tam JW, et al. 2020. Multi-omics analyses of radiation survivors identify radioprotective microbes and metabolites. *Science* 370(6516):eaay9097
89. Domínguez Conde C, Xu C, Jarvis LB, Rainbow DB, Wells SB, et al. 2022. Cross-tissue immune cell analysis reveals tissue-specific features in humans. *Science* 376(6594):eabl5197
90. Chapman NM, Chi H. 2022. Metabolic adaptation of lymphocytes in immunity and disease. *Immunity* 55(1):14–30
91. Llufrio EM, Wang L, Naser FJ, Patti GJ. 2018. Sorting cells alters their redox state and cellular metabolome. *Redox Biol.* 16:381–87
92. Fessenden M. 2016. Metabolomics: small molecules, single cells. *Nature* 540(7631):153–55
93. Bonilla DL, Reinin G, Chua E. 2021. Full spectrum flow cytometry as a powerful technology for cancer immunotherapy research. *Front. Mol. Biosci.* 7:612801
94. Spitzer MH, Nolan GP. 2016. Mass cytometry: single cells, many features. *Cell* 165(4):780–91
95. Hartmann FJ, Bendall SC. 2020. Immune monitoring using mass cytometry and related high-dimensional imaging approaches. *Nat. Rev. Rheumatol.* 16(2):87–99
96. Ahl PJ, Hopkins RA, Xiang WW, Au B, Kaliaperumal N, et al. 2020. Met-Flow, a strategy for single-cell metabolic analysis highlights dynamic changes in immune subpopulations. *Commun. Biol.* 3:305
97. Levine LS, Hiam-Galvez KJ, Marquez DM, Tenvooren I, Madden MZ, et al. 2021. Single-cell analysis by mass cytometry reveals metabolic states of early-activated $CD8^+$ T cells during the primary immune response. *Immunity* 54(4):829–44.e5
98. Hartmann FJ, Mrdjen D, McCaffrey E, Glass DR, Greenwald NF, et al. 2021. Single-cell metabolic profiling of human cytotoxic T cells. *Nat. Biotechnol.* 39(2):186–97
99. Shay T, Kang J. 2013. Immunological Genome Project and systems immunology. *Trends Immunol.* 34(12):602–9
100. Stephenson E, Reynolds G, Botting RA, Calero-Nieto FJ, Morgan MD, et al. 2021. Single-cell multi-omics analysis of the immune response in COVID-19. *Nat. Med.* 27(5):904–16

101. Gainullina A, Mogilenko DA, Huang L-H, Todorov H, Narang V, et al. 2023. Network analysis of large-scale ImmGen and Tabula Muris datasets highlights metabolic diversity of tissue mononuclear phagocytes. *Cell Rep*. 42(2):112046
102. Wagner A, Wang C, Fessler J, DeTomaso D, Avila-Pacheco J, et al. 2021. Metabolic modeling of single Th17 cells reveals regulators of autoimmunity. *Cell* 184(16):4168–85.e21
103. Fitzgerald KC, Smith MD, Kim S, Sotirchos ES, Kornberg MD, et al. 2021. Multi-omic evaluation of metabolic alterations in multiple sclerosis identifies shifts in aromatic amino acid metabolism. *Cell Rep. Med.* 2(10): 100424
104. Lee JW, Su Y, Baloni P, Chen D, Pavlovitch-Bedzyk AJ, et al. 2022. Integrated analysis of plasma and single immune cells uncovers metabolic changes in individuals with COVID-19. *Nat. Biotechnol.* 40(1):110–20
105. Gilmore IS, Heiles S, Pieterse CL. 2019. Metabolic imaging at the single-cell scale: recent advances in mass spectrometry imaging. *Annu. Rev. Anal. Chem.* 12:201–24
106. Taylor MJ, Lukowski JK, Anderton CR. 2021. Spatially resolved mass spectrometry at the single cell: recent innovations in proteomics and metabolomics. *J. Am. Soc. Mass Spectrom.* 32(4):872–94
107. Buchberger AR, DeLaney K, Johnson J, Li L. 2018. Mass spectrometry imaging: a review of emerging advancements and future insights. *Anal. Chem.* 90(1):240–65
108. Cornett DS, Reyzer ML, Chaurand P, Caprioli RM. 2007. MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. *Nat. Methods* 4(10):828–33
109. Rappez L, Stadler M, Triana S, Gathungu RM, Ovchinnikova K, et al. 2021. SpaceM reveals metabolic states of single cells. *Nat. Methods* 18(7):799–805
