

**Immune regulatory metabolites in the tumor microenvironment suppress T cell function in
ovarian cancer**

by

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University of Victoria

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Abstract

Immune regulatory metabolites are key features of the tumor microenvironment (TME), yet with a few exceptions, their identities remain largely unknown. Here, we profiled tumor and T cells from tumor and ascites of patients with high-grade serous carcinoma (HGSC) to uncover the metabolomes of these distinct TME compartments. Cells within the ascites and tumor had pervasive metabolite differences, with a striking enrichment in 1-methylnicotinamide (MNA) in T cells infiltrating the tumor compared to ascites. Despite the elevated levels of MNA in T cells, the expression of nicotinamide N-methyltransferase, the enzyme that catalyzes the transfer of a methyl group from S-adenosylmethionine to nicotinamide, was restricted to fibroblasts and tumor cells. Functionally, MNA induces T cells to secrete the tumor promoting cytokine tumor necrosis factor alpha. Thus, TME-derived MNA contributes to the immune modulation of T cells and represents a potential immunotherapy target to treat human cancer.

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List of Abbreviations

2-NBDG – 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose

2-PY – 1-methyl-2-pyridone-5-carboxamide

4-PY – 1-methyl-4-pyridone-5-carboxamide

ACT – adoptive cell therapy

AOX1 – aldehyde oxidase 1

ATiM – Advanced Tissue Management Application

BH4 – tetrahydrobiopterin

BRAC1 – breast cancer gene 1

CAFs – cancer associated fibroblasts

CAIX – carbonic anhydrase IX

CAR T cells – chimeric antigen receptor T cells

CLL – chronic lymphocytic leukemia

CRISPR – clustered regularly interspaced short palindromic repeats

CTCs – circulating tumor cells

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

CTRNet – Canadian Tissue Repository Network

DMFO – difluoromethylornithine

ELISA – enzyme linked immunosorbent assay

EpCAM – epithelial cell adhesion molecule

FDG-PET –fluorodeoxyglucose-positron emission tomography

FAUST – Full Annotation Using Shape-constrained Trees

FMO – fluorescence minus one

FR α – folate receptor alpha

GC – gas chromatography

HILIC – hydrophilic interaction liquid chromatography

HPLC – high performance liquid chromatography

HRAM – high-resolution, accurate mass

ICI – immune checkpoint blockade

IDH – isocitrate dehydrogenase

IDO – indolamine-2,3-dioxygenase

IFN γ – interferon gamma
IL2 – interleukin 2
Lag3 – lymphocyte Activating 3
LC – liquid chromatography
m/z – mass to charge ratio
MDSCs – myeloid derived suppressor cells
MFI – median fluorescence intensity
MNA – 1-methylnicotinamide
MS – mass spectrometry
MS/MS – tandem mass spectrometry
MT DR – MitoTracker Deep Red
NFAT – nuclear factor of activated T cells
NMR – nuclearmagnetic resonance
NNMT – Nicotinamide N-methyl transferase
OCT – organic cation transporter
OR – operating room
OV – oncolytic viruses
OXPHOS – oxidative phosphorylation
PBMCs – peripheral blood mononuclear cells
PCA – principal component analysis
PD-1 – programmed cell death protein 1
QC/QA – quality control and quality assurance
qPCR – quantitative polymerase chain reaction
Q-TOF – quadrupole-time of flight
R-2-HG – (R)-2-hydroxyglutarate
RNA – ribonucleic acid
RNA-seq – RNA sequencing
RP – reverse phase
SAH – S-adenosylhomocysteine
SAM – S-adenosylmethionine
scRNA-seq – single-cell RNA sequencing
Sp1 – specificity protein 1
TCA – tricarboxylic acid cycle

Tcm – central memory T cell
Teff – effector T cells
Tem – effector memory T cell
TIC – total ion count
TIL – tumor infiltrating lymphocytes
TME – tumor microenvironment
TMRE - tetramethylrhodamine methyl ester perchlorate
TNBA – triple negative breast cancer
TNF α – tumor necrosis factor alpha
Tregs – regulatory T cells
TTR – Tumor Tissue Repository

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Preamble

This work is a compilation of two manuscripts. Chapter 2 is adapted from Kilgour *et. al.* (2022) *Nature Protocols (Accepted)*, while Chapter 3 and Chapter 4 have been adapted from Kilgour *et. al.* (2021) *Science Advances*. The contributions of all authors are indicated at the beginning of each chapter.

Our manuscript in *Nature Protocols (Accepted)* has a unique “recipe style” format in accordance with the guidelines for their Protocol publications. The manuscript has been presented in this unique format in Chapter 2.

This work involved the use of human specimens. We have Research Ethics Board (REB) approval for this work through my protocol at the University of Victoria (#18-161), the Immune Response to Ovarian Cancer (IROC) protocol (#H07-00463), the BC Cancer TIL-M protocol (#H18-01783), and the University of Victoria biosafety protocol (#19-0067).

Chapter 1: Exploiting advances in understanding cell-specific metabolic perturbations within the tumor microenvironment for improved cancer immunotherapy

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Figure 1 was created by BioRender.com.

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1.1 Prologue

Cancer cells develop the capability to adapt their metabolism to fuel proliferation and growth, driving a profoundly dysregulated metabolic landscape in the tumor microenvironment (TME). Solid tumors present unique challenges because of these metabolic perturbations that impact not only the tumor, but also the metabolism of the surrounding tissues (e.g., stromal cells, fibroblasts) and immune cells (e.g., lymphocytes, monocytes/macrophages). While methods for analyzing metabolism in bulk samples have elucidated key metabolic vulnerabilities of tumor cells, current protocols and approaches are limited and do not fully resolve the cellular heterogeneity of the TME. The recent development of techniques to profile the metabolism of the TME at the resolution of enriched populations or near single cells have enabled new insights into the metabolic contributions and interactions of specific cell-subclasses. These techniques have provided cell type specific information on metabolic pathways and uncovered potential therapeutic targets. Thus, a growing body of literature has implicated cellular metabolism as a primary factor contributing to the efficacy of cellular-based immunotherapies.

Here, we review the technological developments that have enabled these advances, highlighting cell type specific metabolic studies. We suggest how these techniques can be harnessed to discover potential targets for improving the effectiveness of immunotherapies for solid cancers. We will also look at these studies in the context of ovarian cancer, a solid tumor that could benefit from these types of scientific advancements.

1.2 Introduction to understanding the metabolic heterogeneity of the tumor microenvironment

The TME is a complex ecosystem of different cell populations (1). Moreover, the proportions of these cell populations in the TME are vastly different for each type of cancer, and even between individual patients with the same type of cancer. Different cell populations that typically make up the TME include stromal cells, fibroblasts, endothelial cells, mesenchymal stem cells, adipocytes, and diverse immune cells (2). Immune cells can include both immune-stimulatory and immune-suppressive cells that can alternatively hinder or support tumor growth. These cells

include lymphocytes (T cells, B cells, natural killer [NK] cells), and myeloid cells (tumor-associated macrophages [TAMs], myeloid-derived suppressor cells [MDSCs], tumor-associated neutrophils [TANs], dendritic cells [DCs], etc.) (2). All of these cell sub-classes are defined by cell type specific gene expression programs, enzymes, and transporters, and more, setting the stage for disparate metabolic interactions among cell types (3). Finally, there is increasing recognition that the spatial distribution of each of these cell sub-classes can be indicative of the magnitude of the endogenous anti-tumor response or responsiveness to immunotherapy interventions (4).

The recent revolution in cancer immunotherapy (stimulating the immune response to treat disease) has highlighted the urgent need to further understand the key metabolic relationships between cancer cells and immune cells (5–15). It is generally accepted that tumor cells compete with immune cells for essential nutrients while also producing immunosuppressive metabolites that limit their function (16). Meanwhile, these changes in metabolism can support immune cells that promote tumor progression such as regulatory T cells (Tregs) or MDSCs. For example, Tregs can use lactate, produced abundantly through the Warburg effect by cancer cells, as a fuel source (17). MDSCs may cooperate with tumors to deplete certain key nutrients such as tryptophan to induce a state of metabolic exhaustion (18). This dynamic relationship is further complicated by nutrient availability in the TME that can influence cell metabolism (19, 20).

Despite the complexity of the TME, most metabolomic studies are carried out on bulk samples that are a composite of many different cell sub-classes, thus providing an average metabolic profile of the sample rather than of any one cell type. In many ways, this is analogous to the first bulk RNA sequencing (RNA-seq) technologies, prior to single-cell techniques. When uncovering tumor metabolism, the metabolic profile normally refers to the cells that make up the bulk of the tumor. For example, many metabolic observations made regarding the TME, such as the Warburg effect, refer to an aggregated phenotype and do not take into account the heterogeneity of the TME (21). This is a concern because heterogenous characteristics of the tumor will be lost. For example, the extent that adipose stromal cells and fibroblasts contribute to the metabolic profile will in no longer be distinguishable. This is important for tumors that form in areas of the body that have a high adipocyte or fibrotic composition, e.g., ovarian omentum *versus* hepatocellular carcinoma, respectively (21).

Identifying and understanding key metabolites and metabolic pathways in different cell types within the TME is an outstanding challenge (22). For example, the widespread analysis of tumor samples by mass spectrometry (MS) measures the abundance of metabolites present in the tumor but cannot tell which cells they came from. Alternatively, metabolic studies that, by the nature of the technique, provide single-cell resolution, such as flow or mass cytometry, have also been limited by the antibodies available against metabolic enzymes and transporters, or dyes to infer metabolism. These techniques have been limited in the sense that we do not have reliable targets for all the metabolic enzymes and transporters of interest. In addition, measuring the abundance of a protein does not always correlate to its activity. These methods typically offer a surrogate measure of metabolism rather than a direct measurement. Nevertheless, recent technological advances in cell sorting and instrument sensitivity have made single-cell and population-specific analyses possible. Thus allowing for new insights into the metabolic heterogeneity and diversity of the TME, and promising to reveal new targets for immune-based interventions (23).

Given the complex nature of the TME, cell type restricted proteins and metabolites, and resulting metabolic dependencies, would make optimal targets to improve the efficacy of cancer therapies. These cell type determined pathways are intrinsically specific, allowing functional improvements directed at the cells that promote tumor regression, while inhibiting the function of cells that promote tumor progression and metastasis. For example, targeting glutamine metabolism using an antagonist can hinder tumor growth by suppressing cancer cells and MDSCs, while promoting effector T cells based on the metabolic dependencies of these cells (24, 25). Understanding the cell type specific metabolic programs in the TME is the first step to developing these types of therapies.

1.3 Basics of cancer

The transition from a healthy cell to a malignant cell is a multi-step process, supported by number of acquired cellular characteristics (26). These characteristics include sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics, and avoiding immune destruction. These characteristics provide avenues for the

cancer cell to bypass the normal checks and balances that regulate the life of a healthy cell. For example, mutations in genes such as TP53 (responsible for regulating cell division), allow for sustained proliferative signaling. In addition, cancer cells can use cellular processes, such as autophagy, for protection during times of stress. These characteristics not only support the growth, proliferation, and dissemination of the cancer cells themselves, but also the formation of a TME. For example, cancer cells can induce angiogenesis (e.g., the formation of blood vessels) to create tumor vasculature that supports the metabolic needs of the tumor as it grows.

To support the energy and synthetic demands that are required during rapid proliferation, cancer cells rely on a dysregulated metabolism. Here on, we will focus on the key hallmark deregulating cellular energetics.

1.4 Basics of cell metabolism in the context of cancer immunology

Cancer cells, immune cells, and other cell types of the TME rely on common metabolic pathways to support their growth and proliferation (27). Some of the key metabolic pathways used to generate ATP include glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS). Glycolysis involves the conversion of glucose to pyruvate, where pyruvate will subsequently enter the TCA cycle (Figure 1). These pathways typically operate in the presence of oxygen, whereas in the absence of oxygen, pyruvate is converted to lactate and does not support OXPHOS. Glycolysis and the TCA cycle generate high energy intermediates that undergo OXPHOS. OXPHOS occurs via the electron transport chain in the mitochondria and is essential for generating ATP to support cellular processes. Importantly, recent studies have highlighted that tumor cells can enhance both glycolysis and OXPHOS allowing them to support enhanced growth and proliferation (27).

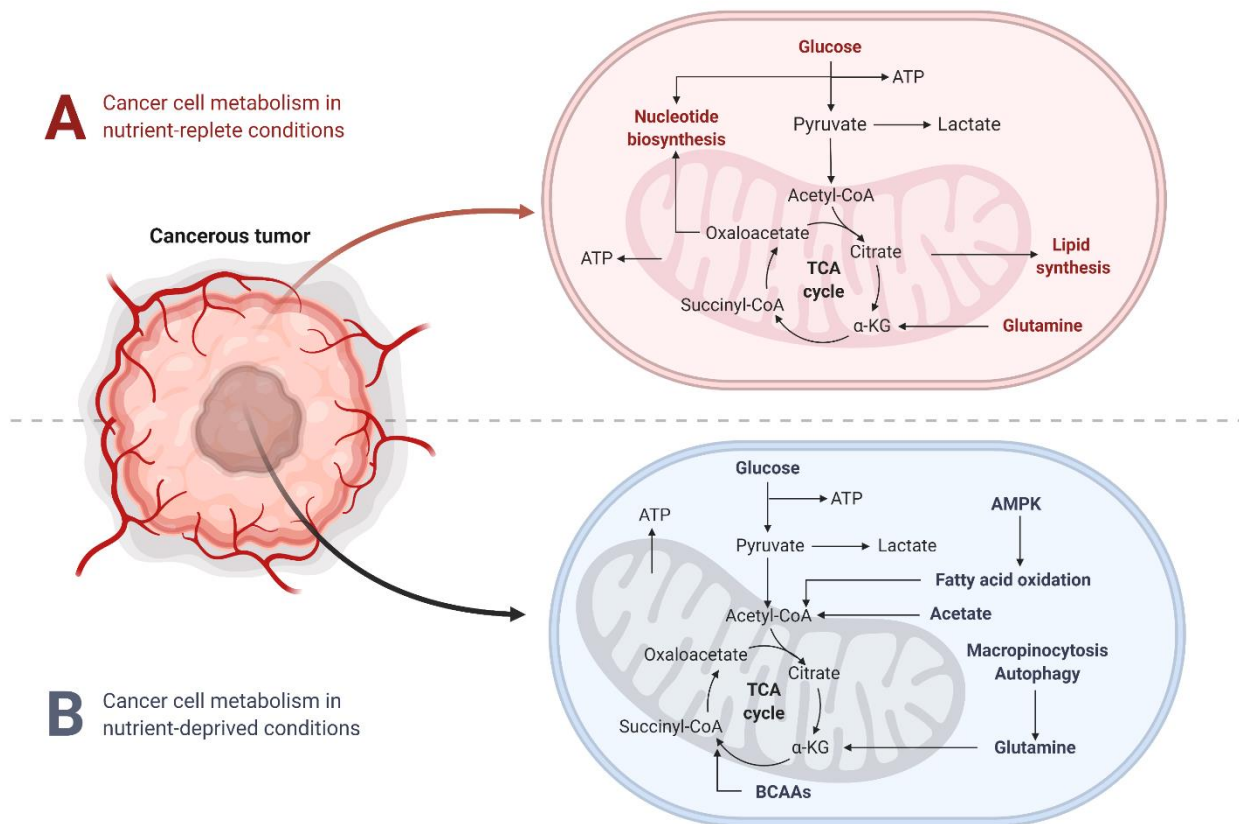


Figure 1. Cancer cell metabolism in nutrient replete and nutrient depleted conditions.

In nutrient replete conditions, cells rely on anabolic metabolism including nucleotide biosynthesis and lipid synthesis to support growth and proliferation. In nutrient depleted conditions, cells rely on catabolic metabolism including fatty acid oxidation and autophagy. AMP kinase (AMPK); branched-chain amino acids (BCAAs). Created by BioRender.com.

Glucose and glutamine are important carbon sources that fuel many metabolic pathways. While glucose is a key source of energy to support glycolysis, the TCA cycle, and OXPHOS, other metabolites can also enter these cycles during intermediary steps. One example of a key nutrient is glutamine. Glutamine can enter the TCA cycle as alpha ketoglutarate. The production of TCA cycle metabolites not only supports the bioenergetics of the cell but can also supply intermediates for lipid synthesis. For example, citrate can be converted to acetyl-CoA for lipid synthesis and acetylation. Oxaloacetate can also leave the TCA cycle to become involved in nucleotide synthesis.

Throughout solid tumor progression, metabolism switches from anabolic to catabolic metabolism (27). In nutrient-replete conditions, cells use processes including glycolysis, nucleotide biosynthesis and lipid synthesis to fuel proliferation and growth. These metabolic pathways are supported through phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) and MYC signaling. The PI3K-Akt-mTOR signaling pathway is commonly upregulated in cancer through mutations in regulatory proteins such as epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) (28). However, within the core of solid tumors where nutrients are scarce, the cells switch to processes including fatty acid oxidation, and macromolecular degradation through autophagy and macropinocytosis (Figure 1). This switch in metabolism is supported by AMP kinase (AMPK) to increase ATP production.

Immune cells have different energy requirements depending on the stage of activation. T cells are a prime example of shifting metabolism based on biosynthetic and bioenergetic requirements during activation and differentiation. For example, naïve T cells have low energy requirements; however, once activated, they rely heavily on glycolytic metabolism to fuel their effector functions. The transition from effector to memory cell also results in a shift from glycolytic to oxidative metabolism (29). In the TME, this metabolic shift is important to recognize as different nutrients may not be available to support this change.

A key metabolic pathway in both T cells and tumor cells is one-carbon metabolism (30, 31). One-carbon metabolism involves the transfer of one-carbon units, or methyl groups, between metabolites. These one-carbon units, through the methionine cycle, play an important role in gene expression. The universal methyl donor S-adenosylmethionine (SAM) regulates gene expression through histone and DNA methylation. Treatment with SAM has been shown to improve responses to cancer therapies, and even work synergistically with immunotherapy treatments such as checkpoint blockade (32, 33). We will explore this pathway further in later chapters of this thesis.

1.5 Considerations for metabolomics

One of the most direct methods for studying cellular metabolism is through metabolomics. Metabolomics is the study of metabolites and their interactions in a biological system.

Metabolomics relies on MS to measure the relative or quantitative abundance of metabolites within a sample. From these measurements, we can infer the reliance on different metabolic pathways. While metabolomics is typically carried out on pure *in vitro* cultures or bulk *in vivo* samples, advancements in cell sorting and low-input sample requirements have helped facilitate studies that analyze specific cell types in the TME. In these experiments, several parameters need to be addressed. These include accounting for sample collection and storage, the impact of delays in processing associated with cellular enrichment, and the extraction of metabolites of interest. Furthermore, the choice of mass spectrometer and method of analysis can change the biological implications of the study. Below we cover each of these considerations in additional detail (Figure 2). We also cover complementary methods (such as flow cytometry) that can help us to unravel the metabolic relationship between cancer and the immune system.

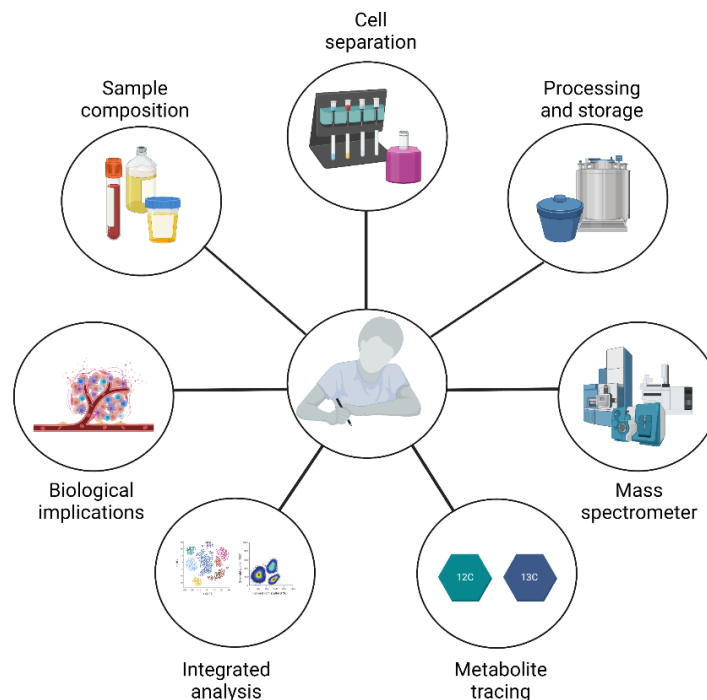


Figure 2. Considerations for metabolomics studies.

Sample collection and composition, cell separation and preparation techniques, processing and storage, choice of mass spectrometer for targeted or untargeted analysis, metabolite tracing, integrated analysis with other techniques (flow cytometry, transcriptomics), clinical and biological implications.

1.5.1 Pre-analytic variable considerations for sample collection and storage

Metabolomics research presents unique challenges compared to genomics and proteomics particularly when considering labile metabolites that have a high rate of turnover (34). For most cell culture experiments, these factors can be mitigated by efficient sample processing. However, careful planning is required to account for pre-analytical variables when collecting samples for metabolomics analysis that are obtained from human clinical samples or animal experiments (35–37). Samples that are either entirely liquid (such as serum, urine, or other cell-free fluids) or entirely solid (such as a tumor or cell culture dish) can be snap-frozen to quench metabolism relatively quickly (technical aspects of metabolic quenching are discussed below) (38).

Conversely, samples containing cell suspensions, such as blood, ascites (accumulation of fluid) or disaggregated tumor (mechanical or chemical disaggregation) require additional preparation steps (39, 40). Although it is a requirement for biobanking to freeze samples, the freezing process and time delay prior to freezing can impact metabolism (41). Furthermore, using a portion of a liquid specimen (i.e., serum, urine) is representative of the entire sample, but this is not the case for tumor or samples with heterogeneous cell suspensions. Selecting a portion of tissue, although often necessary, can introduce bias into the data. Finally, storage length and conditions can impact the integrity of the sample and also require consideration (42).

A key reducing non-biological variable in the data is the need for effective metabolic quenching, a term that refers to the rapid cessation of cellular metabolism to prevent metabolite turnover (43). Effective metabolic quenching is an essential component of any metabolomics assay to capture an accurate representation of the metabolic state without confounding factors of continued metabolism for high-turnover metabolites and metabolite interactions (e.g., degradation products). Efficient arrest of enzymatic activity is therefore a necessary component of any metabolomics workflow. In addition, optimal extraction methods can vary between metabolites due to differences in their chemical properties (e.g., water solubility and polarity). Ultimately, a variety of solvents and sample freezing strategies are commonly utilized depending on the type of metabolites to be measured and the desired level of metabolic quenching, as discussed extensively in previous reviews (34). One commonly used approach is hot or cold organic solvents, such as ethanol or formic acid (34).

1.5.2 Cell type enrichment

Given the critical importance of sample collection on metabolite detection, cell type enrichment steps must also be taken into consideration. Methods to enrich for pure populations of cells are limited to either bead-based sorting or flow cytometric sorting. Magnetic bead-based isolation methods have gained popularity since they require less time and exert minimal mechanical strain on the cells than sorting by flow cytometry. Despite the benefits over flow cytometric sorting, the extra step of a bead-isolation has been shown to change the relative abundance of some metabolites within a sample. However, with careful planning, the advantages of metabolomics on cell type enriched samples can still be extremely beneficial. Recent publications leveraged magnetic bead-based enrichment methods to study the metabolism of immune cells in the TME (44, 45).

One disadvantage of magnetic bead-based isolations is that they require a cell surface antigen and a specific high-affinity antibody conjugated to a magnetic bead. If there is no magnetic bead targeting the protein of interest, indirect staining approaches may be required which can increase the time to isolate the cell population of interest and thus increase the probability of metabolite changes. Additionally, many immune cell populations require simultaneous detection of multiple antigens to distinguish cell types, limiting the capabilities of bead-based methods.

Flow cytometric sorting can overcome some of the limitations of bead-based methods. It is possible to sort on multiple markers at once, including intracellular proteins. However, the process of flow cytometric sorting has also been shown to alter cellular metabolism (46, 47). Some studies have been able to optimize methods to overcome this limitation and show that the metabolism of cells sorted by flow cytometry is similar to matched unsorted cells. For example, DeVilbiss *et. al.* were able to detect 160 metabolites in 10000 cells sorted by flow cytometry (48). This opens the possibility of metabolomics on flow cytometry-based sorted cells, but further optimization is required before this can be a viable strategy on a larger scale.

1.5.3 Choice of a mass spectrometer

Metabolomics-based assays can involve nuclear magnetic resonance (NMR), gas or liquid chromatography (GC or LC) paired MS, and tissue imaging techniques (e.g., MALDI-TOF), to name a few. On the surface, NMR has a major advantage compared to other techniques as it does

not involve processing or destruction of the experimental material. However, NMR sensitivity is an order of magnitude lower than MS (49), and access to NMR devices are prohibitive in most laboratory settings (50). Consequently, GC- or LC-MS are currently the most common technique utilized for metabolomics.

Although GC-MS is a valuable technique for measuring volatile organic acids and fatty acids, technical constraints limit this approach from reaching a broad range of metabolites (see (51, 52) for review). Thus, the wider metabolite detection capabilities of LC-MS have resulted in its use as the predominant analytical procedure for metabolomics. Briefly, in LC-MS, sample metabolites in a liquid phase are separated while flowing across a solid phase column along an imposed gradient of aqueous and organic mobile phases. In this way, the metabolite mixture (biological sample) can be separated into its component parts depending on their elution time from the column. Metabolites then require ionization (most commonly via electrospray ionization (ESI) (53), before entering the mass spectrometer to provide a charged molecule that can be detected by the mass spectrometer to determine the range of mass/charge (m/z) ratios in a given sample. The chemical structure of a given metabolite will determine if optimal detection will occur in positive (+) or negative (-) mode detection on the mass spectrometer, a consideration that may be of importance when choosing a targeted metabolite screen. Pairing the resultant column retention time of a metabolite with its corresponding MS m/z signature in reference to known standards provides a robust method for precise and quantifiable metabolite detection. As the field of metabolomics has developed, typically the LC phase is coupled to tandem MS instruments (discussed below) to achieve more accurate results.

Optimization of the LC component within a metabolomics workflow is not trivial, as multiple methods are required to adequately cover a wide range of metabolites (such as amino acids, lipids, glycolysis/TCA components). Reverse phase (RP) chromatography on silica/ C_{18} columns is used effectively to separate non-polar and weakly polar metabolites, however highly hydrophilic metabolites pass rapidly through the column with this technique disabling its precision for certain metabolites (51). To counteract this issue, hydrophilic interaction liquid chromatography (HILIC) has been developed with columns using an organic mobile phase (as opposed to an aqueous mobile phase for RP) to improve the retention time and subsequent detection of polar metabolites (51). Commonly, a combination of RP-LC and/or HILIC with +/-

mode switching approaches are necessary to provide a complete profile of the metabolite pool of interest in a given study. Myriad columns are commercially available for optimization of detecting metabolites of interest. For a detailed table comparing these metabolomics techniques please refer to the summary by Ren and colleagues (54).

1.5.4 Targeted vs. untargeted metabolomics

Targeted analysis requires the user to know what metabolites they are interested in before they start their analyses. A targeted approach can provide powerful data as technical variations can deliver sample quantification (relative or absolute) and metabolite tracing (^{13}C labeling as discussed below) to determine relative metabolic pathway enrichment or metabolic flux analyses. The quantifiable nature of targeted metabolomics requires tightly controlled comparisons between biological samples and internal standards. The choice of mass spectrometer may be similar between targeted and untargeted assays, however the data generated are typically used for distinct purposes (i.e., hypothesis driven *versus* discovery based, respectively). Choosing a mass spectrometer to match an experimental interest is an essential consideration. A number of excellent reviews have been recently published on this topic (53, 55). Therefore, here we will limit our discussion to a brief overview of the techniques currently being utilized for studying cell subtype-specific metabolomics.

Targeted analysis can be carried out by coupling a high-resolution LC phase, as mentioned above, to a tandem MS (MS/MS) instrument. This has become a commonly used technique for robust and precise metabolite detection. For example, a triple quadrupole arrangement (Q1, Q2, Q3) is routinely utilized whereby scanning of selected m/z ratios occur in Q1, ion fragmentation occurs in Q2 and finally precise detection of the various m/z fragments corresponding to the metabolite of origin first detected in Q1 takes place in Q3. These instruments are desirable as they generally have a high sensitivity, accuracy, and ability to cover a broad range of metabolites. This ability to detect metabolites from a relatively small amount of starting material make it a desirable choice for isolated cell experiments.

Untargeted analysis scans a range of m/z ratios to try and capture a global picture of the metabolites found in a particular setting. Extensive review of the development of untargeted metabolomics including its advantages and limitations have been provided in past articles (55–

57). Briefly, untargeted experimental approaches may invoke a variety of instruments (GC-MS, LC-MS etc.) coupled to an extensive database mining to enable metabolite identification. Although this approach has a labor-intensive identification component which is recognized as a significant bottleneck in the experimental workflow, the ability to observe a wide range of metabolites has proven to be highly beneficial for a number of discovery-based research programs. For example, Weiss *et. al.* used untargeted MS (in combination with bead-based separation) to identify a macrophage-specific metabolite, itaconic acid, that can regulate tumor progression (10). However, given the broad range of metabolites detected, these analyses can be less sensitive than targeted analysis and require a library for identification of metabolites that still requires validation.

1.5.5 Single-cell or low-cell-input techniques

Despite the advancements in cell sorting that now allow for the enrichment of specific cell populations for metabolomics, there are still limitations to this method beyond the changes in metabolism that occur during the enrichment process. Primarily, MS still requires a substantial population of pure or enriched cells that can be difficult to obtain from primary tumors which contain limited numbers of cells or where the target cell population is lowly abundant. Secondly, the enrichment creates a sample of cells with a common marker or phenotype, but many single-cell techniques have shown that these populations can be broken down into smaller and smaller sub-populations, technically maintaining a bulk analysis. Still, advancements in MS methods that rely on low cell numbers can overcome the limitations of studying rare populations.

Single-cell and low-cell-input metabolomics methods are still in development and there are only a few labs worldwide that are known to have this capacity. A study by Abouleila *et. al.* utilized a microfluidics-based cell enrichment technique coupled to LC-MS to metabolically profile single-cells by analyzing circulating tumor cells (CTCs) from liquid biopsies (58). This method relied on differences in size and deformability to create a label free approach that could overcome the typical multistep process of sample preparation. This is a critical advance as CTCs are of incredibly low abundance in patient blood samples (i.e., <100 CTCs/10⁹ blood cells), making it extremely difficult to obtain sufficient material for metabolic analyses (59). Technical advances of this nature should enable expansion into metabolic profiling of other cell populations, including lymphocyte subsets as single lymphocytes were also utilized for comparison purposes

with CTCs in the report of Abouileila *et. al.* (58). Microdroplet/nanodroplet processing in one pot for trace samples (microPOTS or nanoPOTS) techniques have been developed that enable proteomic changes to be observed via LC-MS in as few as 10-200 cells (60, 61). The advantages of these ‘POTS’ strategies for LC-MS detection from low cell numbers should drive exciting technical development for metabolomics using similar methods. Recent advances in sample processing have also made single-cell lipidomics possible (62). Combinations of techniques to allow for spatially resolved single-cell MS for both proteomics and metabolomics have been recently discussed (63) and future studies are now well positioned to exploit near-single cell metabolic profiling as a result.

1.5.6 Experimental design: to label, or not to label, that is the question

Isotopic labelling experiments have become increasingly popular to trace the uptake and utilization of specific nutrients in the body. ^{13}C is a common isotope used in metabolites, where the carbon carries one extra neutron, creating a molecule with a unique mass/charge (m/z) signature that is detectable by MS (64). Exposing cells to ^{13}C -metabolites for varying periods of time, either *in vitro* or *in vivo*, can indicate metabolic flux (quick exposure – seconds to minutes) or metabolism at steady state (longer exposure - minutes to hours). Both techniques have limitations and assumptions that are reviewed elsewhere (64). Combining ^{13}C tracing with bead or flow cytometric-based cell enrichments will provide new and interesting data on immune cell metabolism in the TME (65). However, to take full advantage of the opportunities offered by ^{13}C tracing experiments, cell enrichment methods need to be developed to minimize changes in metabolism and loss of labelled metabolites.

1.6 Other techniques to study metabolism; a multi-omics approach

While metabolomics provides key information about metabolite abundance, and isotopic labeling provides information on metabolic flux, other methods can be leveraged to study cell metabolism. Transcriptomic and cytometry-based approaches provide ways of understanding metabolism through the expression of genes or proteins from key metabolic pathways. Cytometry-based methods are also frequently combined with metabolic dyes that provide indirect measurements of cell metabolism. The recent broad-scale development of single-cell

RNA sequencing (scRNA-seq) can provide transcriptomic data to complement methods such as flow cytometry and mass cytometry which also, by their nature, provide data at single-cell resolution. Combining these datasets into one coherent analysis is still a challenge, but these approaches can thus provide information on metabolism at the single-cell level that is not typically possible through other methods.

1.6.1 Single cell transcriptomics

Whereas metabolomic approaches measures the abundance of metabolites, transcriptomic approaches rely on quantifying messenger RNA (mRNA) expression to define cellular states and thus infer metabolism. Understanding the cross-talk between genetic and metabolic alterations can help to uncover metabolic vulnerabilities and heterogeneity in tumors (66). The inherent heterogeneity of the TME has led to the widespread uptake of single cell RNA sequencing (scRNA-seq) in cancer research, and numerous studies have now defined landscapes of cell types across diverse malignancies (67–69). In ovarian cancer, scRNA-seq is used to distinguish cell subtypes in the ovarian TME and differences in treatment responders (70, 71). In metabolic studies, scRNA-seq has recently been employed in numerous ways. For example, scRNA-seq studies in ovarian cancer were able to show differential expression of metabolic transcripts in different cell types, and even within different tumor subclusters (72). Collectively, these advances suggest that better understanding of cell-specific metabolism through scRNA-seq will continue to reveal new facets of disease (68).

1.6.2 Bioinformatic considerations for multi-omics analysis

As an emerging cross-discipline, bioinformatics combines biological data with computational analysis. However, the bioinformatics analysis of metabolomics data is often under-used, especially as the size and complexity of datasets continues to increase. Integrating complementary datasets, such as sequencing or proteomics data with metabolomics data is complicated. Consequently, the development of computational systems biology approaches to handle metabolomics data, and associated datasets, can contribute to the integration and analysis of large datasets that will provide significantly more information (73).

Metabolomics datasets can be quite large, with hundreds to thousands of metabolites across different samples. Creating a robust experimental protocol, and analyzing and interpreting the

data requires many considerations (74, 75). Commonly used methods for visualizing patterns in metabolomics data include tools such as heatmaps and principal component analysis (PCA), and metabolites of interest can be identified by determining if there is significant change in abundance between samples. While these methods have identified metabolites of interest in previous studies, other more refined analysis methods such as pathway or flux analysis provide information on pathway-level metabolic changes. As metabolomics data sets become more complicated, for example analyzing multiple different cell types from a single sample, a method to combine this data could provide extremely beneficial in understanding the metabolic relationship between different populations of cells.

1.6.3 Cytometry-based methods

Advances in flow cytometry and mass cytometry have led to the development of high dimensional (20-40 color) panels that can tease apart nuanced metabolic states at a single-cell resolution. Flow cytometry uses antibodies conjugated to different fluorophores, and mass cytometry uses antibodies conjugated to different metals (76). Both techniques rely on having antibodies specific to the protein of interest. For metabolism studies, these are typically enzymes or transporters that are in different metabolic pathways. Both techniques have used antibody panels targeted at enzymes and transporters developed to capture the metabolism of cancer and immune cells (77–80). Flow cytometry has also been used *in vitro* to screen for metabolic vulnerabilities in cancer cells (81), and for liquid-biopsy-based metabolic phenotyping for lung cancer (82). Additionally, new tools like Full Annotation Using Shape-constrained Trees (FAUST) (83) make the integration of large flow cytometry panels with metabolic dyes more streamline and feasible to analyze (45).