110. Manzo T, Prentice BM, Anderson KG, Raman A, Schalck A, et al. 2020. Accumulation of long-chain fatty acids in the tumor microenvironment drives dysfunction in intrapancreatic $CD8^+$ T cells. *J. Exp. Med.* 217(8):e20191920
111. Comi TJ, Neumann EK, Do TD, Sweedler JV. 2017. microMS: a Python platform for image-guided mass spectrometry profiling. *J. Am. Soc. Mass Spectrom.* 28(9):1919–28
112. Castro DC, Xie YR, Rubakhin SS, Romanova EV, Sweedler JV. 2021. Image-guided MALDI mass spectrometry for high-throughput single-organelle characterization. *Nat. Methods* 18(10):1233–38
113. Wang L, Xing X, Zeng X, Jackson SR, TeSlaa T, et al. 2022. Spatially resolved isotope tracing reveals tissue metabolic activity. *Nat. Methods* 19(2):223–30
114. Winograd N. 2018. Gas cluster ion beams for secondary ion mass spectrometry. *Annu. Rev. Anal. Chem.* 11:29–48
115. Yuan Z, Zhou Q, Cai L, Pan L, Sun W, et al. 2021. SEAM is a spatial single nuclear metabolomics method for dissecting tissue microenvironment. *Nat. Methods* 18(10):1223–32
116. Passarelli MK, Pirkl A, Moellers R, Grinfeld D, Kollmer F, et al. 2017. The 3D OrbiSIMS—label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power. *Nat. Methods* 14(12):1175–83
117. Ganesh S, Hu T, Woods E, Allam M, Cai S, et al. 2021. Spatially resolved 3D metabolomic profiling in tissues. *Sci. Adv.* 7(5):eabd0957
118. Pareek V, Tian H, Winograd N, Benkovic SJ. 2020. Metabolomics and mass spectrometry imaging reveal channeled de novo purine synthesis in cells. *Science* 368(6488):283–90
119. Kompauer M, Heiles S, Spengler B. 2017. Atmospheric pressure MALDI mass spectrometry imaging of tissues and cells at 1.4-μm lateral resolution. *Nat. Methods* 14(1):90–96
120. Geier B, Sogin EM, Michellod D, Janda M, Kompauer M, et al. 2020. Spatial metabolomics of in situ host-microbe interactions at the micrometre scale. *Nat. Microbiol.* 5(3):498–510
121. Neumann EK, Djambazova KV, Caprioli RM, Spraggins JM. 2020. Multimodal imaging mass spectrometry: next generation molecular mapping in biology and medicine. *J. Am. Soc. Mass Spectrom.* 31(12):2401–15
122. Kaya I, Sämfors S, Levin M, Borén J, Fletcher JS. 2020. Multimodal MALDI imaging mass spectrometry reveals spatially correlated lipid and protein changes in mouse heart with acute myocardial infarction. *J. Am. Soc. Mass Spectrom.* 31(10):2133–42

123. Borodinov N, Lorenz M, King ST, Ievlev AV, Ovchinnikova OS. 2020. Toward nanoscale molecular mass spectrometry imaging via physically constrained machine learning on co-registered multimodal data. *npj Comput. Mater.* 6:83
124. Capolupo L, Khven I, Lederer AR, Mazzeo L, Glousker G, et al. 2022. Sphingolipids control dermal fibroblast heterogeneity. *Science* 376(6590):eabh1623
125. Ravi VM, Will P, Kueckelhaus J, Sun N, Joseph K, et al. 2022. Spatially resolved multi-omics deciphers bidirectional tumor-host interdependence in glioblastoma. *Cancer Cell* 40(6):639–55.e13
126. Doudna JA, Charpentier E. 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346(6213):1258096
127. Adli M. 2018. The CRISPR tool kit for genome editing and beyond. *Nat. Commun.* 9(1):1911
128. Huang H, Zhou P, Wei J, Long L, Shi H, et al. 2021. In vivo CRISPR screening reveals nutrient signaling processes underpinning $CD8^+$ T cell fate decisions. *Cell* 184(5):1245–61.e21
129. Sugiura A, Andrejeva G, Voss K, Heintzman DR, Xu X, et al. 2022. MTHFD2 is a metabolic checkpoint controlling effector and regulatory T cell fate and function. *Immunity* 55(1):65–81.e9
130. Huang B, Phelan JD, Preite S, Gomez-Rodriguez J, Johansen KH, et al. 2022. In vivo CRISPR screens reveal a HIF-1α-mTOR-network regulates T follicular helper versus Th1 cells. *Nat. Commun.* 13(1):805
131. Adamski J. 2012. Genome-wide association studies with metabolomics. *Genome Med.* 4(4):34
132. Kastenmüller G, Raffler J, Gieger C, Suhre K. 2015. Genetics of human metabolism: an update. *Hum. Mol. Genet.* 24(R1):R93–101
133. Nath AP, Ritchie SC, Byars SG, Fearnley LG, Havulinna AS, et al. 2017. An interaction map of circulating metabolites, immune gene networks, and their genetic regulation. *Genome Biol.* 18(1):146
134. Burgener A-V, Bantug GR, Meyer BJ, Higgins R, Ghosh A, et al. 2019. SDHA gain-of-function engages inflammatory mitochondrial retrograde signaling via KEAP1-Nrf2. *Nat. Immunol.* 20(10):1311–21
135. Verbeeck N, Caprioli RM, Van de Plas R. 2020. Unsupervised machine learning for exploratory data analysis in imaging mass spectrometry. *Mass Spectrom. Rev.* 39(3):245–91
136. van der Maaten L, Hinton G. 2008. Visualizing data using t-SNE. *J. Mach. Learn. Res.* 9(86):2579–605
137. McInnes L, Healy J, Saul N, Grossberger L. 2018. UMAP: Uniform Manifold Approximation and Projection. *J. Open Source Softw.* 3(29):861
138. Abdelmoula WM, Lopez BG-C, Randall EC, Kapur T, Sarkaria JN, et al. 2021. Peak learning of mass spectrometry imaging data using artificial neural networks. *Nat. Commun.* 12(1):5544
139. Zhang W, Claesen M, Moerman T, Groseclose MR, Waelkens E, et al. 2021. Spatially aware clustering of ion images in mass spectrometry imaging data using deep learning. *Anal. Bioanal. Chem.* 413(10):2803–19
140. Ovchinnikova K, Stuart L, Rakhlin A, Nikolenko S, Alexandrov T. 2020. ColocML: machine learning quantifies co-localization between mass spectrometry images. *Bioinformatics* 36(10):3215–24
141. Alexandrov T, Bartels A. 2013. Testing for presence of known and unknown molecules in imaging mass spectrometry. *Bioinformatics* 29(18):2335–42
142. Paine MRL, Kim J, Bennett RV, Parry RM, Gaul DA, et al. 2016. Whole reproductive system non-negative matrix factorization mass spectrometry imaging of an early-stage ovarian cancer mouse model. *PLOS ONE* 11(5):e0154837
143. Moerman AM, Visscher M, Slijkhuis N, Van Gaalen K, Heijs B, et al. 2021. Lipid signature of advanced human carotid atherosclerosis assessed by mass spectrometry imaging. *J. Lipid Res.* 62:100020
144. Sun C, Li T, Song X, Huang L, Zang Q, et al. 2019. Spatially resolved metabolomics to discover tumor-associated metabolic alterations. *PNAS* 116(1):52–57
145. Tuck M, Grélard F, Blanc L, Desbenoit N. 2022. MALDI-MSI towards multimodal imaging: challenges and perspectives. *Front. Chem.* 10:904688
146. Van de Plas R, Yang J, Spraggins J, Caprioli RM. 2015. Image fusion of mass spectrometry and microscopy: a multimodality paradigm for molecular tissue mapping. *Nat. Methods* 12(4):366–72
147. Goossens P, Lu C, Cao J, Gijbels MJ, Karel JMH, et al. 2022. Integrating multiplex immunofluorescent and mass spectrometry imaging to map myeloid heterogeneity in its metabolic and cellular context. *Cell Metab.* 34(8):1214–25.e6
148. Argelaguet R, Velten B, Arnol D, Dietrich S, Zenz T, et al. 2018. Multi-omics factor analysis—a framework for unsupervised integration of multi-omics data sets. *Mol. Syst. Biol.* 14(6):e8124

149. Velten B, Braunger JM, Argelaguet R, Arnol D, Wirbel J, et al. 2022. Identifying temporal and spatial patterns of variation from multimodal data using MEFISTO. *Nat. Methods* 19(2):179–86
150. Sindelar M, Patti GJ. 2020. Chemical discovery in the era of metabolomics. *J. Am. Chem. Soc.* 142(20):9097–105
151. Stancliffe E, Schwaiger-Haber M, Sindelar M, Patti GJ. 2021. DecoID improves identification rates in metabolomics through database-assisted MS/MS deconvolution. *Nat. Methods* 18(7):779–87
152. Pomyen Y, Wanichthanarak K, Poungsombat P, Fahrmann J, Grapov D, Khoomrung S. 2020. Deep metabolome: applications of deep learning in metabolomics. *Comput. Struct. Biotechnol. J.* 18:2818–25