Metabolism can be inferred by flow cytometry using dyes that measure general metabolic characteristics (45, 84–88). For example, glucose uptake can be measured using 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose), oxidative phosphorylation can be approximated by measuring membrane potential (MitoTracker dyes, tetramethylrhodamine methyl ester perchlorate [TMRE], DiIC₁, etc.), and fatty acids can be measured using the BODIPY dyes (89). These dyes should be used with caution as different cell types and conditions may impact their usefulness (89–91). Despite these limitations, these dyes can easily measure key processes that can be manipulated to improve the efficacy of cancer immunotherapy

(92). For example, glucose uptake and mitochondrial activity can be used to distinguish T cell phenotypes of interest, and as a method to sort for cells that would function better in the TME (93, 94).

Metabolic dyes can elucidate key differences between (A) different cell types in the same environment, (B) the same cell type in different microenvironments, and (C) changes in function.

(A) Tumor cells, T cells and myeloid cells have been shown to have different metabolic requirements across different cancers. Our lab demonstrated that CD4⁺ tumor infiltrating lymphocytes (TIL) had similar mitochondrial activity to CD45-EpCAM⁺ tumor cells in samples from human ovarian cancer tumor and ascites (45). Baumann *et. al.* demonstrated that MDSCs had lower glucose uptake and low mitochondrial membrane potential compared to monocytes (84). These differences in cell metabolism were confirmed using other methods, such as FDG-PET and nanostring sequencing in other studies (89).

(B) These metabolic dyes can also elucidate metabolic changes between different microenvironments. For example, when cells move from the nutrient rich lymphatic tissue or blood to the tumor or ascites, they can experience a decrease in glucose uptake and mitochondrial activity (85, 87).

(C) Stunted cellular energetics can also be seen in *in vitro* co-culture systems and can correlate with changes in cell function. For example, T cells co-cultured with monocytes or tumor cells had decreased glucose uptake alongside a suppression in function (84, 86). Another indicator of T cell function is the expression of immune checkpoints such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein (CTLA-4), and lymphocyte activating 3 (Lag3). Interestingly, elevated inhibitory receptors have been noted to negatively correlate with glucose uptake (85).

1.7 Clinical context: manipulating cellular metabolic dependencies to improve the efficacy of cancer immunotherapies

Immunotherapy is a treatment that relies on manipulating the body's immune system to treat a patient's cancer. Many types of cancer immunotherapies, such as adoptive cell therapy (ACT)

(including chimeric antigen receptor [CAR]-T cell therapy, and TIL therapy), oncolytic viruses (OVs), and immune checkpoint inhibitors (ICI), exploit the relationship between cancer cells and immune cells (95). ACT involved that collection of an apheresis product from a patient which is expanded and re-infused. TIL therapy relies on endogenous recognition between T cells and tumor cells through neoantigens (tumor specific antigens generated by mutations in tumor cells), cancer-germline antigens, or cancer-overexpressed antigen. CAR-T cells can be engineered to recognize a target antigen, overcoming the obstacle presented by growing up T cells that recognize a tumor from a unique patient sample. OVs, viruses that preferentially target cancer cells, can sensitize a tumor to the immune system. Lastly, ICI work to prevent T cells from shutting-down through activation of their checkpoint molecules such as PD-1, Lag3 and CTLA-4 presented on tumor cells or other immune suppressive cells.

While striking results have been obtained by the use of CAR-T cell therapy in certain hematological malignancies, the outcomes in other diseases, particularly solid tumors, have been less spectacular (96, 97). Targeting cellular metabolism provides a way to overcome the restrictions of the TME and improve the efficacy of immunotherapies. Attempts to improve the efficacy of immune cell-based therapies by manipulating metabolism have been reviewed extensively elsewhere (5–15, 98–100). Here, we will focus on key results that demonstrate how we can manipulate cell type-specific metabolic dependencies to improve the efficacy of immunotherapies.

1.7.1 Rationale for targeting the metabolism of ovarian cancer

Ovarian cancer is the deadliest gynecological malignancy for people with ovaries (101). Early detection is difficult because patients present with vague symptoms. Thus, most diagnoses do not occur until patients reach late stage, at which point the cancer is highly advanced and aggressive. Current treatment strategies involve debulking surgery in combination with platinum-based chemotherapy (101). While most patients respond initially to this treatment, approximately 70% will relapse, leaving the patient with minimal options, such as nonplatinum-based chemotherapy regimens (101, 102). The current 5-year survival for ovarian cancer is approximately 45% (101) and has increased less than 10% over the last 50 years (Ovarian Cancer Research Foundation). Given the grim prognosis for patients diagnosed with ovarian cancer, new strategies for early detection and treatment are needed.

Ovarian tumors can be classified as immunologically “hot” or “cold” based on their immune cell infiltrates (or infiltration status). Patients with high immune cell infiltrates in their tumor are considered “hot”, while tumors with low immune cell infiltrates are considered “cold”. Patients with “hot” tumors containing T cells and B cells have a greater 5-year survival rate than patients with “cold” tumors (103). Given the positive prognostic value of immune cell infiltrates in the tumor, immunotherapies were thought to be a promising treatment option for ovarian cancer; however, this has not shown to be the case thus far. ICI and CAR-T cell therapies have been largely unsuccessful at treating ovarian cancer in clinical trials to date (101, 104–106). The lack of success has led researchers to evaluate immunosuppressive mechanisms that may be preventing these therapies from reaching their full potential in this disease. One such strategy to evaluate immune suppression is to explore the metabolism of the ovarian TME.

Like other solid tumors, the cellular composition of the ovarian TME results in a unique metabolic environment that can be leveraged to improve therapies. Key pathways under investigation include glycolytic and lipid metabolism. Targeting these pathways using metabolic inhibitors has demonstrated positive results in pre-clinical models but only limited efficacy in clinical trials (107, 108). This includes the phase II trial where patients were treated with ionidamine, a drug that inhibits aerobic glycolysis, in combination with cisplatin and paclitaxel (109). This was a single arm study, making determining the efficacy of the combination therapy difficult. Another pathway also starting to be targeted in clinical trials is tryptophan metabolism, using indolamine 2,3-dioxygenase (IDO) inhibitors (110).

A common feature of ovarian cancer is peritoneal dissemination and the presentation of ascites. Ascites is a build-up of cellular fluid in the peritoneal cavity that is associated with advanced disease and poor prognosis (111). Importantly, the ascites exchanges nutrients and material with both the circulatory system and the solid tumor. Thus, the ascites contains cellular and non-cellular components from the TME that contribute to an immune suppressive microenvironment. How the metabolic environment of the ascites leads to tumor dissemination and T cell reprogramming is under investigation (112).

1.7.2 Targeting metabolism for systemic immunotherapy treatments

The same metabolic pathways are universal to all cells in the TME, but different cell populations have different preferences to support their energy demands (89). As a therapeutic, targeting cell metabolism in a cell-specific manner requires a detailed knowledge of these metabolic dependencies. We rely on a metabolic manipulation, especially metabolic drugs, to sensitize specific cell populations. Studies that look at how a metabolic pathway changes across cell populations in the TME can provide insights into how the different cells will respond.

Three of the major metabolic pathways typically targeted using full-body systemic treatments are glycolysis, glutaminolysis, and OXPHOS. Despite the importance of these pathways for all cells, targeting the transporters or enzymes involved in these pathways have shown to preferentially hinder tumor cell growth while promoting the immune response (Figure 3). For example, expression of carbonic anhydrase IX (CAIX), a lactate transporter, is negatively associated with survival in melanoma patients. A small molecule inhibitor of CAIX sensitized melanoma tumors to ICI and led to decreased tumor growth and metastasis (113). Similarly, neutralization of tumor acidity using bicarbonate therapy improved response to ICI and ACT in some mouse models (114). Using a small molecule to block glutamine metabolism in mice led to decreased hypoxia, acidosis, and nutrient depletion, while T cells switched to an oxidative metabolism and adopted a long-lived phenotype. Glutamine blockade sensitized mice to treatment with anti-PD-1 and improved response rates (24). Another metabolic pathway that has been highlighted recently is polyamine metabolism. RNA-seq in glioblastoma demonstrated upregulation of the arginine-ornithine-polyamine pathway in TAMCs but not in CD8⁺ T cells. An inhibitor of the polyamine pathways, difluoromethylornithine (DMFO), worked in combination with ICI and radiotherapy to improve survival of mice (44). Finally, the addition of SAM has been shown to work synergistically with PD-1 therapy (33). These studies highlight the different metabolic dependencies for key cell populations in the TME, even for, what are considered, essential nutrients.

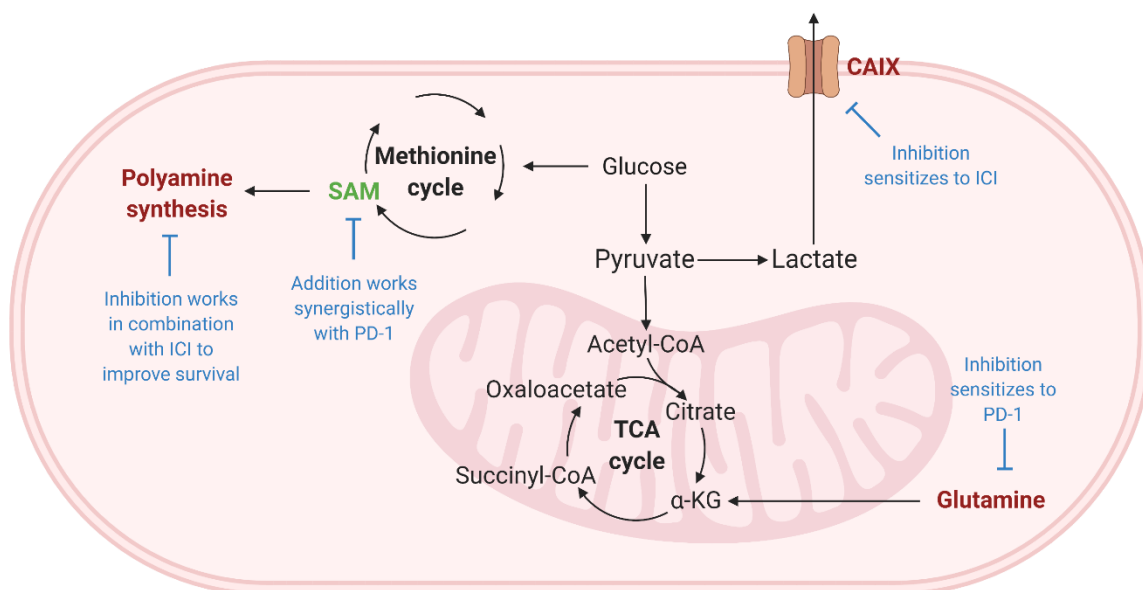


Figure 3. Targeting metabolism in immunotherapy treatments.

Inhibition of cancer cell metabolism can improve therapeutic response in combination with immunotherapy treatment. For example, inhibition of polyamine synthesis, carbonic anhydrase IX (CAIX), and glutamine metabolism or addition of S-adenosylmethionine (SAM) can work synergistically with immune checkpoint inhibition (ICI). Created using BioRender.com

1.7.3 Targeting metabolism for CAR-T cell therapy

CAR-T cell therapy, and more broadly ACT, are two treatments that provide an opportunity to target T cell metabolism *ex vivo*, limiting the effects of full-body metabolic manipulations. Importantly, T cell products with certain metabolic characteristics have been shown to be more efficacious. For example, CAR-T cells with higher mitochondrial biogenesis yields complete response in chronic lymphocytic leukemia (CLL) (115), and CAR-T cells from complete responders had greater OXPHOS (116), supporting the important role of metabolism in CAR-T cell function.

Modifications to CAR-T cell therapy are required for treating cancers that form solid tumors. For these therapies to be successful, infused T cell products or endogenous T cells need to be able to infiltrate and function in the TME. Metabolically, the T cells are not primed to deal with this environment. Two methods to improve T cell function in the TME are by manipulating cell

metabolism of culture conditions during *in vitro* expansion, or genetically engineering the T cells to cope with the TME (9). There are many gene-engineering approaches to manipulate CAR-T cells to cope with the TME. The design of the CAR itself can change the metabolism of the cells. For example, CAR-T cell signaling domains can change the cell's reliance on glucose or oxidative metabolism (117). Additionally, knock-out or overexpression of metabolic genes can increase CAR-T cell efficacy. For example, the TME contains low arginine, so overexpressing enzymes for arginine synthesis in T cells improves their efficacy in mouse models of leukemia and solid tumors (118).

T cells used for ACT or CAR-T cell therapy need to be expanded to reach therapeutic levels prior to re-infusion back into a patient. This provides an opportune time to condition the cells to respond to the TME (119). Clustered regularly interspaced short palindromic repeats (CRISPR) screens in physiological media have elucidated key metabolic genes that could be important in the TME, but were not detected in the super-physiological conditions typically used (120). For example, CD8⁺ T cells cultured in glutamine restricted conditions had enhanced expression of pro-survival transcription factors and were able to expand better in mice upon reinfusion (121). T cells expanded with MEK inhibitors led to decreased tumor burden in mice (122). Understanding the metabolic requirements of cells in the TME through mimicking physiological conditions or human samples could further elucidate conditions to prime T cells prior to infusion in patients.

1.8 Conclusion

In conclusion, the TME is composed of different cell-subclasses, including a variety of types of immune cells. So far, we have been limited by the techniques available, however, new technical advances will provide us with the resources to study metabolism at the level of specific subclasses, or even, eventually, single cells. Understanding the metabolism of these specific cell-subclasses is essential for developing methods to improve the efficacy of immunotherapy treatments.

1.9 Thesis hypotheses

1.9.1 Hypothesis

Immune regulatory metabolites in the tumor microenvironment suppress T cell function in ovarian cancer

1.9.2 Aims

1. Develop and optimize methods to metabolically profile primary ovarian cancer specimens by mass spectrometry and flow cytometry
2. Profile the metabolism of T cells and tumor cells from matched tumor and ascites ovarian cancer specimens
3. Identify immune regulatory metabolites in tumor and T cells
4. Delineate the suppressive mechanisms of identified metabolites with respect to T cells
5. Devise strategies to manipulate metabolism to circumvent T cell immunosuppression

1.9.3 Summary

To begin addressing these questions, we devised a sensitive cell isolation and LC-MS/MS method to interrogate the repertoire of metabolites within and between distinct cell types including CD4⁺ and CD8⁺ T cells as well as tumor cells across the ascites and tumor environments in individual patients with ovarian cancer. We coupled this approach with high-dimensional flow cytometry and scRNA-seq to provide a highly resolved portrait of the metabolic status of these key populations. This approach uncovered profound increases in levels of MNA in T cells in the tumor, while *in vitro* assays revealed a previously unknown immunomodulatory effect of MNA on T cell function. Collectively, this approach revealed reciprocal metabolic interactions between tumor and immune cells and provided a unique insight into immune regulatory metabolites that may yield therapeutic opportunities to manipulate T cell-based immunotherapies for ovarian cancer.

Chapter 2: Principles of reproducible metabolite profiling of enriched lymphocytes in tumors and ascites from human ovarian cancer

Adapted from:

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2.1 Acknowledgements and contributions

2.1.1 Author contributions to the text

M.K.K., S.M., and J.J.L wrote and edited Procedure 0, 1, 2 and 3. J.L., S.B., and P.H.W. wrote, edited, and conducted Procedure 0 and 1. M.K.K. conducted procedure 2 and 3. L.G.Z. and R.J.D. wrote, edited, and conducted the mass spectrometry portion of Procedure 2. P.T.H. wrote and conducted the bioinformatic portion of Procedure 2 and created Appendix 1. S.P., R.D.S., and R.G.J. edited the manuscript.

2.1.2 Author contributions to the tables and figures

M.K.K. and S.M. helped design, and G.K. created Figures 4-6 (using BioRender.com).

M.K.K. created Figure 7, Figure 9 (using iPath3), and Figure 10.

M.K.K. processed the samples, L.G.Z. generated and analyzed the data, and P.T.H. analyzed the data and generated Figure 8. B and D.

M.K.K. processed the samples, L.G.Z. generated and analyzed the data and created Figure 8. C.

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2.2 Abstract

Identifying metabolites and delineating their immune regulatory contribution in the tumor microenvironment (TME) is an area of intense study. Interrogating metabolites and metabolic networks amongst immune cell subsets and host cells from resected tissues and fluids of human patients presents a major challenge, owing to the specialized handling of samples for downstream metabolomics. To address this, we first outline the importance of collaborating with a biobank for coordinating and streamlining workflow for point of care sample collection, processing, and cryopreservation. After specimen collection, we describe our 60-minute rapid bead-based cellular enrichment method that supports metabolite analysis between T cells and tumor cells by mass spectrometry. We also describe how the metabolic data can be complimented with metabolic profiling by flow cytometry. This protocol can serve as a foundation for interrogating the metabolism of cell subsets from primary human ovarian cancer.

2.3 Introduction

The tumor microenvironment (TME) is a complex ecosystem of different cell populations that varies across cancer types but also between individual patients with the same diagnosis (123). Certain cell populations within the TME are well known prognostic and predictive factors of survival in human cancers (123, 124). These cells include stromal cells, fibroblasts, endothelial cells, mesenchymal stem cells, adipocytes, and immune cells (124). Immune cells can include both immune-stimulatory (T cells, B cells, Natural Killer cells) and immune-suppressive cells (T_{reg}, M2 macrophage, myeloid derived suppressor cells (MDSCs)). Thus, careful characterization of the individual populations that comprise a tumor are required to identify factors essential for developing more effective cancer therapies.

The availability of metabolites within the TME can dictate tumor growth and immune cell function (125). Cancer cells are known to outcompete other cell subsets for key nutrients while also producing immunosuppressive metabolites. For example, cancer cells demonstrate increased glucose uptake and aerobic glycolysis, which results in depletion of glucose and excess production of lactic acid. Other known immunosuppressive metabolites produced in the TME

include kynurenine, adenosine, (R)-2-hydroxyglutarate (126–128) and as we recently found, 1-methylnicotinamide (MNA) (129).

To date, mass spectrometry-based metabolomic studies in human cancer are usually carried out on bulk tumor samples. This type of analysis assumes that the tumor is a homogeneous collection of cells; an assumption known to be incorrect. To understand the interactions of metabolites of different cell types, the metabolomes of enriched cell populations need to be considered. However, metabolites vary considerably in their stability which means that it is important to carefully consider the workflow of sample storage and cell isolation processes; and to understand the potential artefactual changes that are likely to occur before the sample is analysed by mass spectrometry and flow cytometry.

In addition to experimental considerations, accessing biospecimens (i.e., tumor, blood, ascites) that are fit for specific research purposes can be logistically and financially challenging. Therefore, it is crucial to partner with a biobank that has experience with these existing established processes that can be adapted to the needs of a specific study, in order to overcome or minimize some of these limitations. Many dedicated biobanks now focus on providing collaborating or contract base biobanking services to researchers (130). This includes assisting with consent, collection, and storage of biospecimens in support of prospective studies, as well as maintaining inventories of previously collection biospecimens for retrospective research study needs.

Here we share our workflow for biospecimen collection, cellular enrichment, and mass spectrometry analysis to identify metabolic differences between tumor and T cells in ovarian cancer tumor and ascites microenvironments (Figure 4). Using our cell enrichment technique followed by liquid chromatography-mass spectrometry (LC-MS), we were able to identify metabolites known to impact T cell function (adenosine and kynurenine) (127, 128), yet we also identified a novel immunomodulatory metabolite MNA (129).

2.3.1 Development of the protocol

Partnering with a Biobank

Biobanking involves a set of linked processes focused on participant enrollment, collection, processing, annotation, storage, and direct use of biospecimens for future research studies. The

field of biospecimen science has expanded our knowledge of a range of pre-analytical variables that alter the intrinsic properties of biospecimens (131). Therefore, variation in the biobanking processes can influence the quality of biospecimens and the interpretation of research performed on them. This is particularly relevant when the analyte(s) are associated with a highly dynamic process such as metabolism. We will highlight and discuss the regulations that were important for our work based in Canada. These may differ depending on jurisdiction and need to be checked with local governing bodies.

To ensure the quality of biospecimens, special attention to protocol details and capturing any variations in the biobanking process is critical. Knowledge of biobanking can be obtained from internationally accessible education courses (e.g. <https://www.edx.org/course/biospecimen-research-methods-6>). In selecting a biobank, be aware that several organizations have created internationally recognized best practices and biobanking standards and associated programs for biobanks. You should look for biobanks that have adopted one of these programs to provide assurance of a known level of expertise and quality relating to the biobanking processes.

In our work, we partnered with the Canadian BC Cancer Tumor Tissue Repository (TTR) Biobank based on the external expertise we needed, and secured their services for patient engagement and informed consent, acquiring biospecimens, and data collection (<https://www.bccrc.ca/services/biobanking-biospecimen-research-services/researchers>). The TTR has standardized operating procedures in place and is externally certified by the Canadian Tissue Repository Certification program and operates under ethics approval from the BC Cancer Research Ethics Board. Partnering with TTR Biobank provided us with: patient confidentiality, standardized biospecimen collection protocols, increased efficiency, and an understanding of the clinical landscape. If not partnering with a biobank, there are many considerations to address prior to initiating patient engagement and biospecimen collections. Resources exist and are available worldwide to assist researchers in the creation of a biobank (e.g. International Society for Biological and Environmental Biorepositories, <https://www.isber.org>; The Biobank Resource Center, <https://biobanking.org>).

Choice of biospecimen

We chose to interrogate the resected primary tumor and the ascites collected from ovarian cancer patients. The primary tumor samples used here were collected at the time of debulking surgery

(herein referred to as tumor), in accordance with the standard of care for the patient. However, collection of material at other timepoints, including tumor recurrence, would allow for the characterization of the tumor in response to therapy or over time. While the tumor is resected from a specific site, the ascites is an immunomodulatory and tumorigenic fluid that accumulates in the intraperitoneal space of ovarian cancer patients. The ascites may be collected at various time points prior to or after surgery depending on the presentation of the patient. In our work, the tumor and ascites samples were matched from the same patient. The power of using two matched biospecimens allows for the comparison between the same cell type within two unique tumor microenvironments. For example, the significance of the metabolite MNA was revealed from the changes in abundance in T cells from the ascites and tumor compartments. The choice of biospecimens to collect is dependent on the scientific question being asked. While we used malignant samples, one could also choose to use adjacent healthy tissue.

Cellular enrichment for mass spectrometry

Recent studies have shown that flow cytometry-based sorting and bead enrichment methods can cause alterations in cellular metabolic profiles (46–48, 65). However, the main issue reported was the flow sort and not the antibody staining (46). Selecting the appropriate separation method requires careful consideration and depends on the purpose of the experiment. Magnetic bead-based isolation methods have gained popularity since they require less time and exert minimal mechano-strain on the cells than sorting by flow cytometry. However, bead-based isolations require a cell surface antigen and a specific high-affinity antibody conjugated to a magnetic bead. If there is no magnetic bead targeting the protein of interest, indirect staining approaches may be required which can increase the time to isolate the cell population of interest, thus increasing the probability of non-specific metabolite changes. Additionally, many immune cell populations require simultaneous detection of multiple antigens to distinguish cell types, limiting the capabilities of bead-based methods. However, used properly, bead-based isolation methods can uncover unique metabolic features of specific immune cells in the TME (129, 132). In this protocol we describe a column-based approach given its rapidness and the availability of high-affinity antibodies for the cell surface markers of interest (CD4, CD8, CD45).

Low cell number limitations of the TME

Metabolomics is typically performed using replicate samples of millions of cells. This can be difficult to achieve due to the cellular composition of the biospecimen or the amount of biospecimen available. Additionally, isolation techniques to enrich specific populations can lead to a reduction in the number of cells analysed: both due to the process of separating the sample and because cells are inevitably lost during the isolation steps. Therefore, the type and size of the biospecimen available needs to be taken into consideration and the separation techniques need to be optimised to account for low cell numbers.

For our analysis of the TME, we focused our resources on performing concurrent high-dimensional flow cytometry and targeted metabolomics. A high-dimensional panel incorporating a collection of antibodies specific for general phenotypic markers, alongside a dye quantifying mitochondrial activity (MitoTracker Deep Red), can be performed on samples in the range of 20,000-100,000 cells depending on the proportion of the cell populations of interest (i.e., the study of rare cell populations will require a higher starting number of cells). In the case that cell numbers within the samples are limited, each phenotypic marker must be evaluated to correctly distinguish the true population of interest.

For our targeted metabolomics panel, we designed a workflow to collect enough cells per replicate to ensure accurate representation of the metabolism of the cells. Therefore, we enriched populations based on broad T cell (CD4⁺ and CD8⁺) and tumor cell (CD45⁻) markers as more rare populations did not meet cell number restrictions. The enrichments can be carried out in parallel if there is enough material, or sequentially if the number of cells is limited. For every experiment, the enriched cell sample was simultaneously assessed for purity of enrichment by flow cytometry analysis thus providing an additional quality control on the separation methodology (129).

Flow-based metabolic profiling studies

Advances in flow cytometry have led to the development of high dimensional (20-40 colour) panels that can tease apart nuanced metabolic states at a single cell resolution. Flow cytometry uses antibodies conjugated to different fluorophores or dyes that fluoresce under different conditions. For metabolism studies, we rely on having antibodies specific to the protein of interest (typically enzymes or transporters) or dyes that measure general metabolic characteristics (129, 133–136).

Nutrient uptake can be measured using 2-NBDG (glucose) or a range of BODIPY dyes for fatty acids (137). While mitochondrial phenotypes can be assessed by measuring membrane potential (MitoTracker Deep Red dyes, TMRE, DilC1, etc.), mitochondrial mass (MitoTracker Green), and Mitochondrial ROS (MitoSOX). These dyes should be used with caution as different cell types and conditions may impact their usefulness, especially 2-NBDG (90, 137). Indeed, there is clear evidence that 2-NBDG does not reflect glucose uptake in lymphocytes and we therefore do not recommend its use (90). Other methods are available that can provide a measure of glucose uptake (137, 138). Here, we optimized our flow cytometry panel to include phenotypic markers for T cells, and the metabolic dye MitoTracker Deep Red.

Mass Spectrometry-based metabolite profiling studies

Liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (MS) instruments are currently the most common techniques utilized for metabolomics (139). Gas chromatography often requires sample derivatization to increase thermal stability, making it more amenable to enter the gas phase and to induce predictable in-source fragmentation patterns (140). In comparison, LC-MS requires minimal up-front sample preparation because samples can remain in their liquid phase (141). In LC-MS metabolomics studies, both Reverse Phase (RP) and normal phase chromatographic platforms are used for separation. The chromatographic method should be chosen based upon the chemical properties of the analytes of interest. For example, a normal phase Hydrophilic Interaction Liquid (HILIC) chromatography might be selected for highly polar, aqueous analytes. Here we use a reverse phase chromatography protocol for metabolite separation.

The ultra-high performance LC system was coupled to a triple quadrupole mass spectrometer for targeted analyses of metabolites. The assay utilized in this study is characterized as targeted metabolomics because of the triple quadrupole's (Q1, Q2, Q3) tandem quadrupole arrangement. Triple quadrupole mass spectrometers are advantageous because the parent/product ion couple in Q1 and Q3 are highly selective and sensitive. This increased sensitivity makes it an ideal instrument for isolated cell populations where sample amount is limited. Thus, we developed a targeted panel of 204 metabolites for intracellular analysis using our LC-MS/MS method. All metabolites included in the targeted panel were verified by injections of 1 ppm standards. Approximately 200 metabolites were chosen to keep instrument cycle time low, while providing

coverage across multiple metabolic pathways. The metabolites in this panel include amino acid, nucleotide, central carbon and vitamin metabolism.

Bioinformatic analysis considerations

Statistical analysis of LC-MS/MS data remains an evolving field. For simple experimental designs, online frameworks such as MetaboAnalyst (142) can help to guide or automate analyses to recover metabolites that differ between experimental or sampled conditions. However, for more complex sampling designs, such as those where multiple sample types are taken from the same patient (e.g., different cell types in tumor vs. ascites) more customized statistical models packages are needed, for example that incorporate the correlations among samples coming from the same individuals. Recent updates to MetaboAnalyst may cover some more complex use cases (142), but other approaches offer more flexible analysis options. Here, we used the R/Bioconductor package *limma*, widely used in transcriptomic analysis to accommodate the multivariate nature of metabolomic data (i.e., numerous metabolites measured per sample) and the typically non-Gaussian distributions of metabolite profiles, as well as complex statistical designs (143, 144). However, we are unaware of benchmarks that rigorously compare *limma* and other analysis frameworks, and we anticipate that future developments will help to refine optimal analysis strategies in metabolomic studies.

Application of the methods

This protocol can be adapted to other tissues and cell types over a wide range of experimental conditions. We found unique differences between T cells and tumor cells in ovarian cancer, however other cell types within the TME would also be interesting to interrogate in different cancer types. Furthermore, these protocols can also be applied to *in vitro* cultures in order to enrich specific T cell populations in co-culture assays with tumor cells or other immune cell subsets.

Comparison with other approaches

There are three main bead-based approaches for T cell isolation: column-based, column free and automated. Column-based methods are known to support high purity and are ideal when isolating a lower cell number population with lower concentrations of antibody compared to column-free. However, column free options have the advantage of being an overall faster technique,

supporting downstream mass spectrometry detection of high flux metabolites (145). Conversely, fully automated systems require the largest amount of time but have the advantage of hands-off processing potentially increasing the reproducibility of this step between samples. With these caveats in mind, careful consideration is required when selecting a cell isolation technique that fits your scientific purposes.

Due to the cell number restrictions in our study, we opted to use the column-based method with sequential separations. This technique involves the isolation of a positive population while the negative cell population continues onto a sequential separation, minimizing the need for separate samples for cell isolation.

2.3.2 Experimental design

Overview

The instructions are divided into three procedures (Figure 4). Procedure 1 describes important considerations for preparing operational protocols for biospecimen collection from cancer patients as well as the collection, transport, and preservation of human tissues. Procedure 2 describes the process of cell enrichment, metabolomics, and data processing from human tumor and ascites samples. Procedure 3 describes a phenotypic and metabolic profiling high-dimensional flow cytometry panel for complex tissues.

Expertise needed

- Ethics education:
 - i.e. Canadian Tri-council Policy Statement: Ethical Conduct for Research Involving Humans – TCPS2 (https://ethics.gc.ca/eng/policy-politique_tcps2-eptc2_2018.html) or the TCPS 2 tutorial Course on Research Ethics (CORE) (https://ethics.gc.ca/eng/education_tutorial-didacticiel.html)
- Biosafety training for researchers and biobank staff working in a biosafety level 2 facility. Protocols include: universal precautions and safe handling of human biospecimens, biological hazards, biohazardous spill control and response, biohazardous waste and sharps disposal.

- For researchers and biobank staff handling research biospecimens, education should include background to biospecimen collection, processing, storage, data management, ethics, privacy, consent and security.
 - Recommended courses include: CTRNet Essentials of Biobanking (<https://biobanking.org/webs/education>) and UBC edX Biospecimen Research Methods (<https://www.edx.org/course/biospecimen-research-methods-6>).
- For researchers and biobanking staff who handle, or transport biospecimens, they must be adequately trained and hold a training certificate or perform those activities under the supervision of a person who is adequately trained and who holds a training certificate.
- Operation of flow cytometry instruments requires specialized training. Can also be completed in collaboration with a nearby laboratory with flow cytometry capabilities.
- Operation of LC-MS instruments requires specialized training. Can also be completed in a metabolomics facility or in collaboration with a laboratory that has the appropriate MS equipment.
- Bioinformatic analysis requires competency with R.

Suggestions for optimal data consistency and quality

In preparation for working with clinical samples, optimization experiments were performed with a mixture of stimulated and unstimulated healthy donor PBMCs (refer to Procedure 3, step 65-69) and tumor. The tumor cell source can come from a cancer cell line expressing the markers of interest, or patient sample if accessible. Using this as a surrogate for the cellular make up of the TME, the selection of flow cytometry markers and fluorophores was optimized to be robust for all cellular populations of interest. Furthermore, this type of sample was also used to assess the feasibility of the bead-based enrichment technique.

To ensure data consistency and quality:

- Perform flow cytometry data analysis with the same number of cells for all patients as variability in cell number may affect the uptake of MitoTracker Deep Red.
- During the magnetic bead sort we found that cells placed on ice versus immediately sorted had similar metabolomic profiles, although some metabolites (e.g. amino acids) were more prone to change (129). Thus, this must be considered when interpreting

results. To minimize metabolic changes and maximize consistency throughout the protocol, the following suggestions should be considered:

- Keep cells on ice, in refrigerated centrifuges and maintain cold media/buffer to minimize metabolic changes.
- Samples should be treated consistently with the same timing and methods to minimize intra-sample variability.
- Metabolites that are highly reactive or have a high cellular turnover are less likely to be detected. Normalize mass spectrometry abundance values to the total ion count (TIC) for the sample.
- Assess isolation purity after every separation. An impure cell population may convolute the metabolomics data.

Metabolite analysis by mass spectrometry

Panel and LC-MS optimization

First, infusion of a 1 ppm standard solution will allow for optimization of precursor ion (Q1), fragment ion (Q3), declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) specific for each metabolite that is desired to be in the targeted panel.

Adjustment of these values during infusion will improve the detectable signal of the analyte. These values are then programmed into the MS acquisition method. Secondly, separation by LC is optimized by building iterative methods that vary column temperature, gradient, and flow rates. Both of these optimization steps were completed prior to creating the LC-MS method previously described (146). Method validation is performed with both quality control (QC) and biological samples for phenotype recapitulation.

MS quality controls

A good quality control (QC) is designed to evaluate the state of both chromatographic and mass spectrometer conditions. A QC will test the LC column status for matching retention time and Gaussian peak shape. The mass spectrometer status is evaluated with the QC by testing for reproducible signal intensity and mass accuracy. QCs should be prepared from biological material (such as tissue or plasma) or a chemical standard mix at known concentrations. Selection of an appropriate quality control sample depends on (1) the ease of acquiring the same

sample material for future use, (2) the concentration must be known and within the linear range of the mass, and (3) it must be easily characterized and detected by mass spectrometer.

Extraction solvent

An extraction solvent of 80% methanol (in water) was chosen to lyse cells and denature protein. Methanol is more effective than other organic solvents such as acetonitrile, or a mixture, at precipitating protein. Methanol is also a volatile solvent that will dry quickly in the SpeedVac so that the samples can be reconstituted in mobile phase A (MPA) for injection.

Mobile phases

For reverse phase chromatography, the organic solvent must be more hydrophobic than the stationary phase for the partition into the mobile phase and elution from the column. Thus, acetonitrile was chosen as mobile phase B (MPB) because it is a non-protic solvent that will not cause additional hydrogen bonding between the analytes, mobile phase, or stationary phase. Water was used as the aqueous solvent for MPA. We added formic acid to both mobile phases to assist with ionization at the source by providing protons.

Limitations

- Multiple reaction monitoring (MRM) data acquisition selects ions for detection that are pre-chosen by the user. Because of this, only ions of known metabolites may be programmed into the mass spectrometer method. Therefore, the data will not contain additional information for metabolites that are not included in pre-chosen metabolite list.
- Unit resolution provided by a triple quadrupole mass spectrometer will not resolve analytes with the same nominal mass that do not have unique MRM transitions in Q3. For example, many small molecules have similar mass to charge (m/z) ratios and lose common functional groups, such as CO₂, upon fragmentation. Therefore it is important to achieve separation with chromatography prior to injection to prevent mixed detection of multiple analytes.
- Low abundant metabolites are often below the limit of detection (LOD). Other enrichment techniques or acquisition techniques should be considered for these analytes.

- An analyte's chemical properties, spatial orientation, and interaction with the stationary and mobile phases determine its retention on a column. Certain chromatographies therefore retain specific classes of molecules differently than others.
- Data from this method is for relative comparison and therefore does not provide quantitative information.
- Patient samples will vary in size and therefore cell number. To prepare your metabolomic analysis for this, we recommend a cell dilution series to assess the sensitivity of your mass spectrometry instrument for low cell number analysis.
- The abundance of metabolites is a static measurement that does not provide the dynamic understanding of cellular metabolism. Therefore, we recommend combining these methods with ¹³C labeling or extracellular metabolite measurements to support that type of analysis. This would be beneficial for pathway analysis.