153. Ji H, Deng H, Lu H, Zhang Z. 2020. Predicting a molecular fingerprint from an electron ionization mass spectrum with deep neural networks. *Anal. Chem.* 92(13):8649–53
154. Palmer A, Phapale P, Chernyavsky I, Lavigne R, Fay D, et al. 2017. FDR-controlled metabolite annotation for high-resolution imaging mass spectrometry. *Nat. Methods* 14(1):57–60
155. Alexandrov T, Ovchinnikova K, Palmer A, Kovalev V, Tarasov A, et al. 2019. METASPACE: a community-populated knowledge base of spatial metabolomes in health and disease. bioRxiv 539478, Feb. 13. **https://doi.org/10.1101/539478**
156. da Silva RR, Dorrestein PC, Quinn RA. 2015. Illuminating the dark matter in metabolomics. *PNAS* 112(41):12549–50
157. Shen X, Wang R, Xiong X, Yin Y, Cai Y, et al. 2019. Metabolic reaction network-based recursive metabolite annotation for untargeted metabolomics. *Nat. Commun.* 10(1):1516
158. Chen L, Lu W, Wang L, Xing X, Chen Z, et al. 2021. Metabolite discovery through global annotation of untargeted metabolomics data. *Nat. Methods* 18(11):1377–85
159. Hastings J, Owen G, Dekker A, Ennis M, Kale N, et al. 2016. ChEBI in 2016: improved services and an expanding collection of metabolites. *Nucleic Acids Res.* 44(D1):D1214–19
160. Bansal P, Morgat A, Axelsen KB, Muthukrishnan V, Coudert E, et al. 2022. Rhea, the reaction knowledgebase in 2022. *Nucleic Acids Res.* 50(D1):D693–700
161. Kanehisa M, Goto S. 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* 28(1):27–30
162. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, et al. 2018. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res.* 46(D1):D608–17
163. O'Donnell VB, Dennis EA, Wakelam MJO, Subramaniam S. 2019. LIPID MAPS: serving the next generation of lipid researchers with tools, resources, data, and training. *Sci. Signal.* 12(563):eaaw2964
164. Jassal B, Matthews L, Viteri G, Gong C, Lorente P, et al. 2020. The reactome pathway knowledgebase. *Nucleic Acids Res.* 48(D1):D498–503
165. MohammadiPeyhani H, Hafner J, Sveshnikova A, Viterbo V, Hatzimanikatis V. 2022. Expanding biochemical knowledge and illuminating metabolic dark matter with ATLASx. *Nat. Commun.* 13(1):1560
166. Gene Ontol. Consort. 2019. The Gene Ontology Resource: 20 years and still GOing strong. *Nucleic Acids Res.* 47(D1):D330–38
167. Liberzon A, Subramanian A, Pinchback R, Thorvaldsdóttir H, Tamayo P, Mesirov JP. 2011. Molecular signatures database (MSigDB) 3.0. *Bioinformatics* 27(12):1739–40
168. Zhang Y, Ma Y, Huang Y, Zhang Y, Jiang Q, et al. 2020. Benchmarking algorithms for pathway activity transformation of single-cell RNA-seq data. *Comput. Struct. Biotechnol. J.* 18:2953–61
169. Li S, Park Y, Duraisingham S, Strobel FH, Khan N, et al. 2013. Predicting network activity from high throughput metabolomics. *PLOS Comput. Biol.* 9(7):e1003123
170. Gaud C, Sousa BC, Nguyen A, Fedorova M, Ni Z, et al. 2021. BioPAN: a web-based tool to explore mammalian lipidome metabolic pathways on LIPID MAPS. *F1000Research* 10:4
171. Pang Z, Zhou G, Ewald J, Chang L, Hacariz O, et al. 2022. Using MetaboAnalyst 5.0 for LC-HRMS spectra processing, multi-omics integration and covariate adjustment of global metabolomics data. *Nat. Protoc.* 17(8):1735–61
172. Wieder C, Lai RPJ, Ebbels T. 2022. Single sample pathway analysis in metabolomics: performance evaluation and application. bioRxiv 2022.04.11.487976, Apr. 12
173. Amara A, Frainay C, Jourdan F, Naake T, Neumann S, et al. 2022. Networks and graphs discovery in metabolomics data analysis and interpretation. *Front. Mol. Biosci.* 9:841373

174. Hafner J, Hatzimanikatis V. 2021. NICEpath: finding metabolic pathways in large networks through atom-conserving substrate-product pairs. *Bioinformatics* 37(20):3560–68