Choice of biospecimens

- Here we chose to use matched ascites and tumor samples (i.e. from the same patients) for analysis. From these samples, we isolated the CD4+, CD8+ and CD45- cell fractions. We have 6 patients, with 2 biospecimens, divided into 3 cell types, creating a 'hierarchical' data set. An experimental design that contains this hierarchical approach may have more statistical power but may require more extensive analysis than if the samples were truly independent (refer to Procedure 3, step 63 - Bioinformatic and statistical analysis of metabolites).
- This protocol may not be applicable for types of cancers where there are no defining surface markers between cancer cells and immune cells, such as leukemias or lymphomas.

2.4 Materials

2.4.1 Biological materials

Human subjects: The tumor and ascites samples are obtained as part of surgical removal of tissue from patients. The considerations and methods are described in detail in Procedure 1.

! CAUTION Collection of human specimens requires ethical approval, conforming to the relevant institutional and national regulations, and patient consent (refer to Procedure 1 for additional details).

2.4.2 Procedure 1

Human sample collection:

- Hard sided coolers: 1 small hard sided container (SAF-T-PAK, cat. no. 35154) with added “Used Medical Equipment” label and 1 large transport container for biospecimens and equipment (MarketLab, cat. no. ML3639)
- Ice packs, frozen (VWR, cat. no. 89049-358)

Tissue Collection:

- 50 mL centrifuge tubes, sterile (Fisher Scientific, cat. no. 05-538-55A)
- Sterile PBS (Fisher Scientific, cat. no. SH3025601)
- Biohazard specimen bags, sealable, 2-3 small (Fisher Scientific, cat. no. 23-700-209)

Tissue collection tool kit (sterilized):

- Sterilization pouch (Fisher Scientific, cat. no. 01-812-54)
- Forceps (VWR, cat. no. 82027-446)
- Scissors (VWR, cat. no. 82027-588)
- Hemostatic forceps (VWR, cat. no. 82027-418)
- Scalpel (VWR, cat. no. 82029-806)
- Scalpel blades (VWR, cat. no. 72044-21)

Ascites collection:

- Suction regulator canisters and tubing, sterile, sourced by participating the hospital (Amico, cat. no. SRX-CN-2000-AC)
- Absorbent sheets (VWR, cat. no. 10306)
- Biohazard specimen bags, sealable, 2 large (VWR, cat. no. 75838-698)
- Sodium Heparin 10,000 U/mL (Pfizer, DIN 02382326)
- Nalgene containers, 1 L, sterilized (VWR, cat. no. 16129-061), marked with gradations

- 3 mL syringes (Fisher Scientific, cat. no. B309656)
- 18-gauge needles (VWR, cat. no. CABD305196)

Tumor sample processing

- Petri Plate, sterile (Fisher, cat. no. FB0875712)
- Scalpel, disposable, No.22 (Fisher, cat. no. 08-927-5F)
- Transfer pipette (Fisher, cat. no. 13-711-20)
- 100 uM cell strainer (VWR, cat. no. CA21008-950)
- 3 mL syringe (Fisher, cat. no. B309586)
- 50 mL Falcon tube (Froogabio, cat. no. TB50-500)
- Cryovials, 2.0 mL (Fisher, cat. no. 03-374-21)
- Human AB serum, heat inactivated and filtered (Sigma, cat. no. H4522-100mL)
- Sample processing medium (Reagent Setup)
- Freezing medium (Reagent Setup)

Ascites sample processing

- 15 mL conical tube (Froogabio, cat. no. TB15-500)
- 50 mL Falcon tube (Froogabio, cat. no. TB50-500)
- 250 mL or 500 mL centrifuge tube, as required (Fisher, cat. nos. 05-538-53, 07-200-621)
- Cryovials, 2.0 mL (Fisher, cat. no. 03-374-21)
- ACK lysis buffer (ThermoFisher, cat. no. A1049201)
- Human AB serum, heat inactivated and filtered (Sigma, cat. no. H4522-100mL)
- Mr. Frosty (or equivalent)
- Sample processing media (Reagent Setup)
- Freezing media (Reagent Setup)

Equipment

- Centrifuge operating at 4 °C
- Balance (Fisher Scientific, cat. no. 01-922-412)

2.4.3 Procedure 2

Cell enrichment

- CD4 MicroBeads, human (Miltenyi, cat. no. 130-045-101)
- CD8 MicroBeads, human (Miltenyi, cat. no. 130-045-201)
- CD45 MicroBeads, human (Miltenyi, cat. no. 130-045-801)
- LS Columns (Miltenyi, cat. no. 130-042-401)
- QuadroMACS Starting Kit (LS) (Miltenyi, cat. no. 130-091-051)
- MACS MultiStand (Miltenyi, cat. no. 130-042-303)
- 40 µm filter (VWR, cat. no. CA21008-949)
- 15 mL conical tube (Froglab, cat. no. TB15-500)
- 50 mL Falcon tube (Froglab, cat. no. TB50-500)
- MACS buffer

Mass spectrometry

- Sodium Chloride (0.9% normal saline) (Fisher Scientific, cat. no. Z1376)
- EppendorfTM Snap-Cap Microcentrifuge Safe-Lock TubesTM (Fisher scientific, cat. no. 05-402-25)
- HPLC grade methanol (Fisher Scientific, cat. no. A456-4)
- Optima Water (Fisher Scientific, cat. no. W6-4)
- Optima Acetonitrile (Fisher Scientific, cat. no. A9554)
- Optima Formic Acid (Fisher Scientific, cat. no. A117-50)
- Mobile Phase A (MPA): Prepare a 1 L solution of 0.03% Formic Acid in Optima grade Water (VWR Scientific cat. No. BJ365-4). Mobile Phase B (MPB): Prepare a 1 L solution of 0.03% Formic Acid in Optima grade Acetonitrile (VWR Scientific cat. No. BJAH015-4). Mobile Phases may be stored at room temperature when not in use. They should be replaced if there is a concern with contamination.

Equipment

- SpeedVac
- Vortex Mixer
- Liquid Nitrogen and Dewar
- Centrifuge and microcentrifuge operating at 4 °C

LC-MS

- Nexera Ultra High Performance Liquid Chromatograph (UHPLC) system (Shimadzu Corporation, Kyoto, Japan)
- Phenomenex Synergi Polar-RP HPLC Column (150 x 2 mm, 4 μ m, 80 Å) (cat. no. 00F-4336-BO)
- AB SCIEX QTRAP 5500 (Applied Biosystems SCIEX, Foster City, CA)
- Vials (MicroSolv Technology, cat no. 9502S-WCV)
- Inserts (MicroSolv Technology, cat. no. 9502S-02N)
- Caps (MicroSolv Technology, cat. no. 9502S-30C-B)
- Analyst 1.6.1 (Applied Biosystems, SCIEX, Foster City, CA)

LC-MS Equipment Setup (refer to step 57)

Standard acquisition program settings include:

- Set the ionization source temperature to 650 °C
- Set Ion Source Gas 1 and Ion Source Gas 2 to 50 psi.
- Set the Curtain Gas at 30 psi
- Set Entrance Potential at 10 V
- Set the positive mode IonSpray Voltage at 5000 V and the negative mode IonSpray Voltage at -1500 V.
- Include polarity switching between positive and negative mode with a dwell time of 3 msec for each metabolite transition.

Software

- MultiQuant 2.1 (Applied Biosystems, SCIEX, Foster City, CA)
- R

2.4.4 Procedure 3

Buffers and media

- Sterile PBS (Fisher Scientific, cat. no. SH3025601)
- FACS buffer (see REAGENT SETUP)
- Complete Media (Reagent Setup)

Flow cytometry

- CD3 monoclonal antibody (OKT3) functional grade (ThermoFisher, eBioscience™, cat. no. 16-0037-85)
- CD28 functional grade human (Miltenyi, cat. no. 130-093-375)
- IL-2 (Proleukin)
- 24-well plate
- Human Peripheral Blood Mononuclear Cells (PBMC) (STEMCELL Technologies, various formats)
- 96 well “U bottom” plate (VWR, cat. no. CA62406-121)
- Fc Receptor Binding Inhibitor Polyclonal Antibody, Functional Grade (ThermoFisher, eBioscience™, cat. no. 16-9161-73)
- Brilliant Stain Buffer (BD, cat. no. 563794)
- UltraComp eBeads Compensation Beads (Fisher, cat. no. 01-2222-42)
- FACS antibodies (Table 1)
- Round-bottom polystyrene test tubes (Fischer, cat. no. 08-771-23)

Equipment

- Centrifuge operating at 4 °C
- Multichannel pipette capable of pipetting 10-200 µL
- Cytex Aurora (3L 16V-14B-8R)

Software

- FlowJo (FlowJo is used for flow cytometry data analysis; <https://www.flowjo.com/>)
- GraphPad Prism 8 (<https://www.graphpad.com/scientific-software/prism/>)
- SpectroFlo (<https://cytekbio.com/pages/spectro-flo>)

2.4.5 Reagent Setup

Sample processing media (store at 4 °C)

- 90% RPMI (Fisher, cat. no. SH3002701)
- 10% Human AB serum, heat inactivated and filtered (Sigma, cat. no. H4522-100mL)

Freezing media (store at 4 °C for short term or -20 °C for long term)

- 80% RPMI (Fisher, cat. no. SH3002701)
- 20% DMSO (Sigma, cat. no. D2650-100mL)

FACS Buffer (store at 4 °C)

Prepare the buffer using:

- 97% Sterile PBS (Fisher Scientific, cat. no. SH3025601)
- 3% Human AB serum, heat inactivated and filtered (Sigma, cat. no. H4522-100 mL)
- Optional: 2 mM EDTA is recommended if the sample is prone to aggregates

Complete Media (store at 4 °C)

This medium is 0.22 µm filtered 50:50 (vol/vol) supplemented RPMI1640:AimV (store at 4 °C).

RPMI (Fisher, cat. no. SH3002701) is supplemented with:

- 10% Human AB serum, heat inactivated and filtered (Sigma, cat. no. H4522-100mL)
- 12.5 mM HEPES (Fisher, cat. no. SH3023801)
- 2 mM L-Glutamine (Fisher, cat. no. SH3003401)
- 1x Penicillin Streptomycin solution (Fisher, cat. no. SV30010)
- 50 µM B-mercaptoethanol (Sigma, cat. no. M3148-100mL)

AIM-V (ThermoFisher, cat. no. 12055-083) is supplemented with:

- 20 mM HEPES (Fisher, cat. no. SH3023801)
- 2 mM L-glutamine (Fisher, cat. no. SH3003401)

These two solutions are combined at a 1:1 ratio.

MACS buffer (store at 4 °C)

- Sterile PBS (Fisher Scientific, cat. No. SH3025601)
- 0.5% Human AB serum, heat inactivated and filtered (Sigma, cat. no. H4522-100 mL)

Optional: 2 mM EDTA is recommended if the sample is prone to aggregates

2.5 Methods

2.5.1 Procedure 1: Establishing a team and workflow for patient enrollment, biospecimen/data collection, and storage of biospecimens

Assemble a team responsible for the enrollment of patients as well as the collection, processing, and storage of biospecimens

1. The specific team members that are needed depend on the expertise of the biobank team and research team. A biobank team might typically consist of the following people:

Person	Responsibilities
Consent nurse/study coordinator	▪ Patient engagement and consent ▪ Documentation ▪ Clinical department engagement and interactions ▪ Sample collection ▪ Coordination between biobank/operating room/pathologist/research team members.
Biospecimen technician	▪ Collection, handling, transporting samples, and documentation
Data manager	▪ Maintaining consent and biobank inventory databases ▪ Mining data from medical records ▪ Updating a secure study database with de-identifiable information

The research team would then consist of:

- Clinicians and healthcare staff identify the patients, introduce the study, and perform the surgery and fluid drainages.
- Pathologists determine availability of biospecimens for research.

- Health care staff including secretaries/medical office assistants (responsible for providing the referral information to the biobank), pre-surgical clinic department clerks, surgical booking department clerks, nurses, and operating room staff.
- Laboratory researchers responsible for the processing and storage of the biospecimens.

CRITICAL STEP If not partnering with a biobank, the responsibilities of the biobank team will need to be assigned to research team members.

Establish team member roles and ensure proper documentation is in place

CRITICAL This is essential in order to conduct research activities within health facilities (e.g., consenting patients or collecting biospecimens).

2. Contact the relevant surgeons and pathologists to introduce the study and discuss feasibility and operational issues. Set a start date once all relevant approvals have been obtained (including research ethics approval – step 5).
3. Obtain permission from the relevant departments for the study. This permission may involve a formal research resources request (e.g. Material and Data Transfer Agreements) and review process submitted to the hospital, individual clinical departments, or the department heads.
4. Organize clinical team meetings to provide the necessary information to the following:
 - Pre-surgical clinic department staff: patients attend appointments to complete all pre-surgical consults and tests; biobank schedules to meet that patient at this appointment.
 - Surgical booking department clerks: note on operating room slate that the patient is a biobank participant to inform the operating room and pathology department on the day of surgery.
 - Operating room staff: responsible for notifying the biobank that the patient is in the operating room and when samples have been sent to the pathology department; documenting pre-analytical time points; sample collection and transport to pathology.
 - Pathology: convey how and why samples are collected.

- Accessioning staff: ensure samples marked for biobanking are immediately sent to the pathology gross room for processing.

Research ethics board submission development

! CAUTION Both the specific research study and the biobank required human subjects research ethics approval to enroll patients into the research study and operate with human biospecimens and data. A biobank may not require ethics review in all jurisdictions.

5. Consider the steps related to the patient recruitment protocol:

- Select appropriate inclusion and exclusion criteria. Our study inclusion criteria included: people with a female reproductive system with a diagnosis of ovarian cancer, any stage or grade of disease, any age, any ethnic background, undergoing surgery as part of their treatment. Our study exclusion criteria included: HIV-positive patients.

CRITICAL STEP Identifying the inclusion criteria for your study is dependent on the experimental question and sample size needed to achieve statistical significance. Appropriate exclusion criteria protect the safety of the participant as well as the team member collecting potentially biohazardous and cytotoxic material.

- Ensure that the consent form adheres to all ethical, legal and regulatory requirements. In Canada this includes: invitation to participate, voluntary participation, what is involved in participating, types of biospecimen collections, types of data collected, benefits and risks, privacy and confidentiality, who will have access to samples and data collected and withdrawal of consent.
- Consider the referral and consent processes.

6. Consider the steps relating to data collection/privacy and confidentiality:

- Data collection, storage, and access.
- Collaborations and sample/data transfer.
- Separate databases to store identifiable data (biobank personnel) and de-identified data (research personnel).
- Implementation of a coding system to de-identify samples and data.
- Declaration that the data remains identifiable for the duration of the study.

CRITICAL STEP This is necessary to be able to link collected samples to clinical outcomes and to specific patients, should a patient choose to withdraw from the study.

Identification and enrollment of eligible patients.

CRITICAL Our processes were conducted in conformity with the Canadian national biobanking standards and followed the Canadian Tissue Repository Network (CTRNet) Participant Recruitment into a Tumor Biobank Program standard operating procedure with the following considerations and steps:

7. Recruit eligible patients. Our workflow, which involves the Biobank, is as follows:
 - Eligible patients are identified, introduced to the study and referred to the biobank by their surgeon (mostly gynecological surgeons).
 - Consent nurse contacts, generally by telephone, the eligible patient and conducts a preliminary consent interview to provide basic study details and determine patient interest in knowing more about the study. It concludes with the scheduling of an informed consent discussion if the patient is interested.
 - An informed consent discussion appointment (to determine the patient's decision) is scheduled with the patient to take place during their hospital pre-surgical clinic appointment. The Subject Information and Consent form is reviewed.
 - A patient may be admitted to the hospital prior to surgery so the consent discussions may take place in their hospital room.
 - Document patient engagement including interactions and the consent decision.
 - Consented study patients may have a copy of the signed signature page added to their medical chart.
 - The biobank will inform the referring clinician of the patient's decision to participate and then proceed to coordinate sample collections for consented patients.

CRITICAL STEP Organizing to meet with the patient during the pre-surgical clinic appointment is a convenience for the patient as they do not need to attend a second appointment for an informed consent discussion.

Data collection of sample information

CRITICAL Data is collected from referral information, sample collections, surgical records, pathology reports, clinician notes, tests, and procedure reports.

8. Put a procedure in place to collect the following data from the surgical and biobanking documents:
 - Date-time points relevant to biospecimen collection: surgery start time, anesthesia time, time of first incision, time sample removed from body, time sample received at pathology department.
 - Ascites: volume, gross appearance (e.g. clear, bloody), colour, time, and amount of heparin added.
 - Tissue: tissue site, weight, and time collected into PBS.

CRITICAL STEP It is important to try to collect and stabilize/preserve biospecimens in as short a time as possible in order to minimize surgical warm (period from initial vascular disturbance to removal from body) and cold (period from removal from body to preservation by freezing, fixation, or other stabilization method) ischemia times. The warm ischemia time cannot be changed for research needs as this is determined by anaesthesia and surgical requirements, but it can be documented. The cold ischemia time can be reduced and minimized by facilitating transport and supporting the pathologist to rapidly designate a portion of a biospecimen as available for research, and this time can also be documented.

9. Put a procedure in place to obtain clinical data from the patient medical records; this can also apply to long-term follow-up. This can include (but is not limited to): date of diagnosis, date of surgery, age at surgery, pathology (tumor site, stage, grade, histology, chemo regimen and time-points, platinum response status, CA-125 lab results and outcomes.

Establish a database for confidential storage of de-identified sample information

! CAUTION Only biobank team members should have access to identifiable study information. Research team members should be restricted to coded materials (data and biospecimens). This is

important to meet the commitment in the consent form to minimize the risk of loss of privacy for participating patients.

CRITICAL Using a database system (as opposed to spreadsheets) allows for enhanced security measures, mitigates the risk of duplicate data entries, and reduces the risk of input error while being critical for tracking the pre-analytical variables associated with patient and sample data and replicating the research methodology. We use the CTRNet Advanced Tissue Management Application (ATiM; <https://www.ctrnet.ca/en/resources/biobanking-software/>). Several general use commercial (e.g. Microsoft Access, REDCap) or dedicated biobanking (e.g. CTRNet ATiM) database software is available.

CRITICAL All databases should have a back-up system in place.

10. To implement a database for de-identified data, consider the following points:

- A study code number system is applied by biobank team members to study patient samples and data that the research team will use.
- The biobank data manager will populate a study database with non-identifiable data by annotating coded study records with information from medical records.
- Data is stored on secure servers that are protected by institutional network security measures and require login details (authenticated username & password).
- Samples and data will be stored indefinitely, and the measures taken by the study and the biobank to ensure security and integrity of samples and data are reviewed annually by the research ethics board.

Here we will discuss the collection of tumor and ascites biospecimens (Figure 4. a).

Prior to the day of surgery

11. Check that the various forms used to collect standardized information for the purpose of annotating tumor and ascites biospecimens are ready.
- Hospital Clinical Form (generated and remains at the hospital): The nurses record the required information in the operating room. The form accompanies the biospecimens to the pathology gross room where the biobank staff will document that research consent was obtained and the biospecimen was collected.
 - Biobank Tissue Handover: Completed by the biobank staff in the pathology gross room and stored by the biobank.

- Biobanking Packing Slip: is completed in the pathology gross room by the biobanking staff and travels with the biospecimens to the research laboratory. A copy of the packing slip remains with the biospecimens for the research team and the original is stored by the biobank.
 - The Ascites and Tissue Processing Summary Report forms are initiated, completed, and stored by the research team.
12. Contact the operating room nurse coordinator to ensure there is a supply of suction regulator canisters and tubing for sterile ascites collections. Time will be required to source and obtain the equipment.
 13. Work with the operating room nurse coordinator to add timepoints related to biobanking collections to the Hospital Clinical Form. We have found that the most reliable timepoints that allow a consistent calculation of overall ischemia time are: “Anesthesia Ready Time”, “Incision Time”, and “Removal of Resected Specimen Time” (refer to step 8).

Start of clinical hours on the day of surgery **Timing** ~ 30 minutes

CRITICAL Not all surgeries begin on-time so active communication is required between the biobank, clinical departments, and the research team. The biobank and research teams need to be flexible.

14. Confirm with the surgical desk that:
 - The time of surgery is on the operating room slate.
 - The patient is marked as a biobank participant.
 - Ascites and tumor will be sent to the pathology department.
 - Biobank staff will be notified at the start of surgery and when the biospecimens have been sent to the pathology department.
15. Contact the on duty pathologist to inform them that this surgery may send ascites and/or tumor and that biobank staff will be in attendance in the pathology department to assist with biospecimen collections.

CRITICAL STEP Ask the pathologist to alert the biobank staff if a biospecimen should arrive before them.

16. Contact the research staff (responsible for processing the biospecimen) to inform them of the surgery and the potential to be provided biospecimens today.

1 hr before surgery start **Timing** ~1 hour

17. Prepare for biospecimen collection:

- For tumor collection: Aliquot ~30 mL of sterile PBS into a 50 mL centrifuge tube (one per tumor site). Record the weight of the tube with the PBS and store the aliquots at 4 °C.
- Pack a hard sided cooler with all equipment and reagents for tumor and ascites collections (see Materials, Procedure 1, Human sample collection).

18. Arrive at the pathology department 30 minutes after surgery has started to be available alongside the pathologist to collect the biospecimens as they arrive.

! CAUTION For safety, wear personal protective equipment including a gown, gloves, and eye protection.

During surgery **Timing** ~1.5 hours

CRITICAL The pathologist determines if the biospecimen can be released for research. This ensures that the biospecimen is primarily available for patient diagnosis and care and enhances the quality of the research material selected.

19. Follow the steps in options A and B for ascites and/or tumor collection respectively.

A Ascites:

- i. Transfer the ascites dedicated for the biobank into the 1 L containers.

CRITICAL STEP The volume of ascites required depends on the patient and the research question. We recommend assessing the cell density and the proportion of the cell population of interest using flow cytometry to ensure that sufficient sample has been collected.

- ii. Add heparin to the ascites (2.0 mL heparin/1000 mL ascites) using a syringe and needle.

! CAUTION For safety, do not recap the needle, dispose of the syringe/needle in a sharps container.

- iii. Label ascites containers with the study code number. The study code number is created by the biobank to de-identify patient sample
- iv. Place 1 container and 4 absorbent sheets per large biohazard bag and seal.
- v. Place next to the frozen ice packs inside the hard sided cooler.

B Tumor:

- i. Once the solid tumor has been received, ask the pathologist to use the sterile tumor collection tool kit and the kit's internal sterile pouch surface to place and examine the tumor.

CRITICAL STEP Sterile tools and work surface are provided by biobank as they are not a clinical requirement.

CRITICAL STEP The use of the pouch surface ensures that there is no contact between the current biospecimen and cellular material from previous specimens processed on the pathologist's work surface. This is important to avoid cross contamination of cellular material from another patient biospecimen.

- ii. Request homogenous, non-necrotic piece(s) of tumor from each tumor site received.

CRITICAL STEP Collect several tumor pieces of 1 cubic cm from each tumor site when possible. This size is simple to manipulate in downstream processing.

CRITICAL The amount of tumor tissue required is dependent on the composition of the tumor tissue and the cell population of interest. For example, we performed immunohistochemistry staining on tumor specimens and selected those that contained elevated immune infiltrates.

- iii. Place the total tissue of one type (e.g. ovarian, omentum) in one tube that was prepared with PBS. Weigh the tube. The difference between this weight and the initial weight in step 17 is the total weight of all the pieces for the one type of tumor.
- iv. Label the tube with the study code number, tumor site, and weight of tumor sample.
- v. Place the tubes in a biohazard bag with absorbent sheets, seal and place in the hard-sided cooler next to the cold packs.

! CAUTION For safety, use forceps or a blade removal box to safely remove the blade from the scalpel and dispose of the blade in a sharps container. Place tumor collection kit tools in a small biohazard bag inside the hard sided container labeled “Used Medical Equipment” during transport.

20. Finalize biospecimen collection.

- Ensure the Biobank Tissue Handover and Biobank Packing Slip are completed.
- Contact the research team to notify them that a biospecimen has been collected and communicate the time it will arrive at the research laboratory.
- Once at the research laboratory, store the biospecimens at 4 °C along with the Biobank Packing Slip.
- Notify the research team that the biospecimens have arrived.
- Record all biobank collection parameters into the database system so that biospecimen collection and release details are traceable.

Preservation and storage of intra-operative specimens and ascites

21. Cryopreservation of tumor and ascites: obtain the Ascites Processing Summary and/or the Tissue Processing Summary forms prior to preservation and storage of biospecimens. Then follow the steps in options A and B for preserving Tumor and ascites specimens respectively.

A Tumor:

- i. Prepare a biological safety cabinet for work.
- ii. Record all notable observations and fill in any associated forms.
- iii. Obtain a sterile Petri plate. Remove lid and place upturned. Pour off the media in which the tumor tissue is stored into the bottom of the Petri plate. The used media can be discarded.
- iv. Into the upturned lid of the Petri plate, slowly slide out the tumor tissue. Discard the container in the waste bag.
- v. Using two disposable scalpels, carefully mince tumor tissue in a scissor-like action. The tumor tissue should be in approximately 0.2 cm² pieces (or resemble minced garlic).

- vi. Cut off the tip of a sterile transfer pipette at the narrowest tip.
 - vii. Place a 100 μm sterile cell strainer in the reserved Petri plate bottom. Use the sterile transfer pipette to place the tumor in the strainer.
 - viii. Using the plunger from a sterile 3 mL syringe, push the tumor fragments through the cell strainer until the entire tumor has been disaggregated.
 - ix. Rinse the cell strainer with 5 mL of sample processing media.
 - x. Transfer the disaggregated tumor suspension from the Petri plate bottom to a fresh 50 mL Falcon tube. To maximize yield, rinse the Petri plate bottom with 5 mL of sample processing media and pool in the Falcon tube with the tumor suspension.
 - xi. Centrifuge the filtrate at 500 g for 10 min at 4 °C.
 - xii. Decant the supernatant into the waste container so as to not disturb the pellet.
 - xiii. Resuspend the pellet in sample processing media and count cells. Prepare cells for storage. Freeze cells at a minimum of 2×10^5 cells/vial and maximum of 1×10^7 cells/vial.
 - xiv. Centrifuge the cells at 500 g for 10 min at 4 °C.
 - xv. Decant the supernatant into the discard beaker so as to not disturb the pellet.
 - xvi. Resuspend the cells in filtered heat inactivated human serum. Pipette up and down 3x to completely resuspend the pellet.
- CRITICAL STEP** If sample freezing is not required, resuspend the sample in processing media and proceed to Procedure 2, Step 25.
- xvii. Dropwise add an equal volume of freezing media (80% RPMI, 20% DMSO) to the cells/human serum mixture. Gently mix by pipetting up and down 2x.
 - xviii. Aliquot 1 mL of cells per cryovial.
 - xix. Immediately transfer cryovials to a Mr. Frosty (or acceptable freezing container) and store at -80 °C for a minimum of 12 hrs and maximum of 24 hrs.
 - xx. Transfer the cryovials to a liquid nitrogen freezer for long-term storage.

PAUSE POINT These can be stored in liquid nitrogen vapor indefinitely.

B Ascites:

- i. Prepare a biological safety cabinet for work.
- ii. Record all notable observations and fill in any associated forms.

- iii. Remove visible pieces of tissue with a pipette and discard.
 - iv. Divide ascites evenly into appropriately sized centrifuge tubes (50 mL, 250 mL, or 500 mL).
 - v. Centrifuge the cells at 500 g for 10 min at 4 °C.
 - vi. If desired, harvest the supernatant and store at -80 °C.
- CRITICAL STEP** It is useful to do this if you are interested in complementing intracellular profile with that of secreted metabolites.
- vii. Very slowly, decant any remaining supernatant into the waste.
 - viii. If the pellet is “bloody”, perform ACK lysis. Otherwise continue to the steps below.

- Resuspend the cells in 5 mL (pellet is <3 mm height from the bottom of the tube) or 10 mL (pellet is 3-6 mm from bottom of the tube) of ACK lysis buffer. Scale accordingly if required.
- Let stand for no more than 5 minutes at room temperature.
- Dilute cell suspension in an equal volume of sample processing media (equal volume to the ACK lysis buffer added above).
- Centrifuge cells at 500 g for 10 min at 4 °C.
- Pellet cells and discard the supernatant.
- If the pellet remains bloody, repeat the ACK lysis.

CRITICAL STEP The presence of red blood cells can decrease the accuracy of cell counts in subsequent steps. ACK lysis should be performed if the pellet is red or the ascites fluid was visibly bloody.

- ix. Resuspend the pellet in sample processing media and count cells. Freeze cells at 1×10^7 cells/mL or up to 5×10^7 cells/mL.
- x. Centrifuge the cells at 500 g for 10 min at 4 °C. Discard supernatant.
- xi. Resuspend the cells in filtered heat inactivated human serum. Pipette up and down 3x to completely resuspend the pellet.

CRITICAL STEP If sample freezing is not required, resuspend the sample in processing media and proceed to Procedure 2, step 25.

- xii. Dropwise add an equal volume of freezing media (80% RPMI, 20% DMSO) to the cells/human serum mixture. Gently mix by pipetting up and down 2x.

- xiii. Aliquot 1 mL of cells per cryovial.
- xiv. Immediately transfer cryovials to a Mr. Frosty (or acceptable freezing container) and store at -80 °C for a minimum of 12 hrs and maximum of 24 hrs.
- xv. Transfer the cryovials to a liquid nitrogen freezer for long term storage.

PAUSE POINT These can be stored in liquid nitrogen vapor indefinitely.

22. Ensure the Ascites Processing Summary Report and/or the Tumor Sample Processing Summary Report is completed.

23. These preserved single-cell suspensions were thawed for metabolomic and metabolite assays described below.

! CAUTION Some metabolites are known to be affected by long term storage.

2.5.2 Procedure 2: Cellular enrichment and metabolite profiling by mass spectrometry

CRITICAL For cellular enrichment and metabolite profiling by mass spectrometry (Figure 4. b), first we will discuss the disaggregation and bead-based protocols used to isolate lymphocytes from tumors and ascites (Figure 5). Then, we will discuss the specific extraction methods and preservation prior to MS analysis (Figure 6), the main MS approaches for metabolite detection including internal standards and QC/QA. Finally, we will describe the analytic and software packages for MS data analysis including normalization strategies and statistical analysis.

CRITICAL Here we use cryopreserved samples. However, we recommend performing the following steps including metabolome extraction on fresh specimens if possible (41).

Sample preparation **Timing** 30 minutes

24. Thaw cryopreserved patient samples. To do this,

- Thaw cryovial in a 37 °C water bath. Immediately after thaw add the contents dropwise to 10 mL of cold complete media in a 15 mL conical tube.
- Centrifuge cells at 500 g for 5 min at 4 °C.
- Resuspend samples in 10 mL of cold complete media.

CRITICAL STEP Use cold complete media and buffers to minimize metabolic changes.

25. Filter the cells.

- Place a 40 μm filter on top of a 50 mL conical tube.
- Place the tip of a 10 mL serological pipette against the 40 μm filter and gently pipette cells through the filter.
- Rinse the filter with an additional 10 mL of cold complete media.

CRITICAL STEP Filtered cells are critical for cellular enrichment. Specimens may have cell aggregates that could clog the column.

26. Spin cells at 500 g, pour off supernatant, and resuspend in 5 mL of MACS buffer.

27. Count cells and record cell number and viability.

CRITICAL STEP Keep cells on ice to minimize metabolic changes.

28. Set aside an aliquot of cells for analysis by flow cytometry on ice (refer to Procedure 3).

Typically, we set aside a minimum of $\sim 30,000$ cells, or ideally 0.5×10^6 - 1×10^6 cells per sample for flow cytometry.

T cell and tumor cell isolation **Timing** 1-4 hrs

CRITICAL Proceed immediately to T cell isolation after sample preparation to minimize metabolic changes due to sitting on ice.

CRITICAL Keep all solutions cold and tubes on ice to minimize changes in metabolism.

CRITICAL Cell aggregates can clog columns. In addition to filtering samples, we use an LS column instead of an MS column to minimize problems.

29. Choose which population of cells you are interested in enriching. The choice of enriched population depends on the experimental question.

30. Perform an initial flow cytometry experiment to determine the proportion of cells that display the extracellular marker of interest.

CRITICAL STEP The purpose of this step is to check that the enrichment step will result in enough cells for mass spectrometry analysis. The minimum number of cells required for mass spectrometry analysis will depend on a number of factors including the instrument used and the metabolites of interest. Here, we aim for a minimum of 100,000 cells.

31. Isolate your chosen cell populations. We describe here steps for isolating CD8⁺, CD4⁺ and CD45⁻ cells. Other extracellular markers can also be used depending on the cell

population of interest. Follow the steps in Option A if care needs to be taken that cells are not wasted, and option B in cases where cell numbers are not limiting (Figure 5). The general approach used in option B is better if there are enough cells, because the total processing time is shorter.

CRITICAL STEP Perform isolations in triplicate to increase confidence of downstream statistical analysis of metabolic data.

A Sequential method (Fig 2. a): Timing ~1-2 hours

- i. Centrifuge 5 mL cell suspension at 500 g for 5 min at 4 °C.
- ii. Remove supernatant and resuspend in 80 µL of MACS buffer per 10⁷ cells.
- iii. Add 20 µL of CD8+ antibody mix per 10⁷ cells.
- iv. Incubate at 4 °C for 15 minutes.
- v. During incubation prepare LS columns in MACS MultiStand for isolation
 - Label 50 ml tube for negative isolation (CD8-) and 15 mL tubes for positive isolation (CD8+).
 - Place the negative isolation tube under the column.
 - Wash column with 3 mL MACS buffer.
- vi. After 15 minutes, add in 1-2 mL of cold MACS buffer per 10⁷ cells to each cell suspension tube.
- vii. Centrifuge cells at 500 g for 5 min at 4 °C.
- viii. Resuspend each cell pellet in 0.5 mL of MACS buffer and pipette the entire cell suspension into the LS column.
- ix. Wash LS columns with 3 x 5 mL MACS buffer.

CRITICAL STEP Wash column within 5 minutes of adding cell suspension to avoid dehydration of the column.

- x. Remove columns from the magnet and place on top of CD8+ 15 mL tube.
- xi. Add 5 mL of MACS buffer and use the plunger to push the solution through the column, eluting the CD8+ population.
- xii. Collect CD8- flow through fraction in the 50 mL tube and keep for sequential separations.

CRITICAL STEP This is kept for isolation of CD4 and CD45- populations do NOT discard.

- xiii. Centrifuge CD8- cells at 500 g for 5 min at 4 °C.
- xiv. Repeat separation protocol (ii-xi) with CD4 magnetic beads.
- xv. From the CD4- separate CD45+ and CD45- cells by repeating separation protocol (ii-xi) with the CD45 magnetic beads.
- xvi. Collect CD45- fraction and keep for metabolome extraction.

CRITICAL STEP This is the tumor fraction, do NOT discard.

B Direct method (Fig 2. b): Timing ~35 minutes

- i. Divide the 5 ml cell solution into three tubes labeled for the enrichment populations of interest: CD8+, CD4+ and CD45-.
- ii. Centrifuge cells at 500 g for 5 min at 4 °C.
- iii. Remove supernatant and resuspend in 80 µL of MACS buffer per 10⁷ cells.
- iv. Add 20 µL of the appropriate antibody mix (CD8+, CD4+ and CD45-) per 10⁷ cells to the corresponding tube.
- v. Incubate at 4 °C for 15 minutes
- vi. During incubation prepare LS columns in MACS MultiStand for isolation.
 - Label 50 ml tubes for negative isolation (CD8-, CD4-, CD45-) and 15 mL tubes for positive isolation (CD8+, CD4+, CD45+).
 - Place negative isolation tubes under their respective columns.
 - Wash column with 3 mL MACS buffer.
- vii. After the 15 minute incubation, add 1-2 mL of cold MACS buffer per 10⁷ cells to each cell suspension tube.
- viii. Centrifuge cells at 500 g for 5 min at 4 °C.
- ix. Resuspend each cell pellet in 0.5 mL of MACS buffer and pipette the entire cell suspension into LS column.
- x. Wash LS columns with 3 x 5 mL MACS buffer.
- xi. Collect CD45- fraction and keep for metabolome extraction.

CRITICAL STEP This is the tumor fraction, do NOT discard.

- xii. Remove CD4 and CD8 columns from the magnet and place on top of their respective 15 mL tube.
 - xiii. Add 5 mL of MACS buffer and using the plunger, push solution through the column, eluting the positive population (CD4+ and CD8+).
32. Count cells from each of the desired cell fractions and record cell number and viability.
33. Set aside an aliquot of cells for analysis by flow cytometry to confirm purity and yield.
Typically we set aside a minimum of 50,000 cells to confirm the enrichment by flow cytometry.
34. Aliquot cells for mass spectrometry in 1.5 mL Safe-Lock™ eppendorf tubes and process immediately. Process samples in triplicate.
- ! CAUTION** Use tubes with snap-cap capabilities to prevent the cap from bursting when the samples are frozen in LN2.

Metabolome Extraction **Timing** 6 hours (Figure 6. a)

35. Prepare 0.9% saline solution cooled at 4 °C, 80% methanol cooled at -80 °C, and LN2.
- CRITICAL STEP** The choice of extraction solvent might depend on the research question.
36. Top up samples with ~1 mL of ice-cold 0.9% saline solution.
37. Centrifuge cells at 500 g for 5 min at 4 °C.
38. Aspirate supernatant and rinse the cells with ~1 mL of ice-cold 0.9% saline solution.
39. Centrifuge cells at 500 g for 5 min at 4 °C.
40. Aspirate supernatant.
- CRITICAL STEP** Be sure to aspirate as much of the media off as possible. Any remaining salt that is left in the sample can cause competing ionization during injection on the mass spectrometer.
41. Quickly add 1 mL of 80% methanol (cooled to -80 °C).
- CRITICAL STEP** The detection of different classes of metabolites may require other extraction methods that the researcher should consider.
42. Pipette briefly to resuspend the cell pellet.
43. Snap freeze in liquid nitrogen.
- PAUSE POINT** Store samples at -80 °C.

44. Subject samples to three freeze-thaw cycles between liquid nitrogen and 37 °C. We recommend using a small liquid nitrogen dewar and water bath at 37 °C.

CRITICAL STEP After the third thaw, pop the lids to relieve pressure prior to proceeding to the following step.

45. Vortex for 1 min at maximum speed.

46. Centrifuge at 20,000 g for 15 min in a refrigerated centrifuge.

47. Transfer the metabolite-containing supernatant to a new Eppendorf tube. Leave ~100 µL in the tube to ensure the pellet is not transferred.

48. Keep the protein pellet to perform protein quantization

- If there is a sufficient sample available, perform a BCA assay to quantify the amount of protein in each sample. This provides an alternative method for sample normalization to cell number.
- SpeedVac the sample to evaporate the remaining methanol and follow manufacturer instructions to perform the BCA assay.

49. SpeedVac the tube containing the supernatant (no heat) until there is no liquid is remaining. **Timing** It takes approximately 4-5 hr to dry 1 mL of methanol. Samples can be dried overnight.

PAUSE POINT The dried metabolite pellet can be kept at -80 °C for one to two weeks.

Sample Preparation for LC-MS/MS **Timing** 20 minutes (Figure 6. b)

CRITICAL Expertise in using mass spectrometry instrumentation is required.

50. Resuspend the metabolite pellet in 100 µL of 0.03% Mobile Phase A (MPA).

51. Vortex thoroughly.

52. Centrifuge at 20,000 g for 10 mins.

53. Transfer to a LC insert with a spring.

! CAUTION – Do not transfer any remaining debris or protein pellet into the LC insert. This could result in clogging the chromatographic column or contamination in the mass spectrometer. If there is visible debris, we recommend that the samples are centrifuged one more time before transfer to the LC insert.

54. Place the LC insert in a LC vial and cap.

Chromatographic Separation and Detection by Mass Spectrometry (MS) **Timing** 28 minutes

55. Ensure that the LC-MS instrument and acquisition method are set up. For our experiments we:

- Install a Phenomenex Synergi Polar-RP HPLC Column (150 x 2 mm, 4 μ m, 80 Å) into the UHPLC system.
- Use 0.1% formic acid in water (Optima grade) for Mobile Phase A (MPA) and 0.1% formic acid in acetonitrile (Optima grade) for Mobile Phase B (MPB).
- Program the following gradient and flow rate for metabolite elution:

Time (mins)	% MPB	Flow Rate (mL/min)
3.00	0	0.5
15.00	100	0.5
17.00	100	0.5
17.10	0	0.5
20.00	Stop	

CRITICAL STEP The phase of chromatography (Normal Phase or Reverse Phase) chosen should be based on the nature of the analytes of interest. Here we use a reverse phase chromatography method that uses a non-polar stationary phase with a high organic mobile phase for analyte separation. Retention of analytes is based on their hydrophobicity and will elute off the column as they partition between the stationary phase and the increasing organic solvent composition of MPB with the more hydrophobic metabolites eluting at a later time in the gradient.

56. Maintain the autosampler at 4 °C and the column compartment at 35 °C.

57. For the mass spectrometer:

- Set the ionization source temperature to 650 °C and the Ion Source Gas 1 and Ion Source Gas 2 both to 50 psi.

- Set the Curtain Gas at 30 psi, the Entrance Potential at 10 V, the positive mode IonSpray Voltage at 5000 V, and the negative mode IonSpray Voltage at -1500 V.
 - Include polarity switching between positive and negative mode with a dwell time of 3 msec for each metabolite transition.
58. Prepare 1 ppm quality control samples from a standard mix stock.
- CRITICAL STEP** Other quality control samples can also be used (e.g. plasma stock, see experimental design section).
59. Load LC vials into autosampler and build sample queue in Analyst 1.6.1 software. Run quality control samples first to verify the performance state of the mass spectrometer.
- CRITICAL STEP** Make sure to check sample names, acquisition methods, file paths, and injection volumes (20uL).
- CRITICAL STEP** After the QC has been run and the status of the instrument is verified, run a single injection of MPA known as a 'Blank,' followed by the samples. It is recommended that the sample order be randomized.
60. Acquire LC-MS sample data.
- In the LC-MS method used here, a total of 204 metabolites are monitored by MRM - with 134 specific to positive mode and 70 specific to negative mode. Metabolites are identified by unique retention time and unique MRM transition(146).

CRITICAL Sufficient numbers of cells are required to obtain a clear signal for each metabolite. The sensitivity of the instrument needs to be determined prior to analysis.

Data analysis **Timing** 1-3 days

61. Using MultiQuant 2.1 review the acquired data and complete the peak area integration.
- Select peaks that are:
- Above baseline with a gaussian distribution.
 - Have an intensity value in the dynamic range of the instrument.
 - Are within 0.3 min of the theoretical retention time of the pure compound.
62. Export raw peak areas from MultiQuant and normalize by the Total Ion Count (TIC).
- CRITICAL STEP** Further normalization by protein amount or cell count may also be performed.

Bioinformatic and statistical analysis of metabolites

63. Further data analysis will vary depending on the precise experimental design. In general, if multiple samples are taken from the same patient (in this case different cell types, from different locations, in different patients), then a so-called ‘hierarchical’ analysis may be required to account for patient and/or location-specific effects. An example of the core analysis we performed can be found at https://github.com/vicDRC/BCCJL01_ovarian or (Appendix 1).

CRITICAL If designs differ substantially from this, it is advisable to consult a statistician.

2.5.3 Procedure 3: high-dimensional flow cytometry for metabolic profiling

Here we will describe the use of multi-spectral flow cytometry methods for evaluating T cell subset and phenotypes, alongside surrogate metabolic markers (Figure 4. c).

CRITICAL Expertise in using flow cytometry instrumentation is required.

CRITICAL We have adapted manufacturer protocols and added additional controls to ensure proper analysis of human biospecimens. If these adaptations conflict with proper operation and analysis of reagents and equipment please use the manufacturer’s recommended procedures.

CRITICAL Staining of extracellular proteins, including metabolic dyes, must be done on the same day the samples are thawed.

CRITICAL These protocols are optimized for up to 1 million cells/well.

CRITICAL The following concentrations have been optimized for the equipment available and may not apply to equipment available in other labs. Optimize all dilutions accordingly.

CRITICAL We recommend using a multichannel pipette for accuracy and efficiency.

CRITICAL Plan a trial experiment where you check that the procedure works, and whether any steps need to be optimised.

CRITICAL If additional markers are required, we recommend that users optimize the panel accordingly.

Prepare control samples

64. The cells for analysis will be stained in a 96 well plate. Decide what samples and controls you will be analysing. These are controls and samples that we typically use (Figure 7):

- Single colour control beads: we use UltraComp eBeads for reference controls for antibodies.
- Single colour control cells: we use a mixture of cells for single colour controls of dyes including MitoTracker Deep Red (MT DR) and viability to ensure the representation across all cell types.
- Unstained cells: are required as a negative control for acquiring usable data. We use a combination of cell types to ensure the representation of the strongest autofluorescence signal.
- Fluorescence minus one (FMO) controls: Using a mixture of cells, we stain the sample with every antibody minus one. FMOs support accurate gating of cell populations.
- Experimental samples: prepared in Procedure 2.
- Quality control samples: samples with known marker expression to aid in data analysis (i.e. PBMCs or cell lines).

CRITICAL The signal from the single colour beads must be the same or higher than that of the cell samples. If the cell samples have a greater signal, these must be used instead of the beads for the single colour controls.

CRITICAL For each FMO and single colour control sample we create a master mix of cells combining cell types to ensure representation across all markers. The cell types include mixtures of activated PBMCs, cell lines, and experimental samples.

CRITICAL Primary biospecimens contain unique cell phenotypes that can be difficult to predict. To ensure you gated accurately, run a quality control sample of PBMCs and cell lines separately to ensure you are able to gate for the predictable cell populations (i.e. quality control samples). For example, activating healthy donor PBMCs the day prior to the experiment to ensure there is a positive control for T cell activation markers such as CD137.

CRITICAL For the single colour viability control, add 50% of the cells to a heat block at 70 °C for 5 min prior to staining to ensure there are dead cells for a clear positive control.

Prepare control cells: Activated PBMCs **Timing** 2 hours

CRITICAL This should be done 1 day prior to analysis.

CRITICAL Ascites and tumor samples can have unpredictable cell populations. To ensure accurate gating, prepare activated PBMCs for gating controls.

65. Mix 2.5 μ L of OKT3 (functional grade 1 mg/ml) with 1 mL of PBS and plate 1 mL/well of a 24 well plate.
66. Incubate at 37 °C for 2 hours or 4 °C overnight.
67. Wash the plate twice with 1 mL of media or PBS.
68. Plate 2 mL of human PBMCs at 2 million cells/well with 300 U/mL IL-2 in T cell medium.
We use RPMI1640:AIM-V.
69. Stimulate cells overnight.

Plate cells for staining **Timing** 10 minutes

70. Prepare a master control cell sample for FMO and single colour control cells (step 64) using a mixture of activated (step 65-69) and non-activated PMBCs, and small amounts of all the experimental samples. The sample should have positive and negative populations for each marker to support correct gating.

CRITICAL Prepare enough for all single colour and FMO controls. Plan to plate cells at a minimum of 20,000 cells, but ideally 1 million cells/well

71. Staining can be carried out in a 96-well U bottom or V-bottom plate. Using the master control cell sample prepared in step 70, aliquot cells for single colour controls (i.e. unstained, viability, MitoTracker Deep Red) and FMOs. Plate control samples (i.e. activated PBMCs) and experimental samples (i.e. aliquoted from Procedure 2) (Figure 7).

Prepare stock solutions **Timing** 20 minutes

72. Prepare a working stock of MitoTracker Deep Red (MT DR). First, create a 500 nM solution in PBS in 1.5 mL Eppi tubes (scale as necessary, vortex well). Then create a 10 nM working stock by adding 1 μ L of the 500 nM MT DR solution to 49 μ L of complete media per well (scale as necessary).

CRITICAL STEP The concentration of MT DR needs to be optimized to ensure it is within range of the detectors on the Aurora. We recommend the following dilutions based on our optimizations.

CRITICAL STEP The complete media we use supports tumor and T cell growth. The type of media may have to be adjusted depending on the cell type analyzed. We used RPMI1640:AIM-V for our experiments. The concentrations of the dyes will need to be optimized in the respective media.

73. Prepare the viability dye by diluting 1:1000 in PBS (1 μ L of dye in 1000 μ L of PBS).

CRITICAL STEP If the viability stain needs to be carried out in a different buffer then optimize the dilution accordingly.

74. Prepare a master mix of Fc Block (5 μ L/well) and Brilliant Stain Buffer (10 μ L/well) in FACS buffer to a total volume of 25 μ L/well.

75. Prepare 2x concentrated antibody master mix in FACS buffer to a total volume of 25 μ L/well.

CRITICAL STEP Optimize the concentration of each antibody prior to analysis.

76. Prepare the antibody solutions that will be used for the FMO samples. This is done by preparing a 2x concentrated antibody master mix in FACS buffer to a total volume of 25 μ L/well with every antibody EXCEPT the antibody of interest (ie. FMO for CD8 will contain every fluorophore except the antibody for CD8).

CRITICAL These can be prepared in separate tubes, or separate wells of a 96 well plate.

Stain with MitoTracker Deep Red **Timing** 40-60 minutes

CRITICAL We recommend including a depolarised mitochondria control using CCCP in order to assess relative staining in the presence of an uncoupler(147).

77. Add 50 μ L/well of the prepared 10 nM MT DR for the respective single colour control wells and samples (prepared in step 72). Add 50 μ L complete media to the respective single colour control and FMO control wells.

78. Pipette to mix.

79. Incubate plate at 37 °C for 30 min.

80. Wash wells by adding 200 μ L of PBS per well and centrifuge plate at 500 g for 5 min at 4 °C. Flick plate to remove supernatant. Repeat twice.

CRITICAL STEP Repeat as necessary to remove dyes.

CRITICAL STEP The flick involves removing the plate lid and flipping the 96 well plate upside down over a bleach bucket with one swift and sure movement to remove the

supernatant. This step requires practice beforehand, improper technique can lead to poor washing or loss in cell numbers.

Stain for viability **Timing** <30 minutes

81. Add 50 μL of diluted viability dye to the respective wells (or PBS for the respective single colour and FMO control samples) and pipette to mix (prepared in step 73).
82. Incubate at 4 $^{\circ}\text{C}$ for 15 min.
83. Wash wells by adding 200 μL of FACS buffer per well and centrifuge plate at 500 g for 5 min at 4 $^{\circ}\text{C}$. Flick plate to remove supernatant. Repeat once.

Stain extracellular proteins **Timing** 60 minutes

84. Resuspend each well in 25 μL /well of the master mix of FC block and brilliant stain buffer in FACS buffer (prepared in step 74).
85. Incubate for 10 min at room temperature.
86. Add 25 μL /well of antibody master mix (prepared in step 75 and 76) to the respective samples and FMO controls (or FACS buffer for the respective single colour control samples) and pipette to mix.

CRITICAL The same master control cell sample (step 70) must be used across FMO samples for an accurate comparison.

87. Incubate plate at 4 $^{\circ}\text{C}$ (in fridge) for 20 minutes
88. Wash wells by adding 200 μL of FACS buffer per well and centrifuge plate at 500 g for 5 min at 4 $^{\circ}\text{C}$. Flick plate to remove supernatant. Repeat twice.
89. Resuspend cells in 200 μL of FACS buffer per well for analysis.
90. Transfer to appropriate tubes for analysis by flow cytometry (ie. round bottom polystyrene test tubes).

Prepare single colour controls **Timing** 15 minutes

CRITICAL Cytel provides guidelines for preparing single colour controls for the Aurora. Please refer to their guidelines. We used UltraComp ebeads for our single colour antibody controls. Please refer to manufacturer instructions. We adapted their protocol to prepare our single colour controls and the instructions are below.

91. Add 1 drop of UltraComp ebeads to a FACS tube (or well of a 96-well plate).
92. Add 1 μL of the respective antibody per tube (or well).
93. Incubate at 4 °C for 10 min.
94. Wash with 1 mL of PBS in tubes (or 200 μL in wells). Centrifuge at 500 g for 5 min at 4 °C. Flick to remove supernatant.
95. Resuspend in 200 μL PBS prior to analysis on the Aurora.

Run flow cytometry samples on the Cytex aurora **Timing** ~1-4 hours

CRITICAL Cytex provides extensive training on operating the Cytex Aurora. Please refer to their training modules for proper operation.

96. Run single colour controls.
97. Confirm correct emission spectra for each fluorophore.
98. Perform spectral unmixing using the SpectroFlo software (following manufacturer instructions) based on the positive and negative populations from single colour controls.
99. Collect data for FMOs, quality control samples, and patient samples.

PAUSE POINT Data can be analyzed at a later time.

Data analysis **Timing** ~1-3 days

100. Perform any extra compensation in the Cytex Aurora workspace.
101. Export FCS files and load into the FlowJo workspace.
102. Clean-up data by creating gates to remove doublets (on single cells) and to remove debris.
103. Gate populations of interest based on FMO controls.

CRITICAL Flow cytometry panel design should be confirmed with FMOs for all fluorophores with minimal variations across cell populations. For example, the percentage of T cells within the sample should not change despite the removal of one antibody or fluorophore.

2.6 Figures and tables

Table 1. Antibodies for flow cytometry

Fluorochrome	Marker	Catalogue number	Clone	RRID
PE	CD326 (EpCAM)	(ThermoFisher) 12-9326-42	1B7	AB_11044497
PerCP	CD8	(Biolegend) 301030	RPA-T8	AB_893421
PerCP-eFluor710	CD25	(ThermoFisher) 46-0257-41	4E3	AB_10548512
PE-Cy7	CD45RO	(ThermoFisher) 25-0457-42	UCHL1	AB_10718534
---	MitoTracker Deep Red	(ThermoFisher) M22426	---	
AF700	CD4	(Biolegend) 300526	RPA-T4	AB_493743
APC/Fire750	CCR7	(Biolegend) 353246	G043H7	AB_2750147
eFlour506	Viability	(ThermoFisher) 65-0866-14	---	
PO	CD45	(ThermoFisher) MHCD4530	HI30	AB_10376143
BV605	CD137	(Biolegend) 309822	4B4-1	AB_2565997
BV650	CD279 (PD1)	(Biolegend) 329950	EH12.2H7	AB_2566362
BV750	CD3	(Biolegend) 344845	SK7	AB_2734352

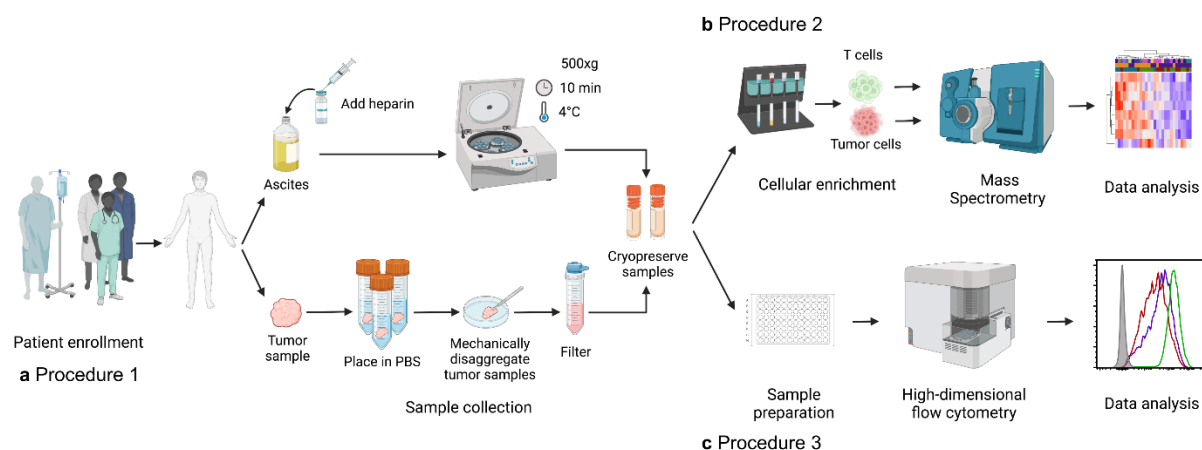


Figure 4. Overall workflow.

a, Procedure 1 – Establishing a team and workflow for patient enrolment and biospecimen/data collection and storage: obtain informed consent from patients. Clinical sample collection: obtain samples and store appropriately. Ascites and tumor samples are collected and processed separately. Heparin is added to ascites fluid and samples are centrifuged. Tumor tissues are mechanically disaggregated using a scalpel and filtered into a single-cell suspension. Both sample types are respectively cryopreserved in 50% heat-inactivated human AB serum, 40% RPMI-1640, and 10% DMSO. **b**, Procedure 2 - Cellular enrichment and metabolite profiling by mass spectrometry: ascites and tumor cell samples are separated by their respective cell types through magnetic bead enrichment. Metabolism of cell types are then analyzed via targeted mass spectrometry. **c**, Procedure 3 - High-dimensional flow cytometry for metabolic profiling: samples are stained and run on a flow cytometer. Created with BioRender.com.

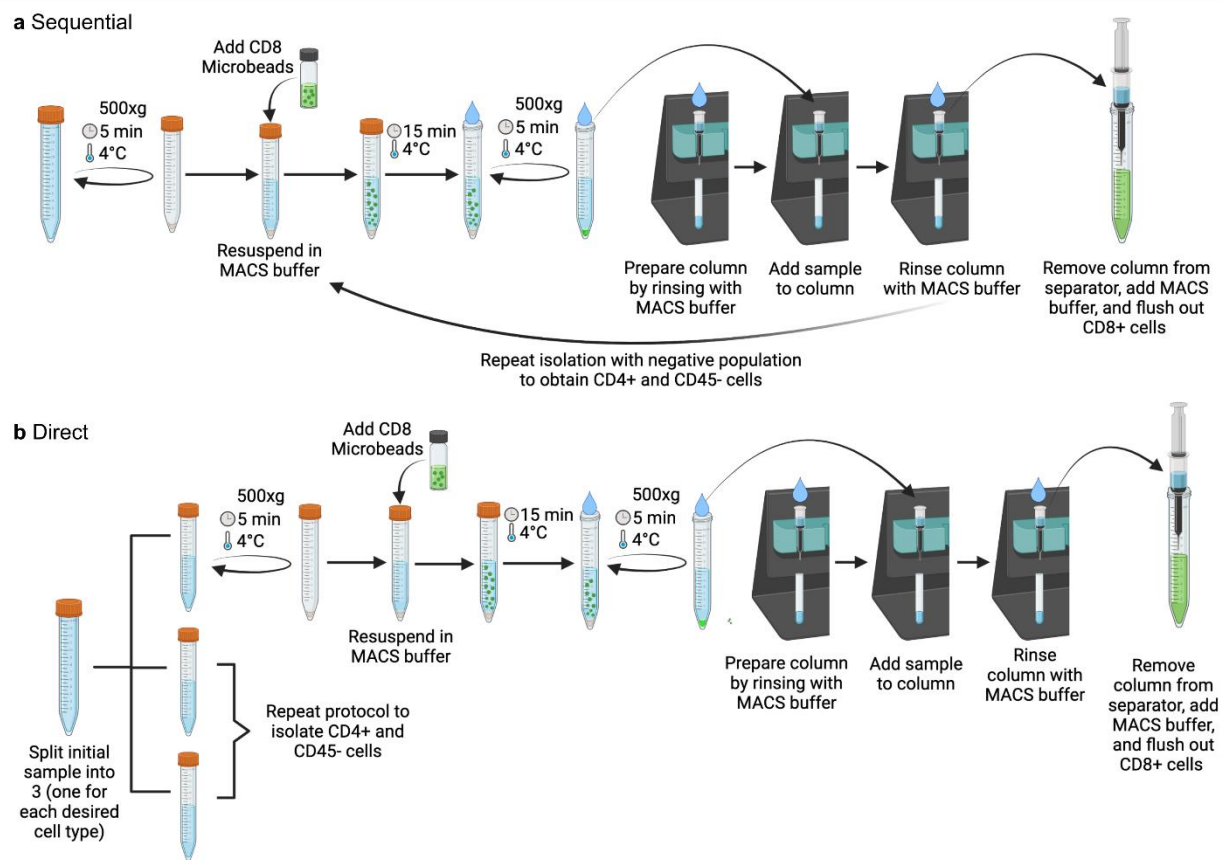


Figure 5. Schematic of cell enrichment and metabolite profiling,

a, Sequential method - the negative flow-through from the first magnetic bead isolation is re-used for the next isolation, and so on. **b**, Direct method - the bulk sample is divided before distinct populations are isolated through concurrent magnetic bead isolations. Centrifugation steps indicated with ellipse arrows. Created with BioRender.com.

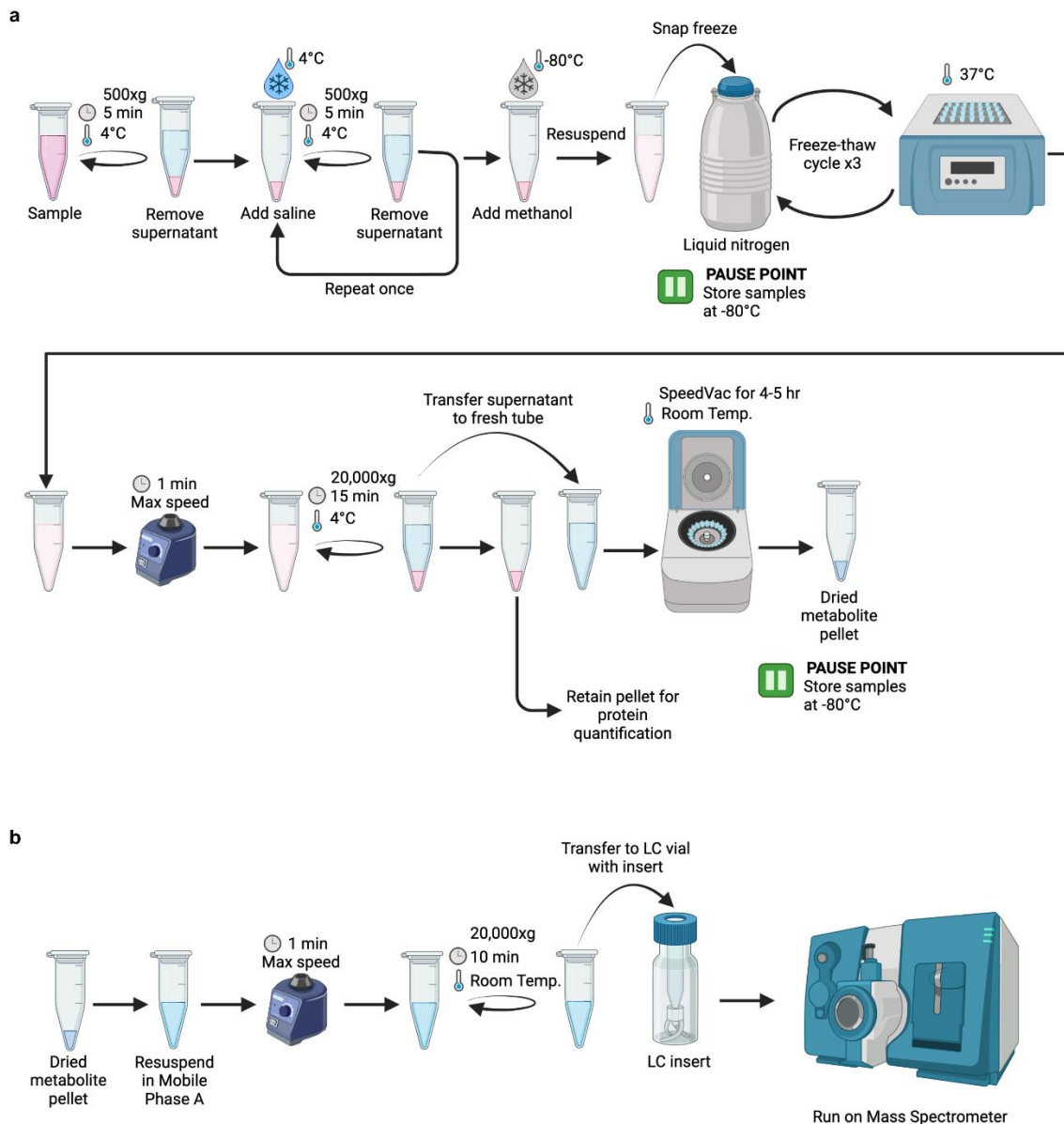


Figure 6. Metabolomics processing workflow.

a, Samples are washed with saline and resuspended in 80% methanol followed by 3 rounds of freeze-thaw and vortexed to lyse cells. The sample is centrifuged to pellet the cellular debris. The metabolite-containing supernatant is transferred to a new tube. **b**, The dried sample is prepared for mass spectrometry. Centrifugation steps indicated with ellipse arrows. Created with BioRender.com.

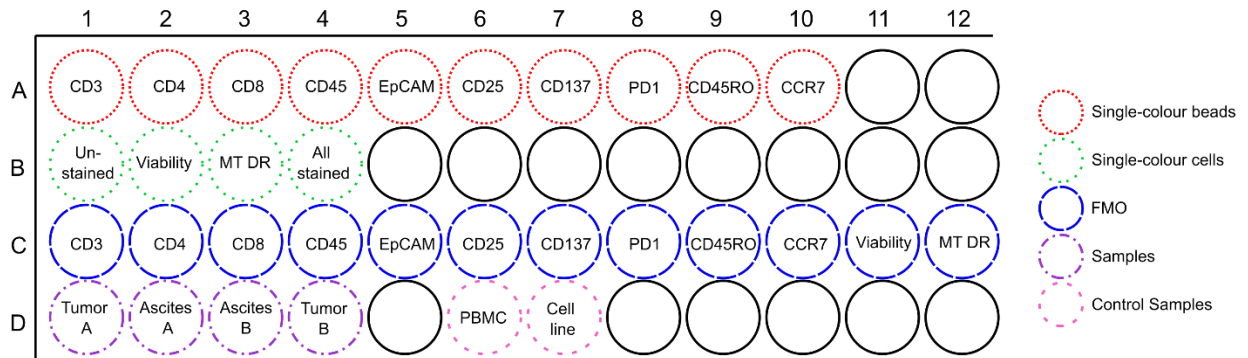


Figure 7. Flow cytometry plate layout.

Example of a 96 well plate with the necessary controls for multi-spectra flow cytometry based on the panel presented in Table 1 (step 64). Rows A-B are used for single-stain controls. Include all antibodies used in panel design which can be prepared using beads in row A or cells in row B. The dye-based stains for MT DR (MitoTracker Deep Red) and viability in row B must be stained with cells. Fluorescence minus one (FMO) controls in row C are prepared using all antibodies in the panel minus the one indicated in the well. An all stained sample control (stained with all antibodies) is used as a reference for the FMOs. Row D is used for patient samples and quality control samples (samples with known marker expression). Samples are stained with a master mix of all panel antibodies.

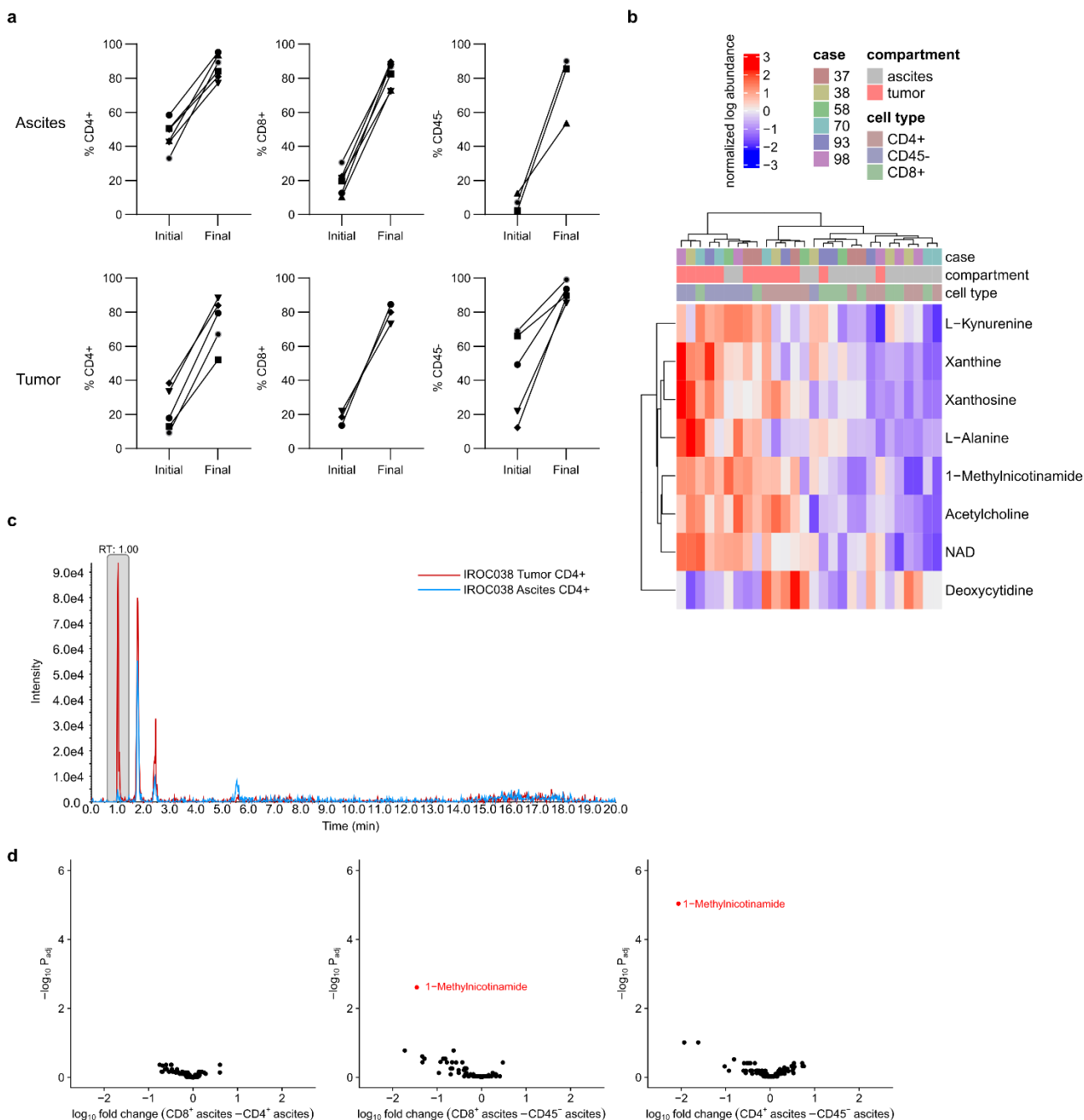
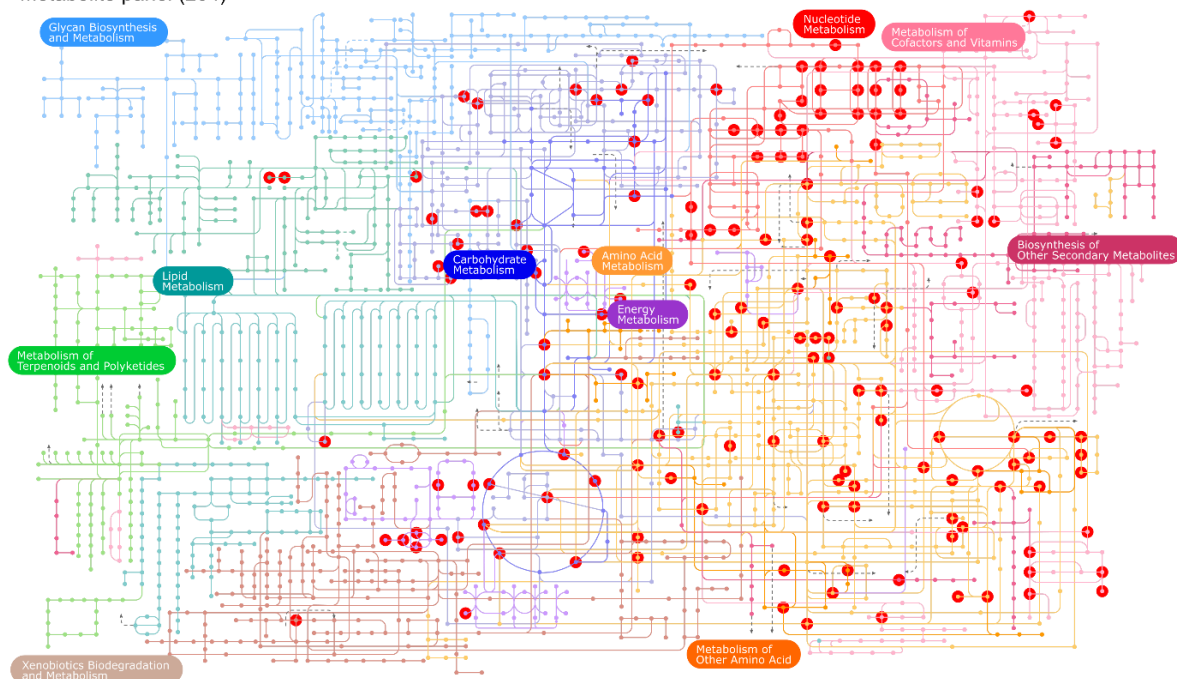


Figure 8. Successful enrichment of tumor and T cell populations from matched ascites and tumor prior to analysis by mass spectrometry reveals key differences between cell types and compartments.

a, Bead enrichment method validation by flow cytometry. CD4⁺, CD8⁺ and CD45⁻ cells are shown as a percentage of live cells prior to bead enrichment (initial) and after bead enrichment (final) for the ascites (top) and tumor (bottom). Symbols represent patients and lines indicate

matched samples. **b**, Heat map of abundance for statistically significant metabolites. Three technical replicates were run for each metabolomics sample. **c**, Chromatogram for MNA (precursor ion: 137.0000 m/z to fragment ion: 94.0000 m/z) detected from patient IROC038 CD4⁺ cells from the ascites and tumor. **d**, Volcano plot of metabolite differences between CD4⁺, CD8⁺ and CD45⁻ cells within the ascites. a, b, d Ascites and/or tumor samples collected from ovarian cancer patients n=6.

a Metabolite panel (204)



b Patient samples (99)

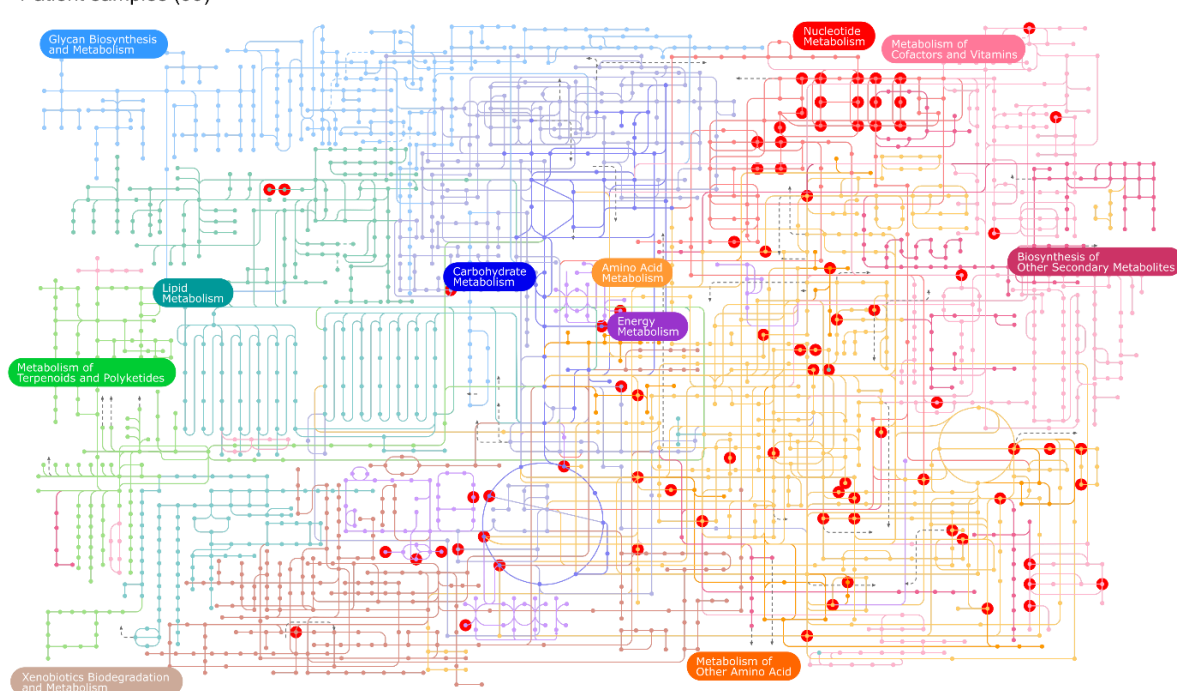


Figure 9. Pathway analysis of measured versus detected metabolites from patient specimens.

a, The red circles represent the 204 metabolites within the optimized panel (using KEGG IDs). **b**, The red circles represent the 99 metabolites detected in the patient samples (using KEGG IDs). Created using iPath3(148).