175. Alcaraz N, Pauling J, Batra R, Barbosa E, Junge A, et al. 2014. KeyPathwayMiner 4.0: condition-specific pathway analysis by combining multiple omics studies and networks with Cytoscape. *BMC Syst. Biol.* 8:99
176. Emelianova M, Gainullina A, Poperechnyi N, Loboda A, Artyomov M, Sergushichev A. 2022. Shiny GATOM: omics-based identification of regulated metabolic modules in atom transition networks. *Nucleic Acids Res.* 50(W1):W690–96
177. Wiwie C, Kuznetsova I, Mostafa A, Rauch A, Haakonsson A, et al. 2019. Time-resolved systems medicine reveals viral infection-modulating host targets. *Syst. Med.* 2(1):1–9
178. Orth JD, Thiele I, Palsson BØ. 2010. What is flux balance analysis? *Nat. Biotechnol.* 28(3):245–48
179. Blazier A, Papin J. 2012. Integration of expression data in genome-scale metabolic network reconstructions. *Front. Physiol.* 3:299
180. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, et al. 2016. Itaconate links inhibition of succinate dehydrogenase with macrophage metabolic remodeling and regulation of inflammation. *Cell Metab.* 24(1):158–66
181. Hrovatin K, Fischer DS, Theis FJ. 2022. Toward modeling metabolic state from single-cell transcriptomics. *Mol. Metab.* 57:101396
182. Alghamdi N, Chang W, Dang P, Lu X, Wan C, et al. 2021. A graph neural network model to estimate cell-wise metabolic flux using single-cell RNA-seq data. *Genome Res.* 31(10):1867–84
183. Winter G, Krömer JO. 2013. Fluxomics—connecting 'omics analysis and phenotypes. *Environ. Microbiol.* 15(7):1901–16
184. Byrd JB, Greene AC, Prasad DV, Jiang X, Greene CS. 2020. Responsible, practical genomic data sharing that accelerates research. *Nat. Rev. Genet.* 21(10):615–29
185. Clough E, Barrett T. 2016. The Gene Expression Omnibus Database. In *Statistical Genomics: Methods and Protocols*, ed. E Mathé, S Davis, pp. 93–110. New York: Springer
186. Kolesnikov N, Hastings E, Keays M, Melnichuk O, Tang YA, et al. 2015. ArrayExpress update—simplifying data submissions. *Nucleic Acids Res.* 43(D1):D1113–16
187. Katz K, Shutov O, Lapoint R, Kimelman M, Brister JR, O'Sullivan C. 2022. The Sequence Read Archive: a decade more of explosive growth. *Nucleic Acids Res.* 50(D1):D387–90
188. Sud M, Fahy E, Cotter D, Azam K, Vadivelu I, et al. 2016. Metabolomics Workbench: an international repository for metabolomics data and metadata, metabolite standards, protocols, tutorials and training, and analysis tools. *Nucleic Acids Res.* 44(D1):D463–70
189. Haug K, Cochrane K, Nainala VC, Williams M, Chang J, et al. 2020. MetaboLights: a resource evolving in response to the needs of its scientific community. *Nucleic Acids Res.* 48(D1):D440–44
190. Alexandrov T. 2020. Spatial metabolomics and imaging mass spectrometry in the age of artificial intelligence. *Annu. Rev. Biomed. Data Sci.* 3:61–87
191. Monge ME, Dodds JN, Baker ES, Edison AS, Fernández FM. 2019. Challenges in identifying the dark molecules of life. *Annu. Rev. Anal. Chem.* 12:177–99
192. Zhou X, Franklin RA, Adler M, Jacox JB, Bailis W, et al. 2018. Circuit design features of a stable two-cell system. *Cell* 172(4):744–57.e17
193. Rossi G, Manfrin A, Lutolf MP. 2018. Progress and potential in organoid research. *Nat. Rev. Genet.* 19(11):671–87
194. Perry WJ, Grunenwald CM, Van de Plas R, Witten JC, Martin DR, et al. 2022. Visualizing *Staphylococcus aureus* pathogenic membrane modification within the host infection environment by multimodal imaging mass spectrometry. *Cell Chem. Biol.* 29(7):1209–17.e4
195. Blanc L, Daudelin IB, Podell BK, Chen P-Y, Zimmerman M, et al. 2018. High-resolution mapping of fluoroquinolones in TB rabbit lesions reveals specific distribution in immune cell types. *eLife* 7:e41115