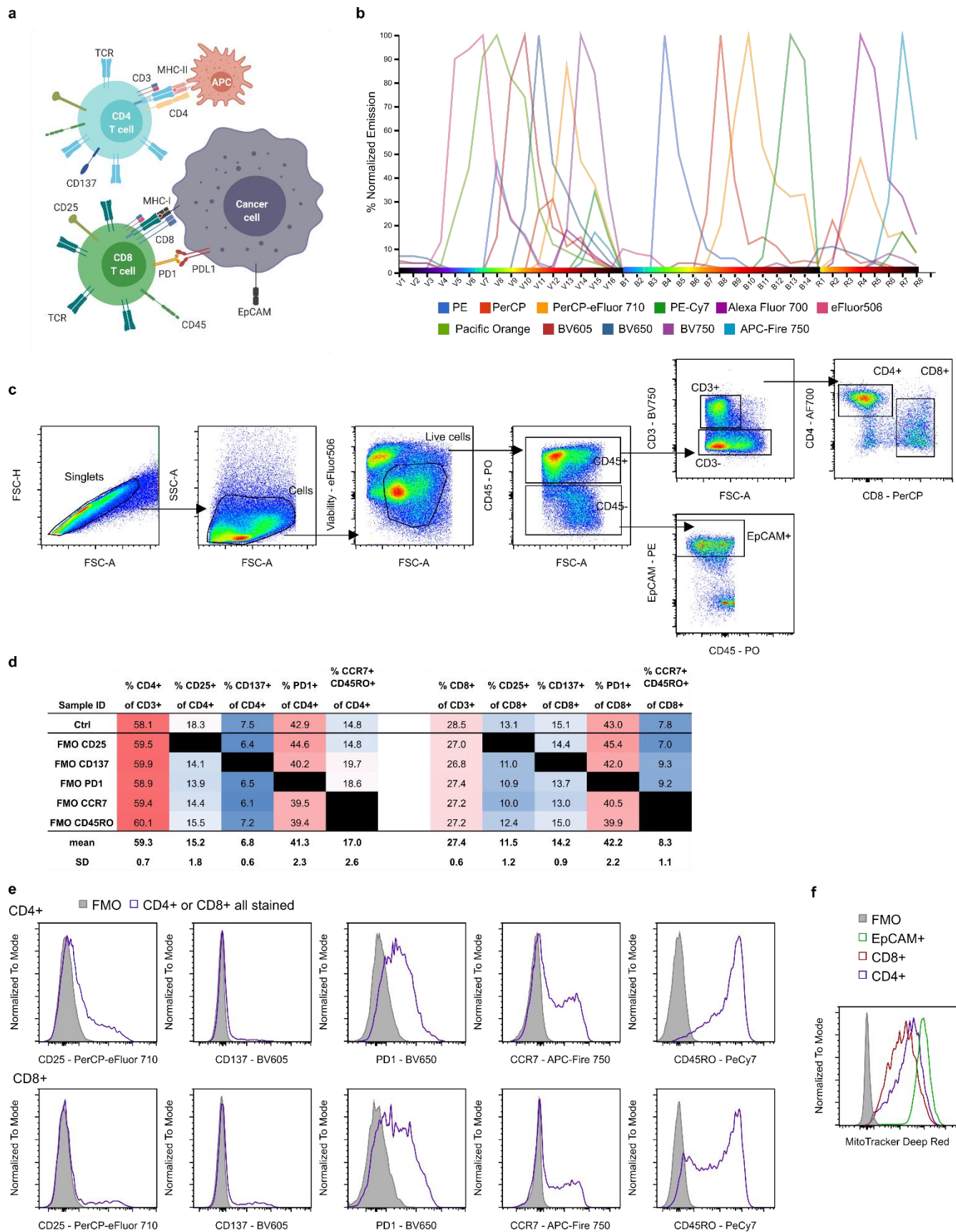


Figure 10. Metabolic profiling by flow cytometry leads to identification of general metabolic characteristics for specific cell populations.

a, Schematic of markers included in the high-dimensional panel. Created with BioRender.com. **b**, Spectral profiles of the chosen fluorophores on the Cytex Aurora (3L-16V-14B-8R). **c**, Flow cytometry gating strategy. **d**, Percentage of indicated cell population in an all stained sample compared to each respective fluorescence minus one (FMOs) control. Mean and standard deviation are shown to ensure the addition or removal of antibodies does not artificially impact cell proportions. **e**, Example flow cytometry histogram plots for each antibody. The respective FMO sample (grey) for each antibody is compared to the all stained sample (purple). In some cases, positive surface expression can be more heterogeneous (PD1) than other markers. **f**, Example plots for the MitoTracker Deep Red. CD4⁺ T cells (purple), CD8⁺ T cells (red) and CD45-EpCAM⁺ cells (green) are shown compared to the FMO control of live cells (grey).

2.7 Timing

Procedure 1 requires the largest amount of time (6-24 months), which can be reduced if working with an established biobank. Factors that contribute to timing include:

- Establishing a team (*<2 months with biobank, >3 months without*)
- Obtain permission to conduct study with clinical team (*<2 months with biobank, >3 months without*)
- Obtain research ethics approval (*<2 months with biobank, >3 months without*)
- Enroll participants (*~1-2 participants/month with or without a biobank*)
- Establish database for stage of de-identified samples

Timing for sample collection can range from ~2.5 hours to 9.5 hours. It is critical for the biobank, clinical, and research teams to communicate and record the timing of each collection step associated with the samples. Factors that may contribute to variation in timing include:

- Surgery start delays due to department clinical needs
- Pathologists busy with stat cases
- Transportation of biospecimens to research lab delays

Due to the dynamic nature of cell metabolism, the processing of patient samples in Procedure 1 and 2 for flow cytometry and metabolomic analysis is a time sensitive process. Therefore, coordinating preparation days before analyzing patient samples is critical. Cell enrichment and metabolome extraction is prioritized as the first experimental step occurring in the morning (9:00 am) and completing within 90 minutes. Samples for flow cytometry analysis remain on ice until enrichment is completed. Around 10:30 am, cells are stained with metabolic dyes followed by the remaining flow cytometry steps outlined above. Depending on the number of samples being assessed, sample preparation can take up to 6 hours (4:30 pm) and 90 minutes for data collection (6:00 pm). Flow cytometry data analysis requires 1-3 days.

Mass Spectrometry samples can remain at -80 °C for 1-2 months until data collection which requires approximately 30 minutes per sample followed by data analysis (1-3 days).

2.8 Anticipated Results

Our approach revealed pervasive metabolic differences between the ascites and tumor environments in ovarian cancer, and the cell types in these environments.

Mass spectrometry

Any sample processing delays prior to metabolomics analysis can result in changes in metabolism within the cells. The sample preparation can also cause changes in the chemical composition of the cells. We performed experiments to find out what effect our column separation method would have on the cell metabolism. We compared eluted cells from the columns to those left on ice for the same period of time (129). We found that most of the changes were in the abundance of amino acids. For example, Binek et al. found changes in amino acids after flow sorting, and similarly we see changes in many amino acids after bead sorting (46). An important point is that the known immunosuppressive metabolites were detected.

Successful cell enrichment prior to analysis by mass spectrometry was confirmed using flow cytometry (Figure 8. a). This ensured the abundance of the metabolites measured was assigned to the correct cell types. In addition, determining the portion of CD4+, CD8+ and CD45- cells in a population prior to enrichment can be important for estimating the final population of enriched cells.

For robust analysis of the metabolomics data, we developed protocols to control for interpatient variability, as well as to account for the hierarchical nature of the data. This revealed strong differences between the cell types within the ascites and tumor (Figure 8. b). The most striking difference was seen for MNA between T cells and tumor cells in the ascites (Figure 8. d).

MNA was one of the 99 metabolites we consistently detected in this experiment. While it is not known why the remaining 105 metabolites were not detected, we speculate this may be due to their low abundance or stability during sample processing. Further experiments are needed to address this question. We were able to observe that some metabolites we were unable to detect were isolated to one pathway (i.e. carbohydrate metabolism), others were dispersed across multiple pathways (Figure 9).

Flow cytometry

Our high-dimensional flow cytometry panel and protocol provided general metabolic characteristics (mitochondrial activity) of specific cell populations (Figure 10. a). This panel was designed through the use of the Cytex panel design tools such as their spectral viewer (Figure 10. b). Optimization of the panel was carried out through iterations of staining on unstimulated T cells, stimulated T cells, and patient ascites cells. During each iteration accuracy was determined through the presence of expected populations and FMO controls. Antibodies and fluorophore combinations that caused issues with unmixing were removed from the panel and replaced (149, 150).

The complexity and unpredictability of patient biospecimens makes accurate detection difficult. The panel must be robust enough to account for different cell sizes and autofluorescence, as well as unpredictable expression of different proteins. Unmixing of the raw data was carried out using beads as positive controls for antibodies, and a mixture of experimental samples for dyes (i.e. viability, MT DR). The accuracy of the panel on the patient samples was ensured through FMOs to guide gating (Figure 10. c-e). The removal of one antibody from the panel did not change the percentage of positive cells for any given fluorophore, attesting to the robustness of the panel (Figure 10. d). The signal from each FMO in the given channel was centered at zero, demonstrating successful panel design and spectral unmixing (Figure 10. e). From this, we were able to demonstrate differences in activation and mitochondrial metabolism between different cell populations (Figure 10. f).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary.

Data availability

Data generated and analyzed in this study are included in previously published article (129). Additional data are available from the corresponding author upon reasonable request.

Chapter 3: Metabolism of tumor cells and T cells in the ascites and tumor microenvironments of ovarian cancer

Adapted from:

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3.1 Acknowledgements and contributions

3.1.1 Author contributions

M.K.K. and P.T.H. designed and performed experiments, analyzed the data, and wrote the manuscript. S.M., S.K., and B.P. helped perform the metabolite profiling experiments. L.G.Z. and R.J.D. generated and analyzed the metabolite profiling data. P.H.W. oversaw biospecimen collection through the IROC-TTR. J.J.L. conceived the project and wrote the manuscript.

3.1.2 Author contributions to tables and figures

M.K.K. created Tables 2-3 and P.T.H. created Table 4.

M.K.K. generated and analyzed the data and created Figures 11-13.

M.K.K. generated the data and P.T.H. analyzed the data and created Figure 14.

M.K.K created Figure 15. A and C using BioRender.com.

M.K.K. processed the samples, L.G.Z. generated and analyzed the data, and P.T.H. analyzed the data and generated Figure 15. B and Figures 16-18. We thank Drs Mary Lesperance and Farouk Nathoo for advice on statistical analysis.

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3.2 Abstract

We devised a sensitive cell isolation and liquid chromatography tandem mass spectrometry (LC-MS/MS) method to interrogate the repertoire of metabolites within and between distinct cell types including CD4⁺ and CD8⁺ T cells, as well as tumor cells across the ascites and tumor environments of the same patients. We coupled this approach with high-dimensional flow cytometry to provide a highly resolved portrait of the metabolic status of these key populations. Collectively, this approach revealed reciprocal metabolic interactions between tumor and immune cells and provided a unique insight into immune regulatory metabolites.

3.3 Introduction

Tumor-derived metabolites can have profound suppressive effects on anti-tumor immunity, with increasing evidence that they can also function as key drivers of disease progression (151). Beyond the Warburg effect, recent work has begun to characterize the metabolic states of tumor cells and their relationship to the immunological state of the TME. Studies in murine models and human T cells have revealed that glutamine metabolism (24), oxidative metabolism (152) and glucose metabolism (153) are able to independently act on various immune cell subsets. Indeed, several metabolites in these pathways inhibit the antitumor function of T cells. Blockade of the enzyme co-factor tetrahydrobiopterin (BH4) was shown to impair T cell proliferation, and augmenting BH4 *in vivo* enhanced CD4 and CD8-mediated antitumor immune responses. Furthermore, the immune suppressive effects of kynurenine could be rescued by administration of BH4 (154). In isocitrate dehydrogenase (IDH) mutant glioblastoma, secretion of the oncometabolite (R)-2-hydroxyglutarate (R-2-HG) suppresses T cell activation, proliferation and cytolytic activity (155). More recently, methylglyoxal, a byproduct of glycolysis, was shown to be produced by myeloid derived suppressor cells (MDSCs) and T cell transfer of methylglyoxal can inhibit effector T cell function. Therapeutically, neutralization of methylglyoxal could overcome MSDC suppressor activity and synergistically enhance checkpoint blockade therapy in mouse models (84). Collectively, these studies highlight the critical role for TME-derived metabolites in modulating the function and activity of T cells.

T cell dysfunction has been widely reported in ovarian cancer (96). This is due in part to the metabolic features inherently associated with hypoxia and aberrant tumor vasculature (156), leading to the conversion of glucose and tryptophan into byproducts such as lactate and kynurenine. Excess extracellular lactate decreases the production of interferon gamma (IFN γ) and drives the differentiation of myeloid suppressor subsets (157, 158). Depletion of tryptophan directly inhibits T cell proliferation and suppresses T cell receptor signaling (159–161). Despite these observations, much of the work surrounding immunometabolism has been conducted on *in vitro* T cell cultures using optimized media or limited to *in vivo* syngeneic murine models both of which do not fully reflect the heterogeneity and physiological macro- and microenvironments in human cancer.

A common feature of ovarian cancer is peritoneal dissemination and the presentation of ascites, a build-up of cellular fluid in the peritoneal cavity that is associated with advanced disease and poor prognosis (111). This unique compartment has been reported to lack oxygen, have high levels of vascular endothelial growth factor (VEGF) and IDO, and be infiltrated by T regulatory and myeloid suppressor cells (111, 162–164). How the metabolic environment of the ascites might differ from that of the tumor itself and consequently reprogramming of T cells in the peritoneal space is unknown. Furthermore, critical differences and heterogeneity between the metabolites that are present within the ascites and tumor environments that might hamper the infiltration immune cells and their function into the tumor requires further investigation.

3.4 Materials and Methods

3.4.1 Patient sample collection and processing

Patient specimens and clinical data were obtained through the BC Cancer TTR, certified by the Canadian Tissue Repository Network. All specimens and clinical data were obtained with either informed written consent or a formal waiver of consent under protocols approved by the Research Ethics Board of BC Cancer and the University of British Columbia (H07-00463). Samples are stored in a certified BioBank (BRC-00290). Detailed patient characteristics are shown in Table 2. For cryopreservation, patient tumor samples were mechanically disaggregated using a scalpel and pushed through a 100 μ m filter to obtain a single cell suspension. Patient

ascites was centrifuged at 1500 rpm for 10 minutes at 4 °C to pellet cells and remove supernatant. Cells obtained from tumor and ascites were cryopreserved in 50% heat inactivated human AB serum (Sigma), 40% RPMI-1640 (Fisher) and 10% DMSO. These preserved single-cell suspensions were thawed for metabolomic and metabolite assays described below.

3.4.2 Cell culture reagents

Complete media consisted of a 0.22 μ m filtered 50:50 supplemented RPMI1640:AimV. RPMI1640 + 2.05 mM L-Glutamine (Fisher) was supplemented with 10% heat inactivated human AB serum (Sigma), 12.5 mM HEPES (Fisher), 2 mM L-Glutamine (Fisher), 1x Penicillin Streptomycin solution (Fisher) and 50 μ M B-mercaptoethanol. AimV (Invitrogen) was supplemented with 20 mM HEPES (Fisher) and 2 mM L-glutamine (Fisher). Flow cytometry staining buffer consisted of 0.22 μ m filtered PBS (Invitrogen) supplemented with 3% heat inactivated AB human serum (Sigma). Cell enrichment buffer consisted of 0.22 μ m filtered PBS supplemented with 0.5% heat inactivated human AB serum (Sigma).

3.4.3 Flow cytometry for metabolic profiling

Cells were stained with 10 nM MT DR and 100 μ M 2-NBDG for 30 minutes in complete media at 37°C. Next, cells were stained with viability dye-eF506 for 15 minutes at 4 °C. Cells were resuspended in F_C block (eBioscience) and Brilliant Stain Buffer (BD Bioscience) diluted in flow cytometry staining buffer (according to manufacturer instructions) and incubated for 10 minutes at room temperature. Cells were stained with a panel of antibodies (Table 3) in flow cytometry staining buffer for 20 minutes at 4 °C. Cells were resuspended in flow cytometry staining buffer prior to analysis (Cytek Aurora; 3L-16V-14B-8R configuration). Cytometry data were analyzed using SpectroFlo and FlowJo V10, and Fig.s were created using GraphPad Prism 8. Median Fluorescent Intensity of 2-NBDG and MT DR were log normalized prior to statistical analysis using paired t-test to account for matched patients. Any population with less than 40 events was removed from the analysis, an median fluorescent intensity (MFI) value of 1 was inputted for any negative values prior to statistical analysis and data visualization.

3.4.4 Unbiased discovery of cell populations in flow cytometry data

To supplement our manual gating strategy for the above flow panel, we used FAUST (165) to automatically assign cells to populations, after dead cell exclusion in FlowJo. We manually curated outputs to merge populations that appeared to be mis-assigned (merged PD1+ with PD1- tumor cells), and retained populations comprising, on average, more than 2% of cells in each sample, for a total of 11 populations.

3.4.5 Cell activation and enrichment for metabolite profiling optimization

PBMCs were isolated from a Leukopheresis product (STEMCELL Technologies) using Ficoll gradient density centrifugation. CD8+ T cells were isolated from the PBMCs using CD8 MicroBeads (Miltenyi) and expanded using TransAct (Miltenyi) for 2 weeks in complete media according to manufacturer's instructions. Cells were rested for 5 days in complete media with 10 ng/ml IL-7 (Peprotech) and then restimulated with TransAct. On day 7, cells were enriched using Human CD45 MicroBeads (Miltenyi) in three rounds of sequential enrichment according to the manufacturer's instructions. Cells were aliquoted for analysis by flow cytometry (described above) and 1 million cells were aliquoted in triplicate for analysis by LC-MS/MS. Samples were processed for LC-MS/MS as described below. We imputed missing metabolite values with an ion count of 1000. Each sample was sum-normalized by total ion count (TIC), log transformed, and auto-normalized in MetaboAnalystR prior to analysis.

3.4.6 Cell type enrichment of patient samples

Single cell suspensions were thawed for each patient and filtered through a 40 µm filter into complete media (described above). Samples were enriched for CD8+, CD4+ and CD45- cells (on ice) using three sequential rounds of positive selection by magnetic bead separation using MicroBeads (Miltenyi) according to the manufacturer protocol. Briefly, cells were resuspended in cell enrichment buffer (described above) and counted. Cells were incubated at 4 °C for 15 minutes with Human CD8 Microbeads, Human CD4 MicroBeads or Human CD45 MicroBeads (Miltenyi) and then washed with cell enrichment buffer. Samples were passed through the LS column (Miltenyi) and both the positive and negative fractions were collected. To reduce the duration and maximize the cell recovery steps, CD8- fraction was subsequently used for a second

round CD4⁺ enrichment, and the CD4⁻ fraction was used for the subsequently CD45⁻ enrichment. Solutions were kept on ice throughout the duration of the separation.

3.4.7 Metabolite extraction for mass spectrometry

To prepare samples for metabolite profiling, cells were washed once with ice-cold saline solution and 1 mL of 80% methanol added to each sample before vortexing and snap freezing in liquid nitrogen. Samples were subjected to 3 freeze-thaw cycles, and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The metabolite-containing supernatant was evaporated until dry. Metabolites were reconstituted in 50 µl of 0.03% formic acid, vortex-mixed, and centrifuged to remove debris.

3.4.8 LC-MS/MS metabolite profiling for patient biospecimens

Metabolite extraction was carried out as described above. The supernatant was transferred to a HPLC vial for the metabolomics study. Each sample was processed with similar numbers of cells using a randomized processing scheme to prevent batch effects. We performed qualitative assessment of global metabolites as previously published on the AB SCIEX QTRAP 5500 triple-quadrupole mass spectrometer (166). Chromatogram review and peak area integration were performed using MultiQuant software version 2.1 (Applied Biosystems SCIEX).

3.4.9 Characterizing metabolic differences across cell types and microenvironments

Missing metabolite values were imputed with an ion count of 1000 and normalized peak area calculated for each detected metabolite using the total ion count from each sample to correct variations introduced from sample handling through instrument analysis. TIC-normalization was followed by log transformation and autoNorm row-scaling using MetaboAnalystR (167) (default parameters). We conducted exploratory analysis of metabolome differences across sample types using PCA with the vegan R package, and conditioned the analysis on patient using partial redundancy analysis. Heatmap dendrograms were constructed using Ward's method to cluster Euclidean distances among samples. We identified differentially-abundant metabolites across cell types and microenvironments using limma (168) on the normalized metabolite abundances. To simplify interpretation, we specified the model using the group means parameterization, treating cell types within microenvironments as each group (n=6 groups); for significance testing

we took the average of triplicate measurements for each metabolite to avoid pseudoreplication, and included patient as a block in the limma design. To examine metabolites that differed across patients we re-fit models in limma including patient as a fixed effect. We reported significance at $P_{adj} < 0.05$ (Benjamini-Hochberg correction) for pre-specified contrasts among cell types and microenvironments.

3.4.10 Quantification and Statistical Analysis

Statistical analysis was carried out as described in the text or methods using GraphPad Prism 8, Microsoft Excel or R v3.6.0. Where multiple samples were taken from the same patient (e.g. ascites and tumor), we used paired t-tests, or included patient as a random effect in linear or generalized models, as appropriate. For metabolomic analysis, significance testing was done on means of triplicate measurements.

3.4.11 Data and materials availability

Processed data files and scripts to reproduce metabolomics and scRNA-seq analyses are available at github.com/vicDRC/onecarbon. Flow cytometry data is deposited at Flow Repository (FR-FCM-Z2NH). Schematics were created with BioRender.com.

3.5 Figures and tables

Table 2. HGSC patient characteristics for metabolic profiling.

Patient	Tumor location	Age at surgery	Survival (months)*	Tumor stage[†]	Tumor grade[†]	Ascites volume (mL)
37	omentum	70	12	3C	3	1800
38	ovary	40	16	T3b	3	1200
70	omentum	50	23	3C	3	80
58	omentum	78	18	4	3	2000
93	fallopian tube	53	82	3B	2	1600
98	omentum	39	13	3C	3	2000

* Survival calculated from the date of diagnosis to the date of death

[†] Tumor stage and grade were determined by a pathologist at the time of surgery

Table 3. Flow cytometry metabolic profiling panel.

Fluorochrome	Marker	Expression	Clone	Company	Catalogue number
---	2-NBDG	Glucose uptake	---	Thermo	N13195
PE	CD326 (EpCAM)	Epithelial cells	1B7	Thermo	12-9326-42
PerCP	CD8	Effector T cells	RPA-T8	Biolegend	301030
PerCP- eFluor710	CD25	Activation/Tregs	4.00E+03	Thermo	46-0257-41
PE-Cy7	CD45RO	Phenotype	UCHL1	Thermo	25-0457-42
---	MitoTracker Deep Red	Mitochondrial activity	---	Thermo	M22426
AF700	CD4	Helper T cells	RPA-T4	Biolegend	300526
APC/Fire750	CCR7	Phenotype	G043H7	Biolegend	353246
eFlour506	Viability	Live/dead cells	---	Thermo	65-0866-14
PO	CD45	Leukocytes	HI30	Thermo	MHCD4530
BV605	CD137	Activation	4B4-1	Biolegend	309822
BV650	CD279 (PD1)	Activation/Exhaustion	EH12.2H 7	Biolegend	329950
BV750	CD3	T cells	SK7	Biolegend	344845

Table 4. Statistical analysis of metabolite profiling data.

Output of limma analysis for differential abundance; blocked on patient with average of replicates per sample used as input. Fold change for each contrast shown, with significance of overall F test (T8.A8 = Tumor CD8 abundance - Ascites CD8 abundance).

Metabolite	T8.A8	T4.A4	T45.A45	T8.T4	T8.T45n	T4.T45	A8.A4	A8.A45	A4.A45	AveExpr	F	P.Value	adj.P.Val
1-Methylnicotinamide	0.937	1.695	0.113	-0.146	-0.649	-0.504	0.612	-1.473	-2.085	-1.98E-18	6.834	0.000	0.000
Xanthosine	0.813	1.081	1.568	-0.169	-0.954	-0.785	0.099	-0.199	-0.298	-1.40E-16	4.450	0.000	0.023
Xanthine	0.845	0.578	0.946	0.552	-0.750	-1.301	0.284	-0.649	-0.933	6.95E-17	4.059	0.001	0.032
L-Alanine	1.197	0.091	0.396	1.308	-0.098	-1.407	0.202	-0.900	-1.102	1.92E-18	3.890	0.002	0.032
NAD	0.879	0.628	0.274	-0.193	-0.900	-0.708	-0.443	-1.505	-1.062	-1.59E-17	3.886	0.002	0.032
L-Kynurenine	1.125	0.327	-0.356	1.084	0.144	-0.940	0.285	-1.338	-1.623	3.17E-17	3.633	0.003	0.045
Acetylcholine	0.983	1.424	0.695	-0.370	-0.304	0.066	0.072	-0.591	-0.663	6.05E-17	3.552	0.003	0.046
Deoxycytidine	-0.342	0.298	-0.407	-1.587	-0.129	1.458	-0.946	-0.193	0.753	-1.46E-16	3.491	0.004	0.046
Butyrylcarnitine	0.018	1.225	-0.232	-0.694	-0.880	-0.186	0.513	-1.130	-1.642	-3.30E-17	3.251	0.006	0.068
L-Proline	1.065	0.881	1.250	-0.421	-0.346	0.075	-0.605	-0.161	0.444	7.45E-17	3.200	0.007	0.068
Thiamine	0.405	-0.244	1.391	0.334	-1.195	-1.529	-0.315	-0.209	0.107	-2.78E-16	3.064	0.009	0.080
Stearoylcarnitine	0.541	0.447	0.231	-0.630	-1.045	-0.415	-0.724	-1.355	-0.631	6.54E-17	3.015	0.010	0.080
Myoinositol	0.960	-0.112	-0.153	1.417	0.253	-1.164	0.344	-0.861	-1.205	-2.37E-16	2.983	0.011	0.080
Xanthylic acid	0.770	0.775	0.795	-0.339	-0.825	-0.486	-0.334	-0.800	-0.466	-1.59E-16	2.958	0.011	0.080
Cytidine	0.821	1.327	0.024	-0.462	-0.174	0.288	0.044	-0.972	-1.016	-7.81E-18	2.880	0.013	0.086
Guanosine	-0.089	1.121	0.440	-1.620	-0.763	0.857	-0.411	-0.235	0.176	2.23E-17	2.857	0.014	0.086
L-Lysine	0.121	0.773	0.343	-1.435	-0.414	1.021	-0.783	-0.192	0.591	3.89E-17	2.819	0.015	0.088
Adenosine	0.447	1.319	1.166	-0.810	-0.251	0.559	0.062	0.469	0.406	6.79E-17	2.781	0.016	0.089
Carnitine	1.057	0.924	0.931	-0.307	0.488	0.795	-0.440	0.363	0.802	-2.42E-16	2.708	0.019	0.095
Deoxyadenosine monophosphate	0.493	0.489	0.354	-0.555	-1.034	-0.479	-0.559	-1.174	-0.615	-1.71E-17	2.700	0.019	0.095
Tetradecanoylcarnitine	0.555	0.216	-0.519	-0.372	-0.667	-0.295	-0.711	-1.741	-1.030	2.42E-18	2.630	0.022	0.098
Deoxyinosine	-0.326	0.094	-0.005	-1.268	-0.137	1.131	-0.849	0.183	1.032	1.57E-16	2.617	0.023	0.098
Creatine	0.426	0.822	0.667	-0.598	-0.993	-0.395	-0.201	-0.752	-0.550	-4.80E-17	2.590	0.024	0.098
L-Hexanoylcarnitine	0.305	0.133	-0.065	-0.944	0.127	1.071	-1.116	-0.242	0.873	9.18E-17	2.575	0.025	0.098
Isovalerylcarnitine	0.852	0.965	0.548	-0.174	-0.445	-0.272	-0.061	-0.749	-0.688	2.08E-17	2.571	0.025	0.098
L-Octanoylcarnitine	0.399	0.219	-0.089	-0.919	0.166	1.085	-1.099	-0.322	0.777	1.17E-16	2.545	0.026	0.099
N-Acetyl-L-aspartic acid	0.635	0.469	1.081	-0.450	-0.895	-0.445	-0.616	-0.449	0.167	-2.27E-17	2.509	0.028	0.102
Uridine	0.912	0.798	0.181	0.143	-0.282	-0.425	0.029	-1.013	-1.042	-2.27E-16	2.495	0.029	0.102
L-Methionine	0.951	1.174	0.744	-0.226	0.581	0.807	-0.003	0.374	0.377	-2.23E-17	2.418	0.034	0.115
1-Methyladenosine	0.226	1.259	0.549	-1.025	-0.696	0.329	0.009	-0.373	-0.382	6.26E-18	2.383	0.036	0.116
2-Ketohexanoic acid	-0.256	-1.074	-0.613	1.184	0.581	-0.603	0.365	0.224	-0.142	3.93E-16	2.378	0.036	0.116
Uridine 5'-monophosphate	0.981	0.578	-0.157	-0.161	-0.240	-0.079	-0.564	-1.378	-0.814	1.98E-17	2.353	0.038	0.118
N-Acetyl-L-alanine	0.846	0.303	0.554	-0.473	0.268	0.741	-1.016	-0.024	0.992	-3.37E-17	2.332	0.040	0.118
Citraconic acid	-0.401	-1.074	-0.620	1.033	0.497	-0.537	0.361	0.278	-0.083	1.11E-16	2.323	0.040	0.118
Betaine	0.913	0.663	0.604	0.225	-0.364	-0.590	-0.025	-0.674	-0.648	6.41E-17	2.280	0.044	0.125
L-Acetylcarnitine	0.182	0.510	1.300	-0.477	0.119	0.596	-0.148	1.237	1.386	-1.58E-16	2.230	0.049	0.133
S-Adenosylmethionine	0.100	0.702	0.909	-1.160	-0.642	0.518	-0.558	0.166	0.725	8.95E-17	2.208	0.051	0.135
L-Malic acid	1.540	0.564	-0.288	0.619	0.798	0.179	-0.357	-1.030	-0.673	7.26E-18	2.171	0.054	0.142
L-Homoserine	0.582	0.674	0.126	-0.849	0.131	0.980	-0.757	-0.324	0.433	9.81E-17	2.109	0.061	0.153
Carbamoyl phosphate	0.174	0.913	-0.580	-1.000	0.439	1.439	-0.260	-0.314	-0.054	-9.85E-17	2.103	0.062	0.153
Propionylcarnitine	1.134	0.857	0.878	0.020	0.176	0.157	-0.258	-0.079	0.178	-4.88E-17	2.081	0.065	0.155
L-Isoleucine	-0.064	0.606	0.330	-1.170	-0.074	1.096	-0.500	0.320	0.821	-3.88E-16	2.071	0.066	0.155
Cytidine monophosphate	0.526	0.502	0.289	-0.782	-0.621	0.161	-0.806	-0.858	-0.052	7.56E-18	2.054	0.068	0.157
Fumaric acid	-0.096	-0.962	0.008	1.187	0.038	-1.149	0.322	0.142	-0.180	-8.67E-18	1.967	0.080	0.180
Guanine	-0.103	0.640	0.129	-1.018	0.235	1.253	-0.274	0.467	0.741	-2.68E-17	1.950	0.083	0.181
N6,N6,N6-Trimethyl-L-lysine	0.445	0.747	0.331	-0.924	-0.067	0.858	-0.623	-0.181	0.442	2.73E-18	1.940	0.084	0.181
Spermidine	0.330	0.796	1.027	-0.808	-0.403	0.405	-0.342	0.293	0.636	-8.18E-18	1.893	0.092	0.188
Hypoxanthine	0.650	0.932	0.593	-0.668	-0.154	0.513	-0.386	-0.211	0.175	1.80E-17	1.888	0.093	0.188
Inosine	-0.392	0.755	0.368	-1.487	-0.958	0.529	-0.339	-0.197	0.142	4.19E-17	1.886	0.093	0.188

Table 4. (continued)

cADP-ribose	1.011	0.088	0.650	0.763	-0.152	-0.915	-0.161	-0.513	-0.352	-1.34E-16	1.863	0.097	0.192
L-Glutamine	0.521	0.106	0.987	-0.479	-0.258	0.221	-0.893	0.208	1.101	-1.98E-18	1.852	0.099	0.192
Creatinine	0.841	0.234	0.602	-0.089	-0.491	-0.401	-0.697	-0.730	-0.033	0.00E+00	1.809	0.107	0.204
L-Asparagine	-0.189	0.439	0.268	-1.202	-0.288	0.914	-0.574	0.169	0.743	-5.42E-17	1.783	0.113	0.208
D-Glutamic acid	0.429	0.593	0.632	-0.813	-0.539	0.274	-0.648	-0.336	0.313	-6.31E-17	1.778	0.114	0.208
Folic acid	0.725	-0.057	-0.338	-0.280	-0.151	0.128	-1.062	-1.214	-0.152	-8.77E-17	1.747	0.120	0.216
Shikimic acid	0.318	0.385	0.977	-0.736	-0.443	0.294	-0.669	0.216	0.885	-3.72E-18	1.738	0.122	0.216
Succinic acid	-0.200	-0.331	-0.007	0.507	-0.664	-1.171	0.376	-0.470	-0.846	9.06E-16	1.710	0.128	0.223
Citric acid	0.299	0.704	-0.839	0.371	0.552	0.181	0.776	-0.585	-1.362	4.47E-16	1.679	0.136	0.232
L-Lactic acid	-0.407	-0.372	-0.102	0.805	0.050	-0.755	0.839	0.355	-0.484	-1.68E-16	1.646	0.144	0.242
N-Alpha-acetyllysine	-0.155	0.445	0.054	-1.085	-0.098	0.987	-0.485	0.111	0.595	-7.93E-18	1.528	0.177	0.292
Pantothenic acid	0.538	0.334	-0.304	-0.442	-0.369	0.073	-0.647	-1.212	-0.565	-4.13E-17	1.520	0.180	0.292
Thymidine	-0.313	-0.498	-0.232	-0.561	0.032	0.593	-0.747	0.112	0.859	-9.05E-17	1.510	0.183	0.292
Guanosine													
monophosphate	0.698	0.735	0.440	-0.494	-0.034	0.460	-0.457	-0.293	0.164	7.19E-17	1.468	0.196	0.304
Guanidoacetic acid	-0.113	0.813	0.456	-1.036	-0.170	0.866	-0.111	0.398	0.509	-1.49E-18	1.468	0.196	0.304
Methionine sulfoxide	-0.932	0.351	0.306	-1.388	-1.299	0.090	-0.105	-0.061	0.044	1.38E-17	1.437	0.207	0.315
Niacinamide	0.574	0.784	-0.165	-0.527	-0.131	0.396	-0.318	-0.870	-0.553	-2.48E-18	1.421	0.213	0.319
L-Valine	0.203	0.435	0.853	-0.606	-0.047	0.559	-0.374	0.603	0.977	4.46E-18	1.387	0.226	0.333
Indole	-0.366	0.597	0.189	-1.179	-0.353	0.826	-0.216	0.201	0.417	6.25E-17	1.274	0.272	0.396
L-Arginine	0.189	0.615	0.867	-0.660	-0.247	0.413	-0.234	0.432	0.665	3.84E-17	1.232	0.291	0.411
Inosinic acid	0.703	0.608	0.501	-0.195	-0.233	-0.038	-0.290	-0.436	-0.145	-1.46E-17	1.232	0.291	0.411
L-3-Phenyllactic acid	0.152	0.455	1.100	0.005	-0.571	-0.576	0.308	0.377	0.069	-3.82E-17	1.222	0.296	0.412
Dimethylglycine	0.420	0.472	1.076	0.029	-0.414	-0.443	0.081	0.242	0.161	1.10E-16	1.185	0.314	0.431
Pipecolic acid	1.100	0.145	-0.272	0.861	0.647	-0.214	-0.095	-0.725	-0.630	-1.34E-16	1.168	0.322	0.437
5'-Methylthioadenosine	0.307	0.220	0.916	-0.459	-0.499	-0.040	-0.546	0.110	0.656	-6.29E-17	1.133	0.340	0.455
Nicotinic acid_Vit B3	-0.010	0.523	-0.389	-0.943	-0.228	0.715	-0.410	-0.607	-0.197	-5.95E-18	1.081	0.368	0.486
2-Hydroxyglutaric acid	0.221	-0.112	-0.026	0.516	-0.301	-0.817	0.184	-0.548	-0.732	5.98E-17	0.997	0.417	0.539
S-Adenosylhomocysteine	-0.169	0.162	0.937	0.150	-0.571	-0.721	0.481	0.534	0.053	2.16E-17	0.994	0.420	0.539
Choline	0.402	0.436	0.901	-0.040	-0.384	-0.345	-0.005	0.115	0.120	-1.75E-16	0.927	0.462	0.585
L-Aspartic acid	0.452	0.255	0.394	-0.373	-0.392	-0.019	-0.569	-0.450	0.120	-1.42E-17	0.914	0.471	0.585
Oxidized glutathione	-0.072	0.655	0.642	-0.467	0.051	0.518	0.259	0.765	0.506	3.62E-18	0.911	0.473	0.585
L-Phenylalanine	0.024	0.411	0.811	-0.566	-0.252	0.314	-0.179	0.535	0.714	-1.15E-16	0.870	0.500	0.611
4-Hydroxybenzoic acid	-0.086	-0.381	0.162	0.614	-0.263	-0.878	0.319	-0.016	-0.335	2.73E-18	0.856	0.510	0.616
Pyridoxamine	0.925	0.127	0.286	0.240	0.519	0.279	-0.558	-0.120	0.438	2.25E-16	0.839	0.521	0.622
Deoxyribose 1-phosphate	-0.183	-0.027	0.601	0.413	-0.117	-0.530	0.569	0.667	0.098	2.75E-16	0.724	0.605	0.713
Putrescine	0.127	-0.051	0.583	0.493	-0.210	-0.703	0.314	0.245	-0.069	-6.67E-17	0.640	0.669	0.764
1-Methylhistidine	0.097	0.760	-0.210	-0.180	0.211	0.392	0.483	-0.096	-0.579	-2.53E-16	0.639	0.670	0.764
Glyceric acid	0.061	-0.233	0.033	0.303	-0.425	-0.728	0.008	-0.453	-0.461	-1.43E-16	0.637	0.671	0.764
Phosphorylcholine	0.843	0.296	0.264	0.544	0.537	-0.007	-0.003	-0.042	-0.039	-1.02E-16	0.609	0.693	0.774
Glycerophosphocholine	0.499	0.634	0.329	-0.059	0.289	0.348	0.076	0.120	0.043	-8.28E-17	0.605	0.696	0.774
Dodecanoylcarnitine	-0.552	-0.450	-0.427	0.095	-0.154	-0.249	0.197	-0.029	-0.226	-4.67E-17	0.569	0.724	0.796
Gamma-Aminobutyric acid	0.063	0.422	0.712	-0.256	-0.449	-0.193	0.103	0.201	0.098	-1.43E-16	0.554	0.736	0.800
Fructose 1,6-bisphosphate	0.074	0.114	0.235	0.445	0.475	0.030	0.485	0.635	0.151	-2.27E-16	0.525	0.758	0.816
L-Tyrosine	-0.013	0.156	0.358	-0.502	-0.300	0.202	-0.332	0.071	0.403	-1.16E-16	0.399	0.850	0.905
Malonic acid	0.325	-0.004	-0.692	0.202	0.414	0.212	-0.127	-0.604	-0.477	-4.03E-17	0.385	0.859	0.905
Adenosine													
monophosphate	0.295	0.330	0.566	-0.052	0.010	0.062	-0.017	0.282	0.299	-1.14E-16	0.348	0.884	0.921
Adenine	0.336	0.457	0.164	-0.161	0.043	0.205	-0.041	-0.129	-0.088	8.43E-18	0.319	0.902	0.930
Taurine	0.079	-0.380	-0.150	0.003	0.013	0.010	-0.456	-0.217	0.239	-4.71E-17	0.273	0.928	0.947
ADP	-0.321	0.278	0.147	-0.394	-0.441	-0.047	0.204	0.027	-0.178	-4.96E-18	0.206	0.960	0.970
Hydroxyisocaproic acid	0.199	-0.019	-0.387	0.310	0.328	0.019	0.091	-0.258	-0.350	-3.87E-17	0.176	0.972	0.972

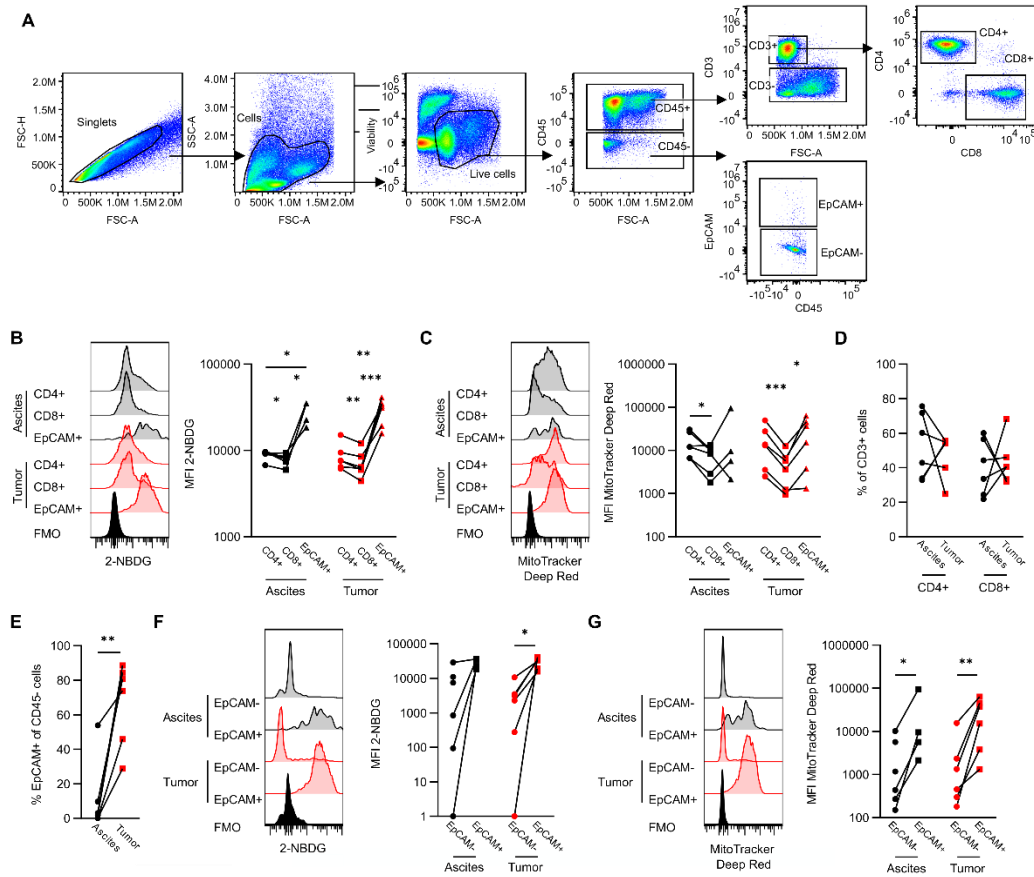


Figure 11. Tumor cells have greater glucose uptake but similar mitochondrial activity to T cells.

(A) Representative gating strategy for analysis by flow cytometry. (B-C) Representative plot (left) and tabulated data (right) for median fluorescent intensity (MFI) of glucose uptake (2-NBDG) (B) and mitochondrial activity (MitoTracker Deep Red) (C) of CD4+ T cells, CD8+ T cells, and EpCAM+CD45- tumor cells from ascites and tumor. (D) Proportion of CD4+ and CD8+ cells (of CD3+ T cells) within ascites and tumor. (E) Proportion of EpCAM+ (of CD45-) tumor cells within ascites and tumor. (F-G) Representative plot (left) and tabulated data (right) for glucose uptake (2-NBDG) (F) and mitochondrial activity (MitoTracker Deep Red) (G) of EpCAM+CD45- tumor and EpCAM-CD45- stromal cells from ascites and tumor. P-values determined by paired t-test (*p<0.05, **p<0.01, ***p<0.001). Lines indicate matched patients (n=6). Fluorescence Minus One (FMO); Median Fluorescence Intensity (MFI).

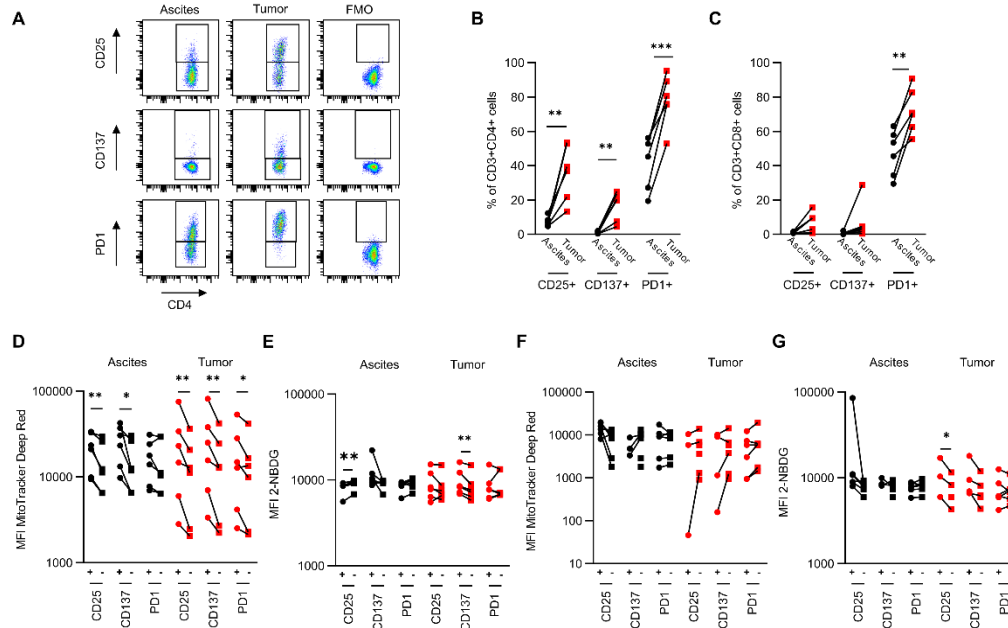


Figure 12. T cell metabolism is minimally impacted by expression of activation markers.

(A) Representative plots for CD25, CD137 and PD1 expression by flow cytometry. (B-C) CD25, CD137 and PD1 expression on CD4⁺ T cells (B) and CD8⁺ T cells (C). Lines indicate matched patients (n=6). Fluorescence Minus One (FMO); Median Fluorescence Intensity (MFI). (D-E) Mitochondrial activity (D) and glucose uptake (E) of CD25, CD137 and PD1 +/- CD4⁺ T cells. (F-G) Mitochondrial activity (F) and glucose uptake (G) of CD25, CD137 and PD1 +/- CD8⁺ T cells. Lines indicate matched patients (B-G). P-values determined by paired t-test (*p<0.05, **p<0.01, ***p<0.001). Lines indicate matched patients (n=6). Fluorescence Minus One (FMO); Median Fluorescence Intensity (MFI).

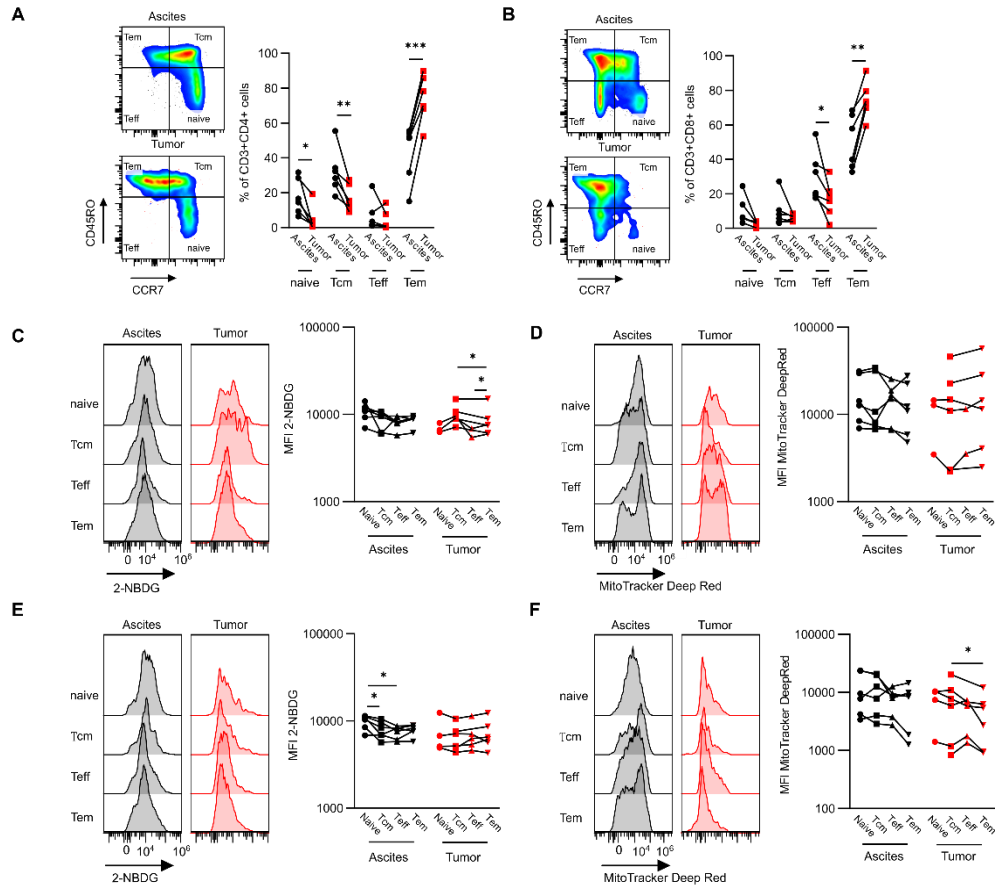


Figure 13. T cell metabolism is minimally impacted by expression of differentiation markers.

(A-B) Naive, central memory (Tcm), effector (Teff), and effector memory (Tem) phenotype based on CCR7 and CD45RO expression. Representative plot (left) and tabulated data (right) for CD4+ T cells (A) and CD8+ T cells (B) from ascites and tumor. (C-D) Representative plot (left) and tabulated data (right) for glucose uptake (C) and mitochondrial activity (D) of naive, Tcm, Teff and Tem CD4+ T cells. (E-F) Representative plot (left) and tabulated data (right) for glucose uptake (E) and mitochondrial activity (F) of naive, Tcm, Teff and Tem CD8+ T cells. P-values determined by paired t-test (*p<0.05, **p<0.01, ***p<0.001). Lines indicate matched patients (n=6). Fluorescence Minus One (FMO); Median Fluorescence Intensity (MFI); Central memory T cells (Tcm); Effector T cells (Teff); Effector memory T cells (Tem).

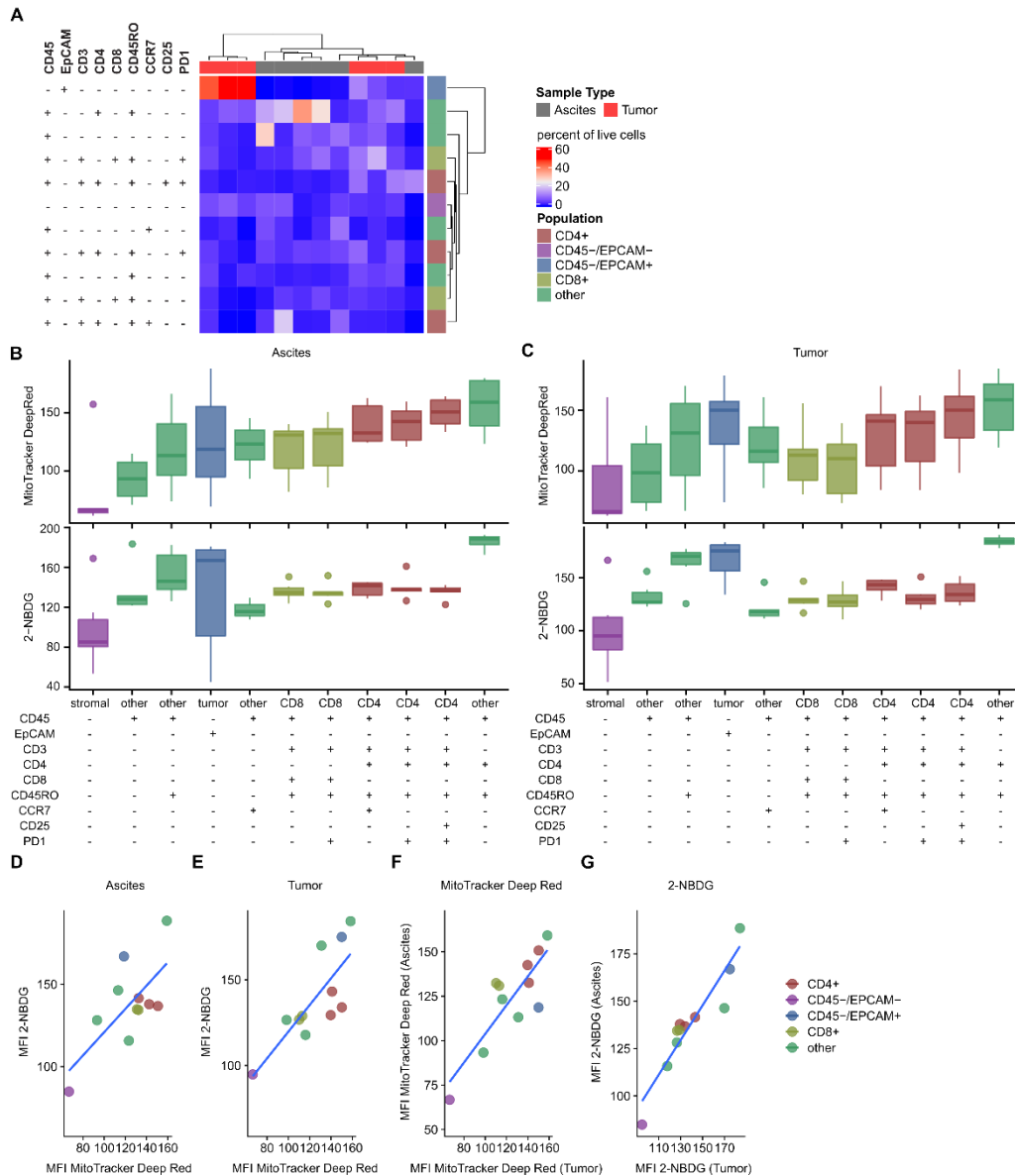


Figure 14. Automated analysis of metabolism and cell type by flow cytometry.

(A) Heat map of cell type abundance. (B-C) Glucose uptake and mitochondrial activity of cell fractions within ascites (B) and tumor (C). Boxplots show medians (lines), interquartile range (box hinges) and range of data (box whiskers; excepting outliers, shown as points). (D-E) Correlation between glucose uptake and mitochondrial activity of cell fractions within the ascites (D) and tumor (E). (F-G) Correlation of glucose uptake (F) and mitochondrial activity (G) of cell fractions between ascites and tumor. Median (per phenotype) of median (per sample) biexponential-transformed Median Fluorescence Intensity (MFI) values shown.

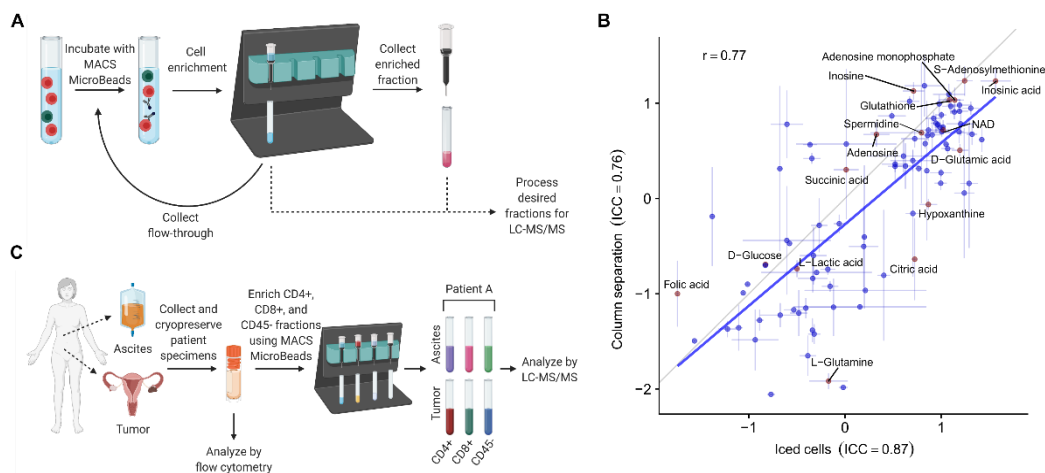


Figure 15. Impact of cell enrichment on metabolomics analysis.

(A) Schematic of magnetic bead enrichment. Cells underwent three consecutive rounds of magnetic bead enrichment or remained on ice prior to analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). (B) Impact of enrichment type on metabolite abundance. Means of triplicate measurements for each enrichment type $\pm$ SE shown. Gray line represents 1:1 relationship. Intraclass correlation for replicate measurements (ICC) shown in axis labels. (C) Schematic of patient metabolite profiling workflow. Ascites or tumor was collected from patients and cryopreserved. A fraction of each sample was analyzed by flow cytometry, while the remaining sample underwent three rounds of enrichment for CD4+, CD8+ and CD45- cells. These cell fractions were analyzed using LC-MS/MS.

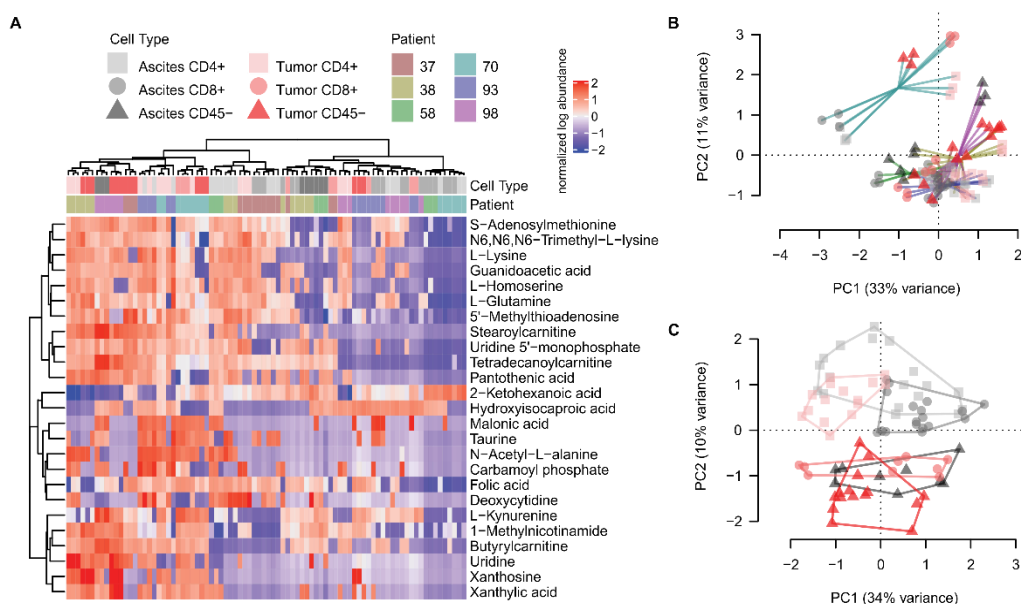


Figure 16. Metabolite profiling of matched ascites and tumor reveals key differences between tumor cells and T cells.

(A) Heatmap of normalized metabolite abundance, with dendrograms representing Ward's clustering of Euclidean distances among samples. (B) Principal components analysis (PCA) of sample metabolite profiles, showing triplicate replicates of each sample, with samples from the same patients joined by lines. (C) PCA of sample metabolite profiles conditioned on patient (i.e., using partial redundancy); sample types are circumscribed by convex hulls.

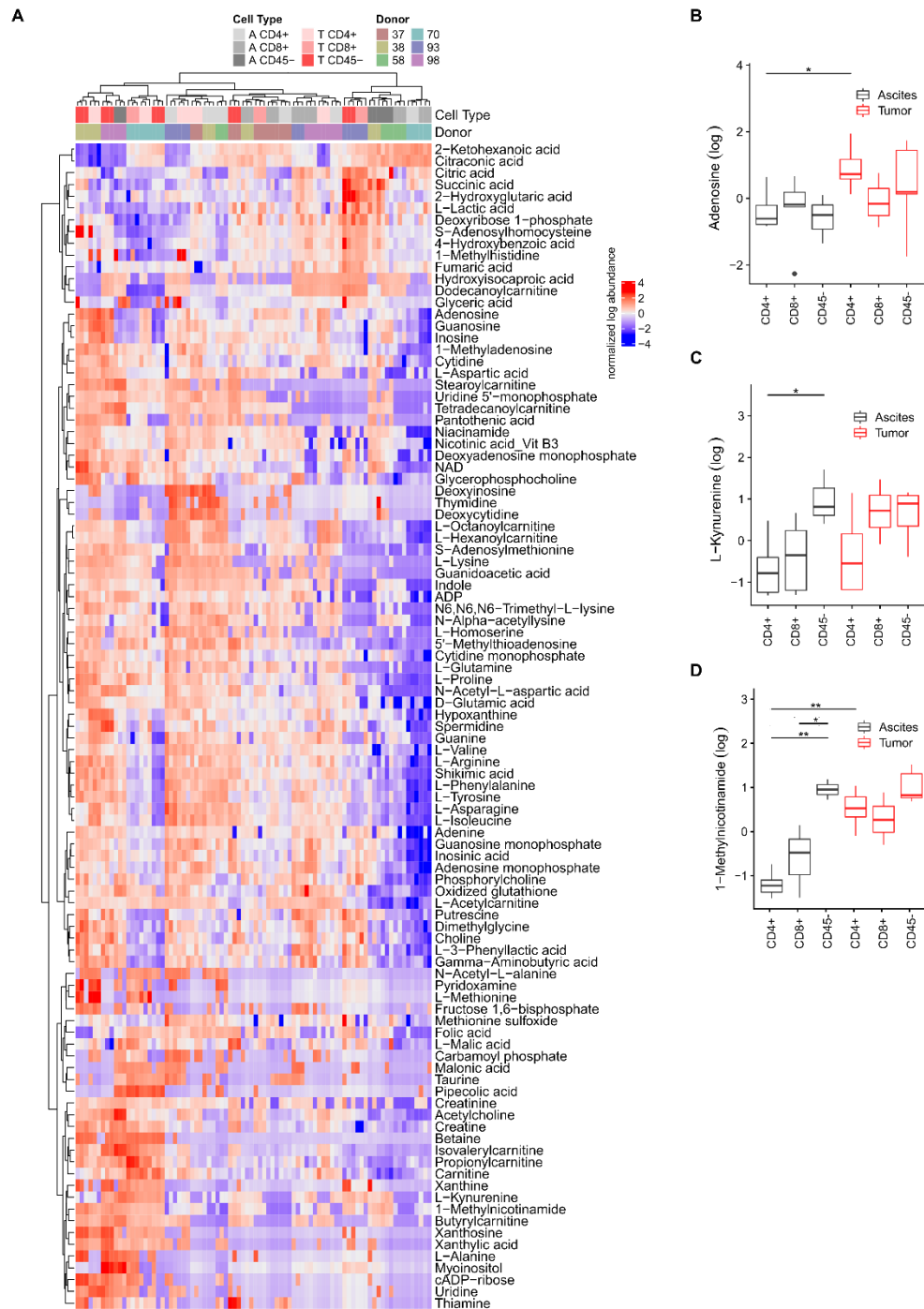


Figure 17. Cell-specific metabolite profiling reveals changes in relative metabolite abundance

(A) Heatmap of normalized metabolite abundance, with dendrograms representing Ward's clustering of Euclidean distances among samples. (B-D) Relative abundance of adenosine (B), L-

kynurenine (C), and 1-methylnicotinamide (D) measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). Boxplots show medians (lines), interquartile range (box hinges) and range of data up to 1.5X interquartile range (box whiskers; outliers shown as points). P-values are determined using *limma* with patient as a random effect, as described in methods (*p<0.05).

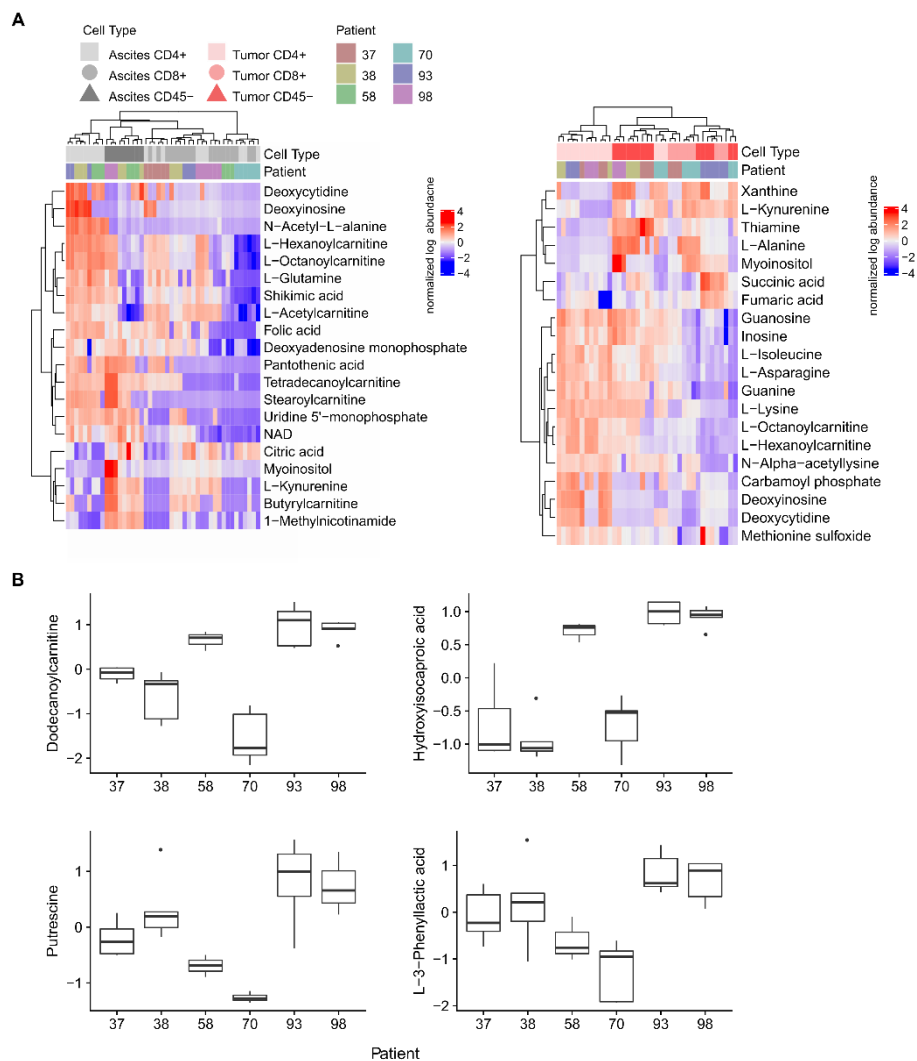


Figure 18. Changes in relative metabolite abundance across cell types within the ascites and tumor.

(A) Heatmap of normalized metabolite abundance, with dendrograms representing Ward's clustering of Euclidean distances among samples. Relative abundance of metabolites in the ascites (left) and tumor (right). (B) Top four significantly differing metabolites across patients (all P_{adj} for F-Test of patient effect in *limma* < 0.05). Boxplots show medians (lines), interquartile range (box hinges) range of data up to 1.5X interquartile range (box whiskers; excepting outliers, shown as points).

3.6 Results

3.6.1 Metabolism of T cells and tumor cells in the ascites and tumor microenvironments

We evaluated the phenotypic and metabolic states of cells in the matched ascites and tumor environments from six patients with HGSC (Table 2) using high-dimensional flow cytometry to synchronously quantify glucose uptake (2-NBDG) and mitochondrial activity (MT DR) (84–86) alongside canonical markers to distinguish immune and tumor cell populations (Table 3, Figure 11. A). This analysis revealed high levels of glucose uptake in tumor cells relative to T cells in both the ascites and tumor, but more modest differences in mitochondrial activity. Tumor cells (CD45-EpCAM⁺) had on average 3–4 times the glucose uptake of T cells, whereas CD4⁺ T cells had on average 1.2 times the glucose uptake of CD8⁺ T cells, suggesting that tumor infiltrating lymphocytes (TILs) have different metabolic requirements even within the same TME (Figure 11. B). In contrast, the mitochondrial activity in tumor cells was similar to CD4⁺ T cells, and both cell types had greater mitochondrial activity than CD8⁺ T cells (Figure 11. C). Collectively, these results reveal a metabolic hierarchy, with tumor cells more metabolically active than CD4⁺ T cells, and CD4⁺ T cells more metabolically active than CD8⁺ T cells. Despite these effects across cell types, there were no consistent differences in the metabolic states of CD4⁺ and CD8⁺ T cells, or their relative proportions, in the ascites compared to the tumor (Figure 11. D).

Conversely, within the CD45⁺ cell fraction, there was an increase in the proportion of EpCAM⁺ cells in the tumor compared to the ascites (Figure 11. E). We also observed clear metabolic differences among EpCAM⁺ and EpCAM[−] cell fractions. EpCAM⁺ (tumor) cells had substantially greater glucose uptake and mitochondrial activity than EpCAM[−] cells, consistent with much higher metabolic activity in tumor cells than fibroblasts in the TME (Figure 11. F-G).

Further analysis revealed other clear differences across highly resolved phenotypic states of T cells. Indeed, activated T cells (Figure 12. A-C) were much more frequent (as a proportion of CD3⁺ T cells) in the tumor than ascites. Resolving phenotypes by the expression of activation markers (CD25, CD137) and exhaustion markers (PD1) revealed that while these populations showed some differences in metabolic features (Figure 12. D-G), no consistently significant metabolic differences were observed. Similarly, effector memory (Figure 13. A-B) T cells were

much more frequent (as a proportion of CD3⁺ T cells) in the tumor than ascites. However, no consistently significant metabolic differences were observed between naïve, effector, or memory subsets (Figure 13. C-F).

These results were confirmed through automated assignment of cell phenotypes using a machine learning method (165), which further revealed an abundant myeloid cell population (CD45⁺/CD3⁻/CD4⁺/CD45RO⁺) predominately in patient ascites (Figure 14. A). This myeloid population displayed the highest glucose uptake and mitochondrial activity of any identified cell type (Figure 14. B-G). These results underscore strong metabolic differences across a broad range of cell types found in the ascites and tumors of HGSC patients.

3.6.2 Cellular enrichment and metabolite profiling by mass spectrometry

A major challenge in understanding the metabolomic profiles of TIL has been the need to isolate samples of T cells of sufficient purity, quality, and quantity from tumors. Recent studies have shown that flow cytometry based sorting and bead enrichment methods can cause alterations in cellular metabolite profiles (46, 47, 65). To overcome this, we optimized a bead enrichment approach to isolate and separate TIL from surgically resected human ovarian cancers prior to analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) (See Methods; Figure 15. A). To assess the overall impact of this protocol on metabolite changes, we compared the metabolite profiles of healthy donor activated T cells following the above bead isolation steps to cells that did not undergo bead isolation but remained on ice. This quality control analysis found high correlation among these two conditions ($r = 0.77$), as well as high reproducibility among technical replicates for this panel of 86 metabolites (Figure 15. B). Thus, these methods enabled accurate metabolite profiling in cells undergoing cell type enrichment, to provide a first high-resolution platform for the identification of specific metabolites in HGSC thereby allowing deeper insight into cell-specific metabolic programs.

3.6.3 Metabolite profiling revealed key differences in metabolite abundance across cell types and compartments across patients

Next, we applied this enrichment method to profile 99 metabolites in CD4⁺, CD8⁺, and CD45⁻ cell fractions from the primary ascites and tumor of six patients with HGSC (Figure 15. C). The populations of interest ranged from 2-70% of live cells of the original bulk sample, with high

variability in cellular proportions between patients. After bead isolations, the enriched fractions of interest (CD4+, CD8+ or CD45-) consisted on average of greater than 85% of the total live cells within the sample.

Our profiling also revealed strong metabolic separation of cell types within and across patients (Figure 16. A). In particular, patient 70 exhibited distinct metabolic profiles compared to the other patients (Figure 16. B), indicating the potential for substantial metabolic heterogeneity among patients. Notably, patient 70 had a smaller total volume of ascites collected (80 mL) compared to the other patients (1.2-2 L) (Table 2). Controlling for inter-patient heterogeneity during principal component analysis (e.g. using partial redundancy analysis) revealed consistent changes among cell types, with clear clustering of cell types and/or microenvironments based on metabolite profile (Figure 16. C). Analyses of single metabolites underscored these effects and revealed dramatic differences among cell types and microenvironments (Table 4).

This enrichment approach allowed us to metabolically profile cell populations from human tumor tissues that would not otherwise be possible from bulk samples (Figure 17. A). Using this protocol, we identified L-kynurenine and adenosine, two well-characterized immunosuppressive metabolites that were elevated in T cells from the tumor or in tumor cells (Figure 17. B-C). Thus, these results demonstrate the fidelity and capacity of our cell isolation and mass spectrometry technique to uncover biologically important metabolites in patient tissues. These data also reveal a promising resource set of immunoregulatory metabolites for further study.

Notably, the most extreme difference observed was for 1-methylnicotinamide (MNA), which was enriched in CD45- cells in general, and in tumor infiltrating CD4+ and CD8+ cells (Figure 17. D). This effect was most pronounced for CD4+ cells, while MNA in CD8+ cells also appeared to be strongly affected by the environment. However, this was not significant as tumor CD8+ fractions were only evaluable for three of the six patients.

In addition to MNA, other metabolites less well-characterized in TIL were found to be differentially abundant across cell types in the ascites and tumor (Figure 18. A). While the majority of the analysis focused on the metabolic differences common to all of the patients, the patients themselves exhibited metabolic heterogeneity. The metabolites dodecanoylcarnitine, hydroxyisocaproic acid, putrescine, and L-3-phenyllactic acid were differentially abundance

across patients (Figure 18. B). These metabolites could provide insight into metabolic pathways specific to individual patients, or subsets of patients.

3.7 Discussion

Flow cytometry-based fluorescent markers have been the primary method used to study the metabolism of TIL. These studies have shown that murine and human TIL have a higher propensity for glucose uptake (93, 153) and a progressive loss of mitochondria function (85, 88) when compared to peripheral blood lymphocytes or T cells from secondary lymphoid organs. Although we observed similar results in this study, a key advance was the comparison of the metabolism of tumor cells and TIL from the same resected tumor tissue. Consistent with some of these previous reports, tumor (CD45-EpCAM+) cells from both the ascites and tumor had greater glucose uptake than both CD8+ and CD4+ T cells supporting the concept that high glucose uptake by tumor cells can compete with T cells in the TME. However, tumor cells had greater mitochondrial activity than CD8+ T cells, but similar mitochondrial activity to CD4+ T cells. These results reinforce the emerging theme that oxidative metabolism is important to tumor cells (169, 170). They also imply that CD8+ T cells may be more susceptible to dysfunctions in oxidative capacity than CD4+ T cells, or that CD4+ T cells may use carbon sources other than glucose to maintain mitochondrial activity (171, 172).

It should be noted that we observed no differences in glucose uptake or mitochondrial activity between CD4+ T effector, effector memory and central memory cells from the ascites. Similarly, the differentiation state of CD8+ T cells in the tumor was not associated with changes in glucose uptake, highlighting the prominent differences between *in vitro* cultured T cells compared with *in vivo* human TIL (65). These observations were also confirmed using an unbiased automated cell population assignment that further uncovered a prevalent yet metabolically active population of CD45+/CD3-/CD4+ cells with greater glucose uptake and mitochondrial activity than tumor cells. This population may represent a putative myeloid suppressor cell subset or plasmacytoid dendritic cells that were identified in the scRNA-seq analysis, both of which have been reported in human ovarian tumors (173) although further work will be needed to delineate this myeloid subset.

While flow cytometry-based methods permit the elucidation of general differences in glucose and oxidative metabolism between cell types, the precise metabolites that are produced from glucose or other carbon sources utilized for mitochondrial metabolism in the TME are largely undetermined. Assigning the presence or absence of a metabolite to a given TIL subset requires purified populations of cells from a resected tissue. Thus, our cell enrichment method combined with mass spectrometry provides insight into metabolites that are differentially enriched in T cell and tumor cell populations from matched patient specimens. Although this approach has advantages over fluorescence-activated cell sorting, some metabolite pools are likely affected, due to inherent stability and/or rapid turnover rates (65). Despite this, our approach was able to identify two well-established immunosuppressive metabolites, adenosine and kynurenine as being differentially abundant across sample types. It also identified MNA, a metabolite with unknown function in cancer, providing avenues to explore novel immune regulatory metabolites in ovarian cancer.

In conclusion, our metabolome profiling of tumor and TIL subsets provides additional insight into the role of the metabolism ovarian TME. First, using flow cytometry, we determined no differences in mitochondrial activity between tumor and CD4⁺ T cells. However, LC-MS/MS profiling uncovered significant changes in metabolite abundance between these populations suggesting that conclusions regarding the metabolism of TIL and their overall metabolic activities require careful interpretation. Second, MNA was the most differentially abundant metabolite between the CD45⁻ cells and T cells in the ascites but not the tumor. Compartmentalization and tumor location may therefore have differential impacts on TIL metabolism, which highlights the heterogeneity that likely exists within a given microenvironment.

Chapter 4: 1-Methylnicotinamide is an immune regulatory metabolite

Partially adapted from:

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4.1 Acknowledgements and contributions

4.1.1 Author contributions

M.K.K. and P.T.H. designed and performed experiments, analyzed the data, and wrote the manuscript. S.M. performed the ChIP. E.L. and G.C. generated the CAR-T cells. E.L. performed the cytolytic assay. P.T.H., J.S, B.A., J.S, and B.H.N. generated the scRNA-seq data. L.G.Z. and R.J.D. generated and analyzed the metabolite profiling data. A.E.E., R.D.S, K.S.W., and R.G.J. generated and analyzed the cellular MNA content data. J.J.L. conceived the project and wrote the manuscript. S.P.^{1,2,3} performed and analyzed the mass spectrometry for the MNA exposure assays at the University of Victoria Proteomics Centre.

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4.1.2 Author contributions to tables and figures

M.K.K. created Table 5, Figure 19. A, Figure 22. A.

M.K.K created Figure 19. C, Figure 26. D, Figure 28. A, and Figure 29 A and B using BioRender.com.

M.K.K. processed the samples, L.G.Z. generated and analyzed the data, and P.T.H. analyzed the data and generated Figure 19. B.

P.T.H., J.S, B.A., J.S, and B.H.N. generated the scRNA-seq data, and P.T.H. analyzed the scRNA-seq data and created Figure 20 and Figure 26. C.

M.K.K. generated and analyzed the data and created Figure 21. A and B, Figure 23, Figure 24, Figure 25, Figure 26. A, Figure 27. C, and Figure 29. C.

A.E.E., R.D.S, K.S.W., and R.G.J. generated and analyzed the data, and M.K.K created Figure 21. C and Figure 22. B-D.

S.M. generated and analyzed the data and created Figure 26. B.

S.P. generated and analyzed the data, and M.K.K created Figure 27. A and B.

E.L. and G.C. generated and analyzed the data and created Figure 28. B.

Note: Figures 22, 23, 25, 27, and 29 contain unpublished data.

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4.2 Abstract

We devised a sensitive cell isolation and LC-MS/MS method to interrogate the repertoire of metabolites within and between distinct cell types including CD4⁺ and CD8⁺ T cells, as well as tumor cells across the ascites and tumor environments in matched patients with ovarian cancer. This approach uncovered profound increases in levels of MNA in T cells in the tumor, while *in vitro* assays revealed a previously unknown immunomodulatory effect of MNA on T cell function. Specifically, exogenous MNA caused an increase in the expression of tumor necrosis factor alpha (TNF α) and a decrease in IFN γ , leading to suppression of CAR-T cell function in a co-culture killing assay. In addition to these functional effects, exogenous MNA led to changes in cell metabolism, including a decrease in SAM. This was surprising as scRNA-seq and qPCR data demonstrated that T cells do not have the enzymes to produce or degrade MNA. Together, these results suggests MNA metabolism as a potential therapeutic target in ovarian cancer.

4.3 Introduction

Nicotinamide N-methyl transferase (NNMT) is a 264 amino-acid (29.6 kDa) methyl transferase that catalyzes the methylation of nicotinamide and other pyridine-containing compounds using a methyl group from SAM. The methylation reaction forms MNA or pyridinium ions, respectively, along with SAH (Figure 20. A). NNMT is mainly expressed in the liver, but it is also detected in low levels in other tissues. In the liver, inhibition of NNMT alters glucose and cholesterol metabolism *in vivo*, suggesting important metabolic roles for NNMT (174, 175). The role of NNMT in human health and disease has been reviewed elsewhere (174, 175).

NNMT is overexpressed in many different cancer types including breast and ovarian, where it has been linked to proliferation, invasion, and metastasis (176–178). NNMT has a role in epigenetic regulation through impairment of the methylation potential of cells by consuming methyl units from SAM to create MNA. Cancer cells that overexpress NNMT have an altered epigenetic state, including histone hypomethylation (176). Interestingly, NNMT also alters the sensitivity of cancer cells to different metabolic drugs. Specifically, BRAC1 depletion, a common mutation in breast and ovarian cancer, leads to upregulation of NNMT that sensitized cells to drugs that inhibit mitochondrial metabolism and glucose import (177). Given the role of NNMT in metabolism and epigenetic regulation, its role in cancer warrants further research.

The main product of NNMT methylation, MNA, is degraded by aldehyde oxidase 1 (AOX1). AOX1 converts MNA to 1-methyl-2-pyridone-5-carboxamide (2-PY) or 1-methyl-4-pyridone-5-carboxamide (4-PY) (Figure 20. A). This oxidation reaction also produces reactive oxygen species (ROS), specifically H_2O_2 . Like NNMT, AOX1 is mainly expressed in the liver, but can also be found at low levels in other tissue types. Interestingly, AOX1 has different variants across the human population as a result of single nucleotide polymorphisms (SNPs) which lead to different levels of activity.

AOX1 has been shown to have conflicting roles in different tumor types. Epigenetic silencing of AOX1 during advanced bladder cancer progression leads to increased invasive properties (179). Alternatively, higher AOX1 expression was associated with decreased progression free survival in colorectal cancer (180). In ovarian cancer, AOX1 has shown to be downregulated. Knockdown of AOX1 led to inhibition of apoptosis in ovarian cancer cell lines (181).

The metabolite itself, MNA, is mainly found in the liver and kidneys where there is high NNMT and AOX1 expression. Although most studies focus on the enzymes, MNA has also shown to play a role in human disease. It has been reported for the treatment of inflammatory skin disorders where it has shown to have anti-inflammatory properties (182–185) and in Sirt1 stabilization (186). Despite a well described role for NNMT and AOX1 in cancer, the literature on MNA is scarce. However, in renal and ovarian cancer, deuterated MNA was found to be stable and the deuterated groups were not transferred to other metabolites (176). Given the cancer literature demonstrating NNMT overexpression, AOX1 silencing and MNA stability, as well as the previous reports on the immune-regulatory roles of MNA, MNA may have an unexplored role in human cancers.

4.4 Materials and Methods

4.4.1 Patient sample collection and processing

Patient specimens and clinical data were obtained through the BC Cancer TTR, certified by the Canadian Tissue Repository Network. All specimens and clinical data were obtained with either informed written consent or a formal waiver of consent under protocols approved by the

Research Ethics Board of BC Cancer and the University of British Columbia (H07-00463). Samples are stored in a certified BioBank (BRC-00290). Detailed patient characteristics are shown in table S1 and table S5. For cryopreservation, patient tumor samples were mechanically disaggregated using a scalpel and pushed through a 100 μ m filter to obtain a single cell suspension. Patient ascites was centrifuged at 1500 rpm for 10 minutes at 4 °C to pellet cells and remove supernatant. Cells obtained from tumor and ascites were cryopreserved in 50% heat inactivated human AB serum (Sigma), 40% RPMI-1640 (Fisher) and 10% DMSO. These preserved single-cell suspensions were thawed for metabolomic and metabolite assays described below.

4.4.2 scRNA-seq

Single cell transcriptome sequencing was performed on total viably-frozen ascites and tumor samples using the 10X 5' Gene Expression protocol, following viability enrichment with the Miltenyi Dead Cell Removal Kit (>80% viability). 5 cases with matched tumor and ascites available were profiled, although low viability from 1 tumor sample prevented its inclusion. To enable multiplexing of patients, we combined samples from each patient in lanes of the 10X Chromium controller, with separate runs for ascites and tumor fractions. Following sequencing (Illumina HiSeq 4000 26x98bp PE, Genome Quebec; mean of 73,488 and 41,378 reads per cell for tumor and ascites, respectively), we assigned donor identities using *CellSNP* and *Vireo* (187) (based on the common human SNP VCF provided by *CellSNP* for GRCh38). We excluded unassigned cells and those identified as doublets, and matched donors between ascites and tumor samples based on the nearest identity-by-state (IBS) of inferred patient genotypes using *SNPRelate* (188). Based on this assignment, we retained 3 cases with abundant cellular representation in both tumor and ascites fractions for downstream analysis. Following quality filtering steps in the *scater* (189) and *scraper* (190) BioConductor packages, this yielded 6,975 cells (2,792 and 4,183 from tumor and ascites, respectively) for analysis. We clustered cells by expression using *igraph*'s (191) Louvain clustering implementation of the shared nearest neighbour network (SNN) based on Jaccard distance. Clusters were manually annotated into presumptive cell types based on marker gene expression and visualized with t-SNE. The cytotoxic T cells are defined by the expression of *CD8A* and *GZMA*, excluding a subcluster with low ribosomal protein expression. We accessed the published data of Izar *et al.* (162), including

their tSNE embedding, to rule overlap in expression between immune cell markers and *NNMT* expression. Raw sequencing data will be deposited at NCBI dbGAP.

4.4.3 T cell functional assay

PBMCs were isolated from a leukapheresis product (STEMCELL Technologies) by Ficoll gradient density centrifugation. CD3⁺ cells were isolated from the PBMCs using CD3 Microbeads (Miltenyi). The CD3⁺ cells were activated with plate bound CD3 (5 µg/ml), soluble CD28 (3 µg/ml), and IL2 (300 U/ml, Proleukin) in the presence or absence of MNA. On the final day of expansion viability (Fixable Viability Dye eFluor450, eBioscience) and proliferation (123count eBeads, Thermo) was assessed by flow cytometry. Effector function was assessed by stimulating cells for 4 hours with PMA (20 ng/ml) and Ionomycin (1 µg/ml) with GolgiStopTM and monitored for CD8-PerCP (RPA-T8, Biolegend), CD4-AF700 (RPA-T4, Biolegend), and TNFα-FITC (MAb11, BD). Cells for qPCR and ChIP were stimulated 4 hours with PMA (20 ng/ml) and Ionomycin (1 µg/ml). Supernatant for enzyme linked immunosorbent assay (ELISA) was collected prior to, and after 4 hour stimulation with PMA (20 ng/ml) and Ionomycin (1 µg/ml).

4.4.4 qPCR

RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN) following the manufacturer protocol. Samples were homogenized using the QIAshredder (QIAGEN). cDNA was synthesized using the High-Capacity RNA-to-cDNA kit (Thermo). TaqManTM Fast Advanced Master Mix (Thermo) was used to quantify gene expression (following the manufacturer protocol) with the following probes: Hs00196287_m1 (*NNMT*), Hs00154079_m1 (*AOX1*), Hs00427552_m1 (*SLC22A1*), Hs02786624_g1 (*GAPDH*), Hs01010726_m1 (*SLC22A2*). Samples were run on the StepOnePlus Real-Time PCR system (Applied Biosystems) in the MicroampTM Fast Optical 96-Well Reaction Plate (Applied Biosystems) with MicroampTM Optical Adhesive Film (Applied Biosystems). Any Ct value over 35 is considered above the detection threshold and identified as undetectable (U.D.).

4.4.5 Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as described previously (192). Briefly, cells were treated with formaldehyde (1.42% final concentration) and incubated at room temperature for 10 minutes. A supplemental swelling buffer (25 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40) was used for 10 minutes on ice before re-suspension in immunoprecipitation buffer as described (192). Samples were then sonicated with the following cycle: 10 cycles of 20, 1-second pulses with 40 second rest periods. ChIP grade IgG (Cell Signaling; 1 µL), Histone H3 (Cell Signaling; 3 µL), NFAT (Invitrogen; 3 µL) and SP1 (Cell Signaling; 3 µL) antibodies were incubated with samples at 4 °C overnight while rocking. Protein A beads (ThermoFisher) were incubated with the samples for 1 hour at 4 °C with gentle rocking followed by DNA enrichment with chelex beads (Bio-Rad) and protein digestion with proteinase K (ThermoFisher). TNF α promoter detected by PCR, Forward: GGG TAT CCT TGA TGC TTG TGT, Reverse: GTG CCA ACA ACT GCC TTT ATA TG (207 base pair product). Images produced by Image Lab (Bio-Rad) and quantification was performed with ImageJ software.

4.4.6 ELISA

Cell culture supernatants were collected as described above. Assays were carried out according the manufacturer protocol for Human TNF- α ELISA Kit (Invitrogen), Human IL2 ELISA Kit (Invitrogen), and Human IFN- γ ELISA Kit (Abcam). Supernatants were diluted 1:100 for detection of TNF α and IL2, and 1:3 for IFN γ according to manufacturer protocol. Absorbance was measured at 450 nm using the EnVision 2104 Multilabel Reader (PerkinElmer).

4.4.7 Cellular MNA content

PBMCs were isolated from a leukapheresis product (STEMCELL Technologies) by Ficoll gradient density centrifugation. CD3⁺ cells were isolated from the PBMCs using CD3 Microbeads (Miltenyi). The CD3⁺ cells were activated with plate bound CD3 (5 µg/ml), soluble CD28 (3 µg/ml), and IL2 (300 U/ml, Proleukin) in the presence or absence of MNA for 3 days. After 3 days cells were collected, washed with 0.9% saline and the pellet was snap frozen. Cell counts were carried out using 123count eBeads by flow cytometry (Cytex Aurora; 3L-16V-14B-8R configuration).

Metabolite extraction was performed as described above. Dried extracts were reconstituted at a concentration of 4000 cell equivalents/ μ L. Samples were analyzed via reversed phase chromatography (1290 Infinity II, Agilent Technologies, Santa Clara, CA) with a CORTECS T3 column (2.1 x 150 mm, 1.6 μ m particle size, 120Å pore size; #186008500, Waters) coupled to a triple quadrupole mass spectrometer (6470, Agilent) with electrospray ionization operated in positive mode. Mobile phase A was 0.1% formic acid (in H₂O) and mobile phase B was 90% acetonitrile, 0.1% formic acid. The LC gradient was: 0-2min 100% A, 2-7.1 min ramp to 99% B, 7.1-8min hold at 99% B. The column was then re-equilibrated with mobile phase A for 3 minutes at 0.6 mL/min. Flow rate was 0.4 mL/min, and the column compartment heated to 50 °C. A neat chemical standard (M320995, Toronto Research Chemicals, North York, ON, CAN) of 1-MNA was used to establish retention time and transitions (RT = 0.882 min, transition 1= 137 $\rightarrow$ 94.1, transition 2 = 137 $\rightarrow$ 92, transition 3= 137 $\rightarrow$ 78). Transition 1 was used for quantitation when all three transitions were present at the correct retention time to ensure specificity. A standard curve for 1-MNA (Toronto Research Chemicals) was generated from 6 subsequent serial dilutions of a 1 mg/mL stock solution to yield 0.1, 1.0, 10, 100 ng/mL, and 1.0, and 10 μ g/mL standards. The lower limit of detection was 1 ng/mL with a linear response between 10 ng/ml and 10 μ g/mL. 2 μ L of each sample and standard were injected for LC/MS analysis, and a pooled quality control sample was run every 8th injection to ensure stability of the analytical platform. The 1-MNA response of all 1-MNA treated cell samples was within the linear range of the assay. Data analysis was completed with MassHunter Quantitative Analysis Software (v9.0, Agilent).

4.4.8 CAR design and vector cloning

The second generation α FR-CAR construct was taken from Song *et al.* (193). In brief, the construct contained the following: CD8a leader sequence, scFv specific to human α FR, CD8a hinge and transmembrane region, CD27 intracellular domain, and CD3z intracellular domain. The full CAR sequence was synthesized by GenScript and then cloned into a second generation lentiviral expression vector upstream of a GFP expression cassette used to assess transduction efficiency.

4.4.9 Lentivirus production for FR α -CAR-T cells

Lentivirus was generated by transfecting HEK293T cells (ATCC; grown in DMEM supplemented with 10% FBS and 1% PenStrep) with CAR-GFP vector and packaging plasmids (psPAX2 and pMD2.G, Addgene) at a 1:1:1 ratio using Lipofectamine (Sigma). Virus-containing supernatant was collected at 48 and 72 hours post-transfection, filtered, and concentrated by ultracentrifugation. Concentrated virus supernatants were stored at -80°C until transduction.

4.4.10 FR α -CAR-T cell generation

PBMCs were isolated from a healthy donor leukapheresis product (STEMCELL Technologies) by Ficoll gradient density centrifugation. CD8⁺ cells were isolated from PBMCs using positive selection CD8 microbeads (Miltenyi). T cells were stimulated with TransAct (Miltenyi) and expanded in TexMACS medium (Miltenyi; supplemented with 3% heat-inactivated human serum, 1% PenStrep, and 300U/ml IL2) according to manufacturer instructions. 24 hours post-stimulation, T cells were transduced with lentivirus (10 μ l concentrated virus supernatant per 10⁶ cells). Cells were assessed for GFP expression 1-3 days following transduction on a Cytex Aurora (gated on FSC/SSC, singlets, GFP⁺) to demonstrate at least 30% transduction efficiency.

4.4.11 Cytolytic assay

CAR-T cells were cultured in Immunocult (STEMCELL Technologies; supplemented with 1% PenStrep) under the following conditions for 24 hours: without treatment, with 250 μ M of adenosine, or with 10 mM of MNA. After pre-treatment, CAR-T cells were washed with PBS and co-cultured with 20,000 SK-OV-3 cells (ATCC; expanded in McCoy 5A medium (Sigma) supplemented with 10% FBS and 1% PenStrep) in supplemented Immunocult media in triplicate at an effector to target ratio of 10:1. SK-OV-3 cells and SK-OV-3 cells lysed with digitonin (0.5 mg/mL, Sigma) were used as negative and positive controls, respectively. Following 24 hours of co-culture, supernatants were collected and LDH measured according to manufacturer's instructions (LDH Glo Cytotoxicity Assay Kit, Promega). LDH supernatant was diluted 1:50 in LDH buffer. Percent killing was measured using the following formula: % killing = corrected killing / maximum killing x 100%. Where corrected killing = coculture - T cells alone, and maximum killing = positive control - negative control.

4.4.12 OCT inhibitor assay

PBMCs were isolated from a leukapheresis product (STEMCELL Technologies) by Ficoll gradient density centrifugation. CD3⁺ cells were isolated from the PBMCs using CD3 Microbeads (Miltenyi). The CD3⁺ cells were activated with plate bound CD3 (5 µg/ml), soluble CD28 (3 µg/ml), and IL2 (300 U/ml, Proleukin). On day 3, MNA was added to the respective wells. On day 3 and day 5 diphenhydramine, verapamil, or fluoxetine (suspended in DMSO) was added to the respective well. On the final day of expansion (day 7) viability (Fixable Viability Dye eFluor450, eBioscience) and effector function was assessed. Cells were stimulated for 4 hours with PMA (20 ng/ml) and Ionomycin (1 µg/ml) with GolgiStopTM and monitored for CD8-PerCP (RPA-T8, Biolegend), CD4-AF700 (RPA-T4, Biolegend), and TNF α -FITC (MAb11, BD).

4.4.13 Histone methylation

PBMCs were isolated from a leukapheresis product (STEMCELL Technologies) by Ficoll gradient density centrifugation. CD3⁺ cells were isolated from the PBMCs using CD3 Microbeads (Miltenyi). The CD3⁺ cells were activated with plate bound CD3 (5 µg/ml), soluble CD28 (3 µg/ml), and IL2 (300 U/ml, Proleukin) in the presence or absence of MNA for 3 days. Cells were fixed and permeabilized with the BD Cytofix/Cytoperm kit. Histone methylation tri-methylation was monitored by flow cytometry (H3K4me3, H3K27me3, H3K9me3; Cell Signalling)

4.4.14 Cancer cell line culture conditions

HEK293T and CaOV3 were grown in DMEM (Fisher) supplemented with 10% heat inactivated FBS (Sigma), and 1x Penicillin Streptomycin solution (Fisher). SKOV3 were grown in McCoy's 5A (Fisher) supplemented with 10% heat inactivated FBS (Sigma), 2 mM L-Glutamine (Fisher), and 1x Penicillin Streptomycin solution (Fisher). EO771 were grown in DMEM (Fisher) supplemented with 10% heat inactivated FBS (Sigma), 20 mM HEPES (Fisher), and 1x Penicillin Streptomycin solution (Fisher). OVCAR3 were grown in RPMI (Fisher) supplemented with 20% heat inactivated FBS (Sigma), 0.01 mg/mL bovine insulin (?), and 1x Penicillin Streptomycin solution (Fisher). PEO1 were grown in RPMI (Fisher) supplemented with 10% heat inactivated FBS (Sigma), 2 mM L-Glutamine (Fisher), 2 mM sodium pyruvate (Fisher), and

1x Penicillin Streptomycin solution (Fisher). OVCAR4 were grown in DMEM/F12 (Fisher) supplemented with 10% heat inactivated FBS (Sigma), and 1x Penicillin Streptomycin solution (Fisher). ID8 were grown in DMEM (Fisher) supplemented with 5% heat inactivated FBS (Sigma), 1 mM sodium pyruvate (Fisher), 0.1% Insulin-Transferin-Selenium (Fisher), and 1x Penicillin Streptomycin solution (Fisher).

4.4.15 MNA in cancer cell lines and media

Media was collected, and spun at 500g for 5 minutes to pellet cellular debris. The supernatant was removed and stored at -80 until further analysis. To prepare the cell culture media samples, the volume was adjusted by combining 100 μ L of culture media with 900 μ L of methanol-chloroform. Samples were vortexed and sonicated for 1-2 minutes, and then left on ice for 30 minutes. Samples were centrifuged at full speed for 10 minutes and 800 μ L of the supernatant was transferred to a fresh tube. 200 μ L of water and 200 μ L of CF was added to each sample. Samples were vortexed, spun for 5 minutes, and then 80% of the upper metabolite fraction was collected (to avoid the phase layer). 656 μ L of the methanol/water containing metabolite was transferred to an LC-MS glass vial (equivalent to ~64 μ L of starting culture media) for nitrogen evaporation (Organomation Multivap Nitrogen Evaporator) in the fume hood. Samples were evaporated for 10-15 minutes or until dry. Samples were resuspended in 50 μ L of 5% methanol, vortexed and sonicated for 1 min, and spun at 15000 rpm for 5 min.

Cells were trypsinized, washed with saline, and snap frozen in 200 μ L of 80% methanol. To prepare the cell extracts, the pellet of 2 million cells was resuspended in 200 μ L of 80% methanol using MS grad water. Two stainless steel beads were added per tube and the samples were shaken (Retsch MM 400 shaker) at 21 Hz for 1 min. Samples were centrifuged at 10,000 rpm for 10 minutes. 100 μ L of the metabolite supernatant was transferred into a LC-MS glass vial for nitrogen evaporation (Organomation Multivap Nitrogen Evaporator) in the fume hood. Samples were evaporated for 10-15 minutes or until dry. Samples were resuspended in 50 μ L of 5% methanol, vortexed and sonicated for 1 min, and spun at 15000 rpm for 5 min.

Samples were run on the Agilent 6495B Triple Quad mass spectrometer. To quantify MNA, a 10 point standard curve was established by making serial $\frac{1}{4}$ dilutions from the original starting point

of 25 μ M (6.25 μ M, 1.56 μ M, 0.39 μ M, 97.7nM, 24.4nM, 6.1nM, 1.53nM, 0.381nM, 0.095nM). The RT for MNA was 1.371.

4.4.16 Lentivirus production and transduction for AOX1 T cells

Lentivirus was produced through co-transfection of 293T cells with an AOX1-2A-YFP plasmid and 2nd generation lentiviral plasmids (psPAX2 and pMD2.G). 293T cells were seeded (3×10^6 cells) in a 10 cm dish to obtain 70% confluency in 24 hours. On day 2, the media was replaced with 9 mL of Opti-MEM (Fisher, supplemented with 5% FBS). Cells were transfected using lipofectamine 3000 (Fisher) following manufacturer instructions. On day 3 media was replaced with 13 mL of DMEM (Fisher, supplemented with 10% FBS and 1% PenStrep). On day 4 and 5 media was collected and sterile filtered (0.45 μ m). Virus was concentrated using an ultra centrifuge (Optima XL-100K) for 1.5 hours at 25000 rpm. The virus pellet was resuspended in 10 μ l per original 10 cm dish of 293T cells.

PBMCs were isolated from a healthy donor leukapheresis product (STEMCELL Technologies) by Ficoll gradient density centrifugation. CD3⁺ cells were isolated from PBMCs using positive selection CD3 microbeads (Miltenyi). T cells were stimulated with CD3/CD28 Activator (STEMCELL Technologies) and expanded in Immunocult medium (STEMCELL Technologies; supplemented with 1% PenStrep and 300U/ml IL2) according to manufacturer instructions. 24 hours post-stimulation, T cells were transduced with lentivirus (10 μ l concentrated virus supernatant per 10^6 cells). Virus containing supernatant was replaced with fresh media on day 3. Cells were assessed for YFP expression 3-7 days following transduction on a Cytex Aurora (gated on FSC/SSC, singlets, YFP⁺).

4.4.17 Data and materials availability

Raw sequencing data are deposited at the European Genome Phenome Archive (EGAS00001004829).

4.5 Figures and tables

Table 5. HGSC patient characteristics for single cell RNA sequencing.

Patient	Tumor location	Age at surgery	Survival (months)*	Tumor stage[†]	Tumor grade[†]
46	ovary	54	24	3C	3
58	omentum	78	18	4	3
59	omentum	68	26	3C	3
69	omentum	59	17	3C	3
109	ovary	77	62	3C	3

* Survival calculated from the date of diagnosis to the date of death

[†] Tumor stage and grade were determined by a pathologist at the time of surgery

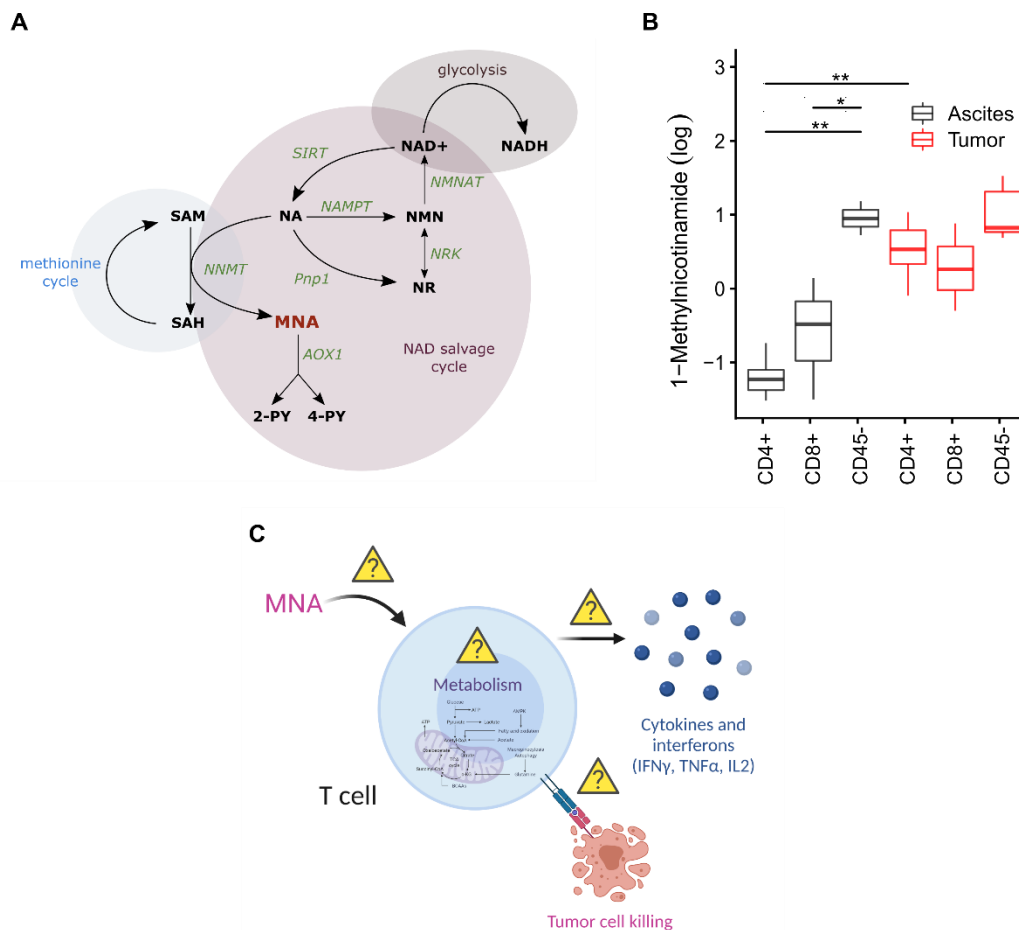
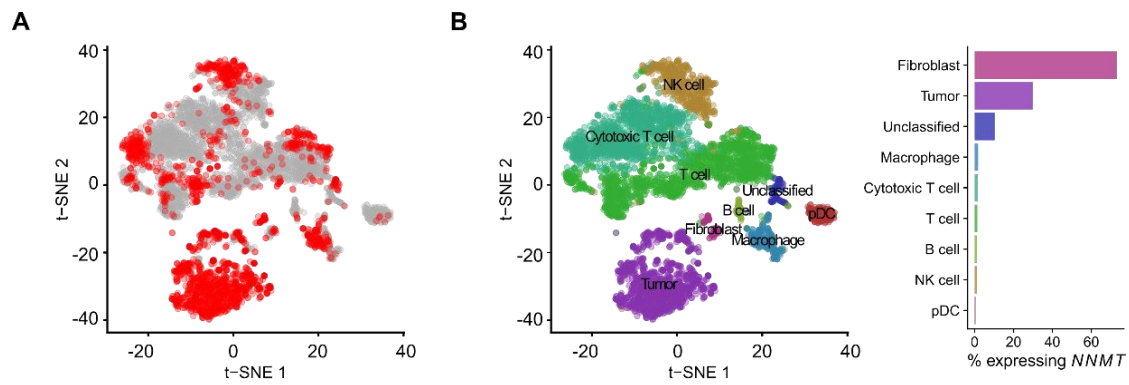
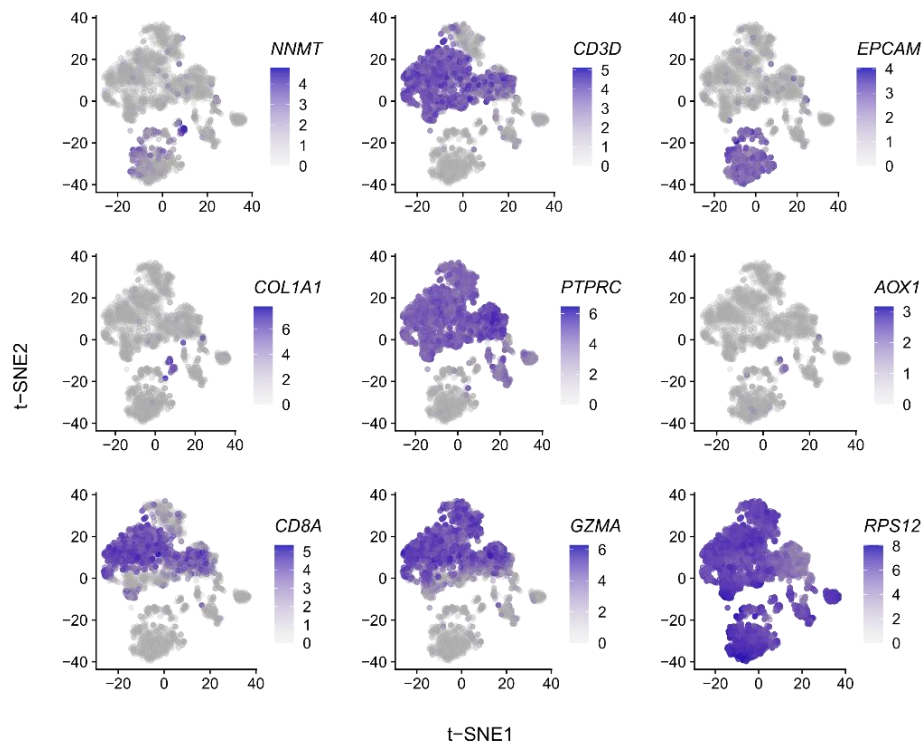


Figure 19. 1-Methylnicotinamide (MNA) is more abundant in T cells from the tumor compared to ascites.

(A) Schematic of MNA metabolism (194). Metabolites: S-adenosyl-L-methionine, SAM; S-adenosyl-L-homocysteine, SAH; Nicotinamide, NA; 1-methylnicotinamide, MNA; 1-methyl-2-pyridone-5-carboxamide, 2-PY; 1-methyl-4-pyridone-5-carboxamide, 4-PY; nicotinic acid, NA; nicotinamide mononucleotide, NMN. Enzymes (green): nicotinamide N-methyl transferase, *NNMT*; sirtuins, *SIRT*; nicotinamide phosphoribosyltransferase, *NAMPT*; aldehyde oxidase 1, *AOX1*; nicotinamide riboside kinase, *NRK*; nicotinamide mononucleotide adenylyltransferase; *NMNAT*; purine nucleoside phosphorylase, *Pnp1*. (B) Normalized abundance of MNA in CD4+, CD8+ and CD45- cells from ascites and tumor. Boxplots show medians (lines), interquartile range (box hinges) and range of data up to 1.5X interquartile range (box whiskers). P-values are determined using *limma* with patient as a random effect, as described in methods (* $p < 0.05$, ** $p < 0.01$). (C) Delineating the impact of MNA on T cells: transport, metabolism, cytokine production, and cytolytic activity.



C Kilgour et al. (n=3)



D Izar et al. (n=6)

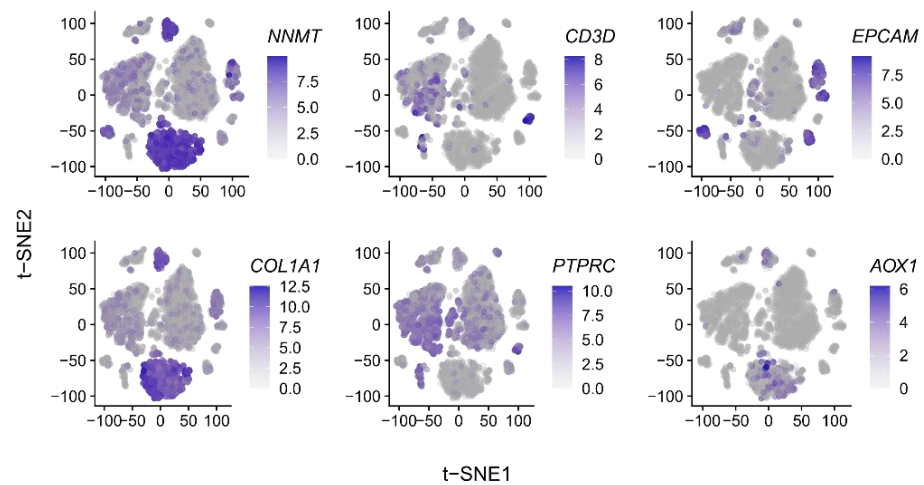


Figure 20. Expression of population defining makers and metabolic genes within the scRNA-seq data.

(A) t-SNE of scRNA-seq of ascites (grey) and tumor (red) (n=3 patients). (B) *NNMT* expression in different cellular populations identified using scRNA-seq. (C-D) Expression of *NNMT*, *CD3D*, *EPCAM*, *COL1A1*, *PTPRC*, *AOX1*, *CD8A*, *GZMA*, and *RPS12* within ascites and tumor, shown as log₂ normalized unique molecular identifier (UMI) counts. Data in (C) was obtained from samples from the BC Cancer Tumour Tissue Repository (n=3), and data from (D) was adapted from Izar *et al.* (n=6) (162) as described in the methods.

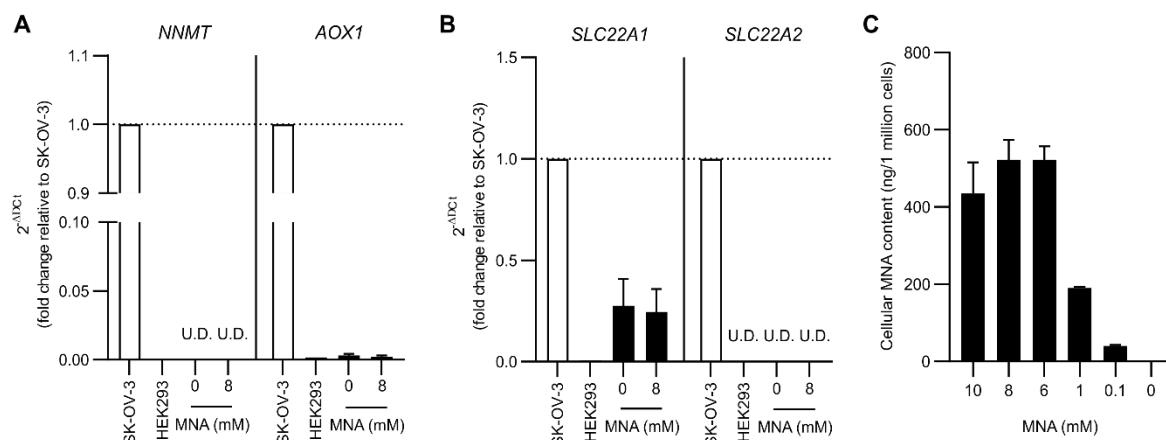


Figure 21. MNA metabolism and transport in healthy donor T cells.

(A) Expression of *NNMT* and *AOX1* in SK-OV-3, HEK 293T, T cells, and T cells treated with MNA. Fold expression is shown relative to SK-OV-3. Means of expression (n=6 healthy donors) with SEM shown. A Ct values greater than 35 is considered undetectable (U.D.). (B) Expression of *SLC22A1* and *SLC22A2* in SK-OV-3, HEK 293T, T cells, and T cells treated with 8 mM MNA. Fold expression is shown relative to SK-OV-3. Means of expression (n=6 healthy donors) with SEM shown. A Ct values greater than 35 is considered undetectable (U.D.). (C) Cellular MNA content in activated healthy donor T cells after 72-hour incubation with MNA. Means of expression (n=4 healthy donors) with SEM shown.

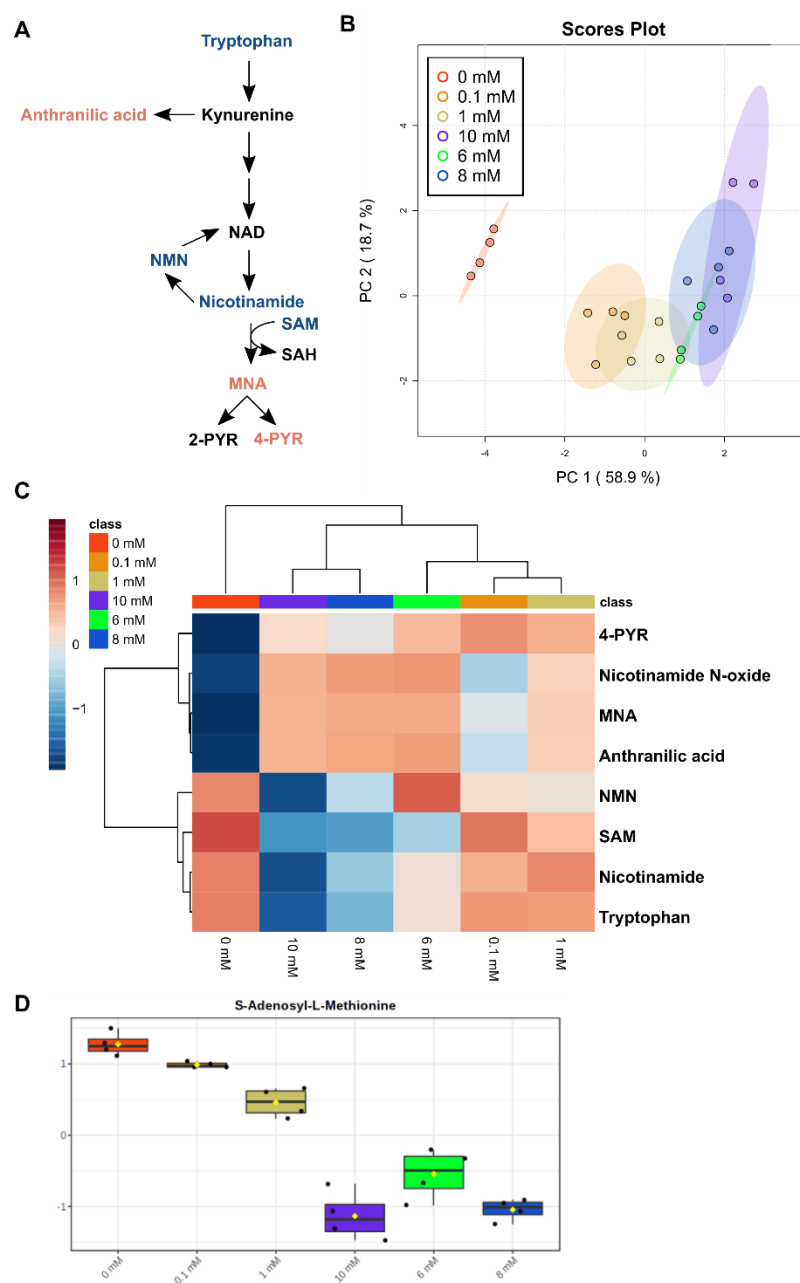


Figure 22. Exposure to MNA impacts T cell metabolism.

(A) Metabolites measured by targeted mass spectrometry. **(B-D)** CD3⁺ cells from healthy donor PBMCs (n=4) were isolated and activated using plate bound CD3 and soluble CD28 and interleukin 2 (IL2) for 3 days with MNA (0-10 mM). Raw abundance values were normalized by cell number, log transformed, and autoscaled. **(B)** PCA analysis of targeted mass spectrometry data. **(C)** Heatmap of metabolite abundance. A Euclidean distance algorithm and Ward clustering algorithm was used. **(D)** Relative intracellular abundance of S-adenosyl-L-methionine.

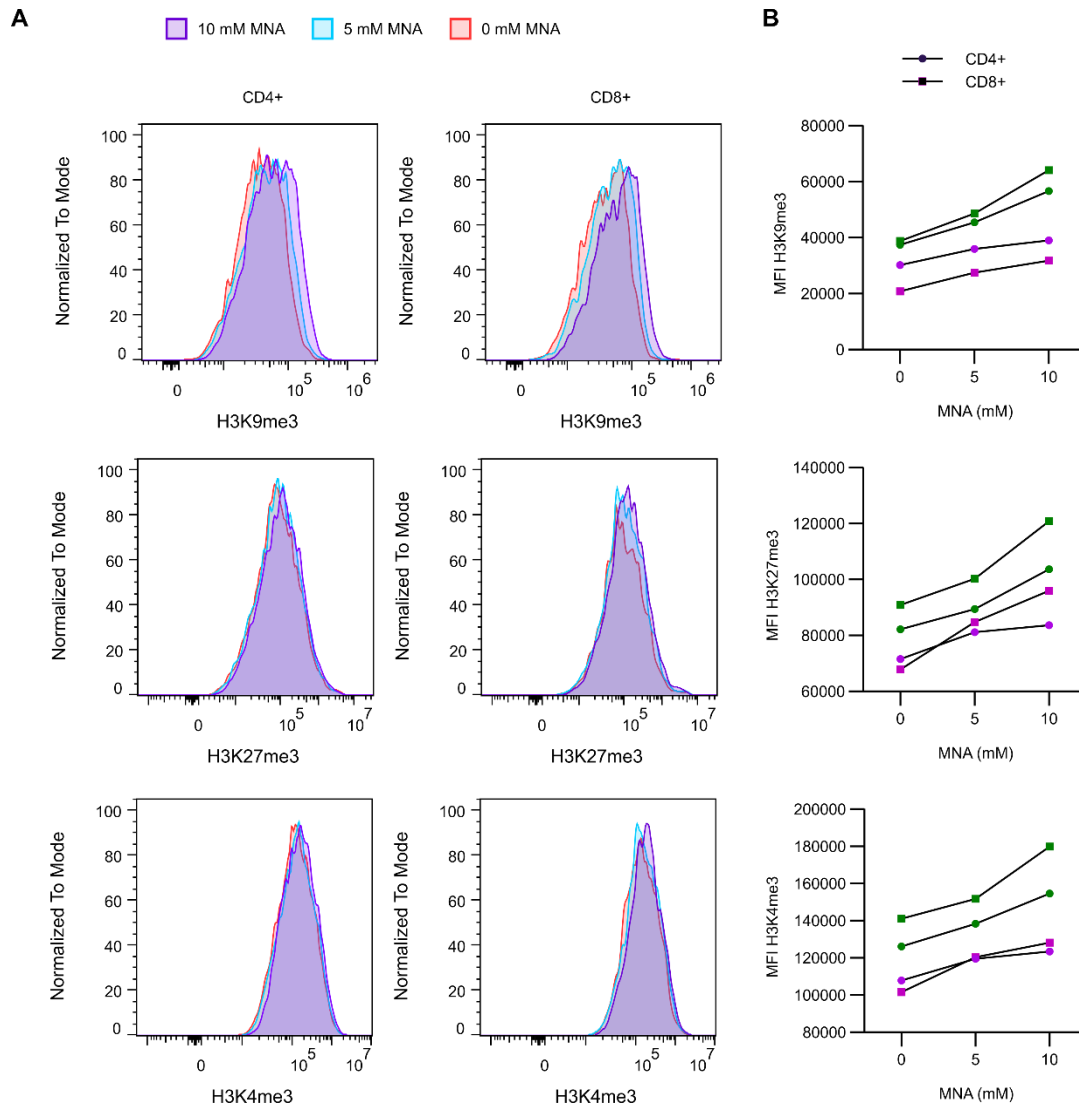


Figure 23. MNA leads to a slight increase in histone tri-methylation on T cells.

(A) Example flow plots for tri-methylation of H3K9, H3K27, and H3K4 of CD4+ (left) and CD8+ (right) T cells. **(B)** Median fluorescent intensity (MFI) of tri-methylation. Lines indicate matched donors (green and pink). Data representative of n=2 independent experiments.

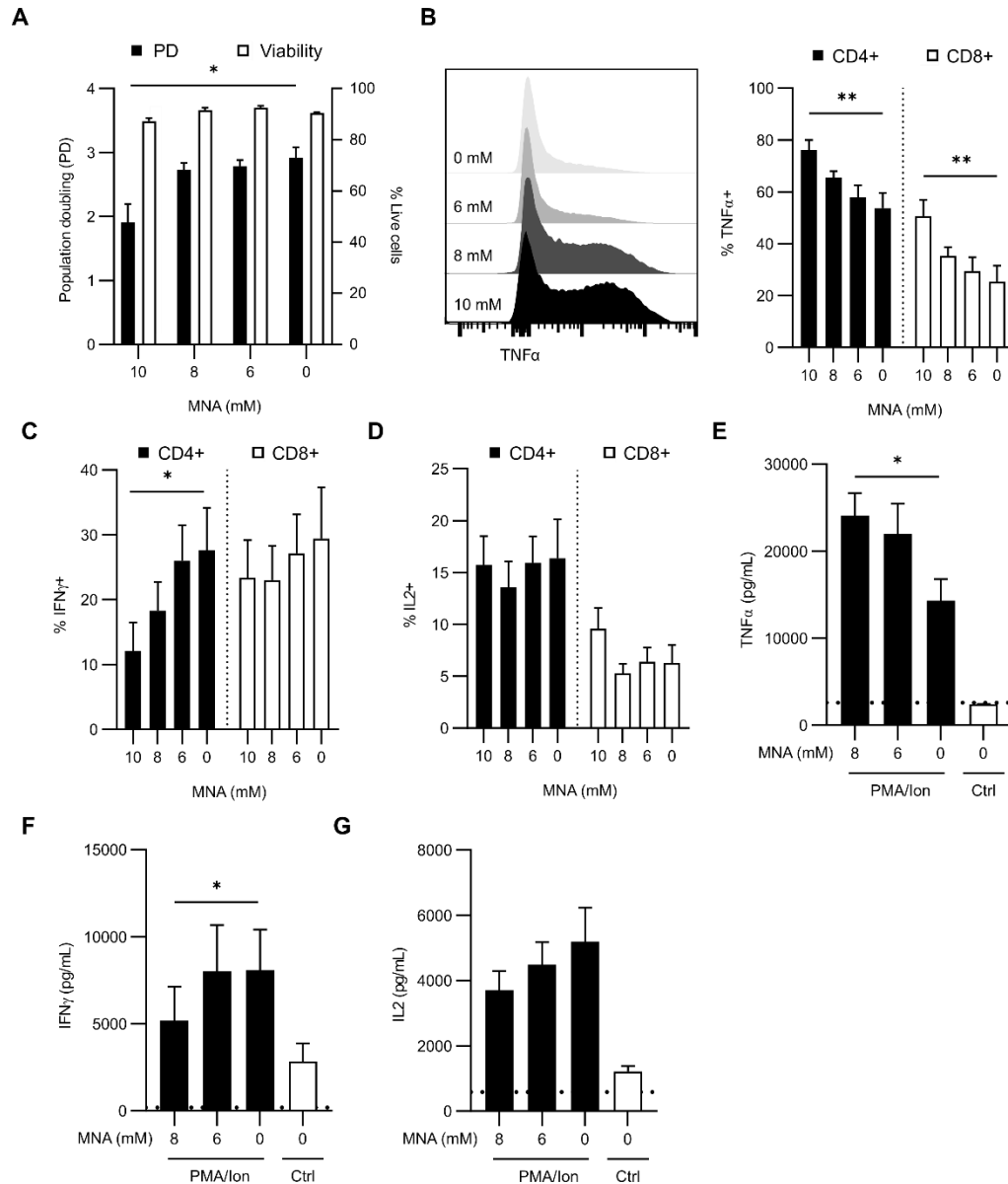


Figure 24. Exogenous MNA enhances TNFα expression and inhibits IFNγ production in T cells.

(A) Total live cell count and population doubling (PD) directly from culture on day 7. Bar graphs represent mean +SEM of 6 healthy donors. Data representative of at least n=3 independent experiments, one experiment shown. (B-D) T cells were activated using CD3/CD28 with interleukin 2 (IL2) in respective concentrations of MNA for 7 days. Cells were stimulated with PMA/Ionomycin with GolgiStop™ for 4 hours prior to analysis. TNFα (B) expression in T cells. Example plot of TNFα expression in live cells (left) and tabulated data (right). IFNγ (C), and IL2 (D) expression in T cells. Cytokine expression was measured by flow cytometry. Bar graphs

represent means (n=6 healthy donors) +SEM. P-value determined using a one-way ANOVA with repeated measures (*p<0.05, **p<0.01). Data representative of at least n=3 independent experiments. **(E-G)** T cells were activated using CD3/CD28 with IL2 in respective concentrations of MNA for 7 days. Media was collected prior to (Ctrl), and after PMA/Ionomycin stimulation for 4 hours. Concentration of TNF α (E), IFN γ (F), and IL2 (G) was measured by ELISA. Bar graphs represent means (n=5 healthy donors) +SEM. P-value determined using a one-way ANOVA with repeated measures (*p<0.05). Dotted line indicates the limit of detection for the assay. P-value determined by Welch's t-test (*p<0.5, **p<0.01).

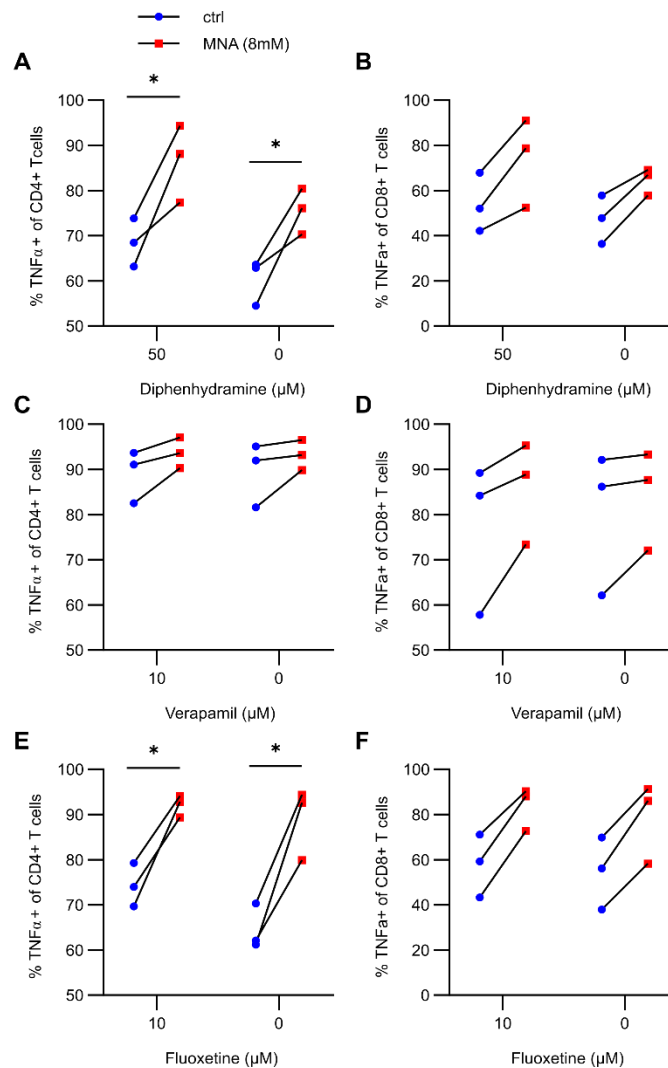


Figure 25. Diphenhydramine, verapamil, and fluoxetine do not impact MNA-induced TNF α expression.

(A-C) T cells were activated using CD3/CD28 with interleukin 2 (IL2) in respective concentrations of MNA for 7 days. On day 3, MNA (8 mM) was added to the respective samples. On day 3 and day 5 diphenhydramine (A), verapamil (B), fluoxetine (C), or DMSO, respectively, were added to the samples. On day 7, T cells were stimulated with PMA/Ionomycin with GolgiStopTM for 4 hours prior to analysis by flow cytometry for TNF α expression. No toxicity was seen at the presented doses. P-value determined by multiple t-tests ($*p < 0.05$).

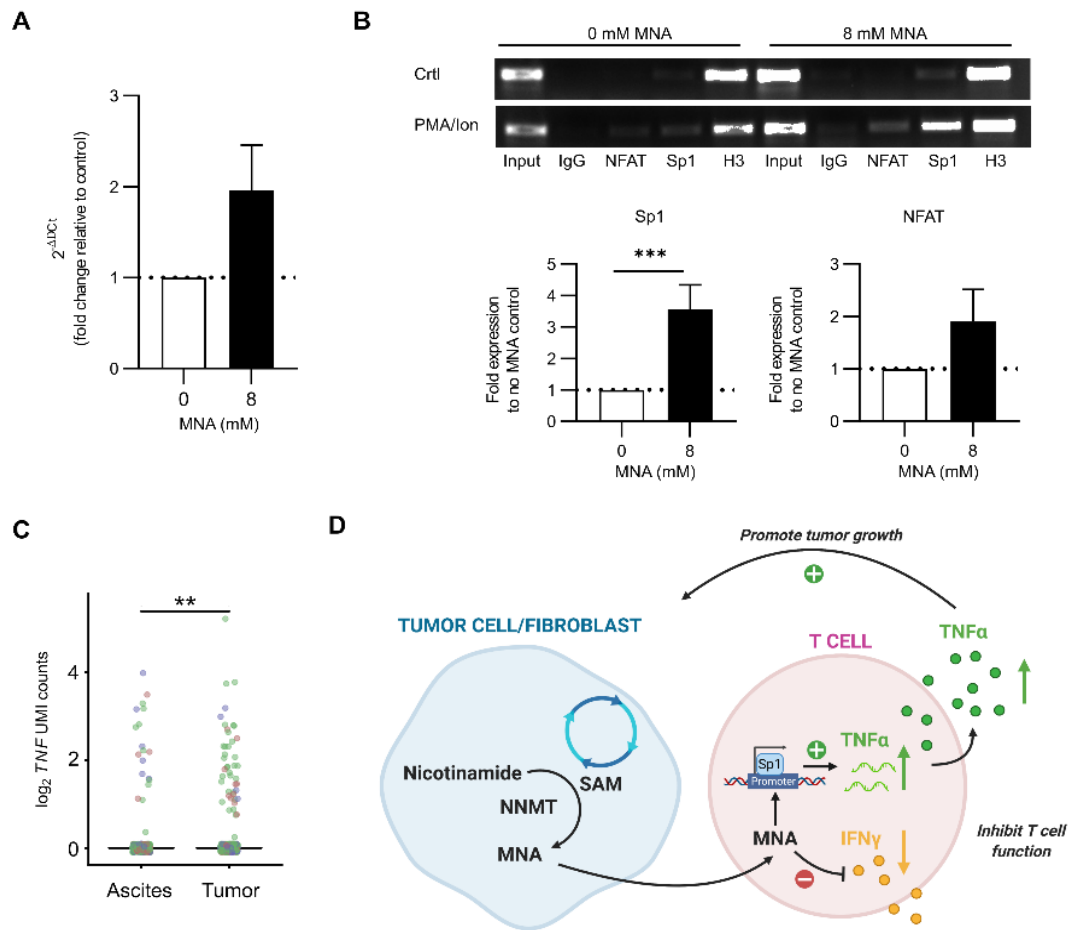


Figure 26. MNA increases binding of Sp1 to the promoter of *TNFα*, increasing *TNFα* transcription and cytokine production.

(A) Fold change in expression of *TNFα* in T cells treated with MNA over T cells cultured without MNA. Means of expression (n=5 healthy donors) with SEM shown. Data representative of at least n=3 independent experiments. **(B)** Nuclear factor of activated T cells (NFAT) and Sp1 binding to the *TNFα* promoter of T cells treated with or without 8 mM MNA prior to (Ctrl) and after 4 hour stimulation by PMA/Ionomycin. IgG and H3 were used as negative and positive controls respectively for the immunoprecipitation. Quantification of ChIP shows the fold increase in Sp1 and NFAT binding to the *TNFα* promoter in MNA treated cells compared to control. Data representative of at least n=3 independent experiments. P-value determined by multiple t-tests (***p<0.01). **(C)** T cells (non-cytotoxic) show increased expression of *TNF* in the tumor relative to the ascites of HGSC. Colors represent different patients. Displayed cells have been randomly subsampled to 300 and jittered to limit overplotting (**P_{adj} = 0.0076). **(D)** Proposed model of

MNA in ovarian cancer. MNA is produced in tumor cells and fibroblasts in the tumor microenvironment and taken-up by the T cells. MNA increases binding of Sp1 to the promoter of *TNF α* , leading to increased transcription of *TNF α* , and cytokine production of *TNF α* . MNA also leads to a decrease in IFN γ . The resulting inhibition of T cell function leads to decreased killing capacity and increased tumor growth.

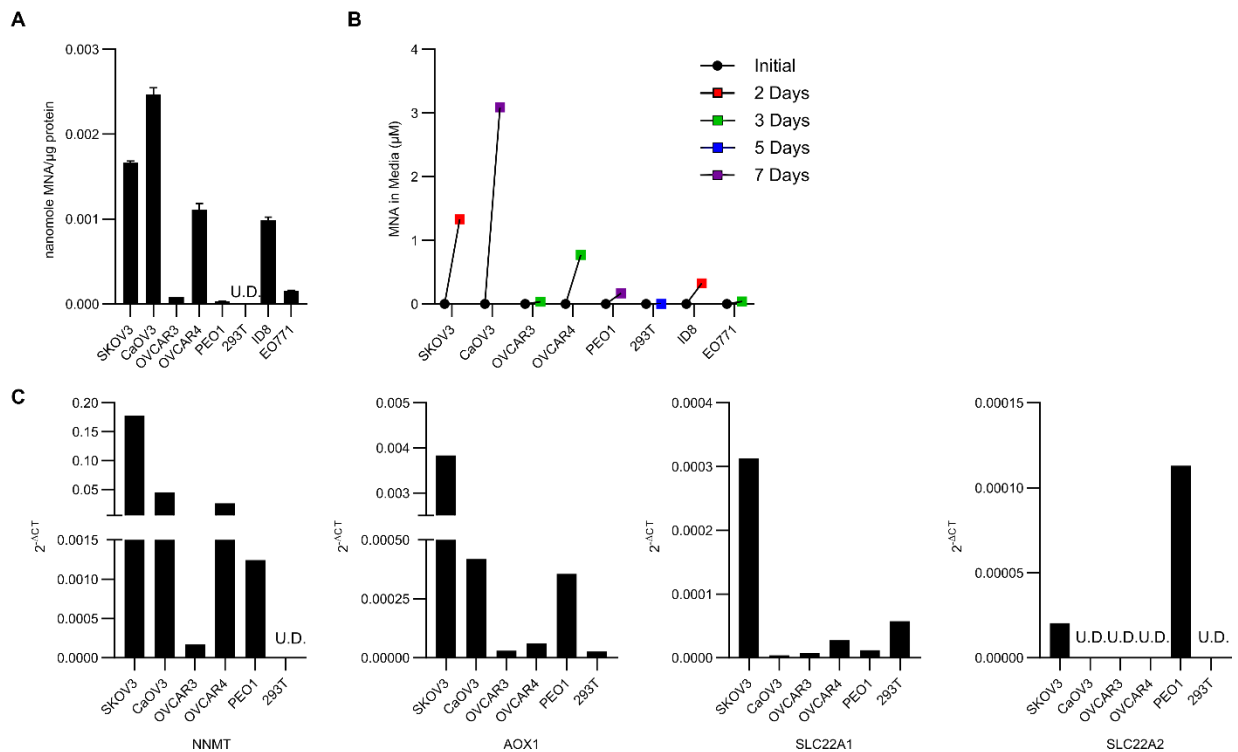


Figure 27. Human and mouse ovarian and breast cancer cell lines produce and secrete MNA *in vitro*.

(A) Cellular MNA content in nanomole of MNA per μg of protein. Means of expression (n=2) with SEM shown. A value of zero is considered undetectable (U.D.). (B) Concentration of MNA in media after 2, 3, 5, or 7 days in culture. 1 million cells were plated in a 15 cm dish with 20 mL of media. Supernatant was collected when the plate reached 90-100% confluent. (C) Expression of *NNMT*, *AOX1*, *SLC22A1*, and *SLC22A2* controlled to *GAPDH*. Means of expression (n=2 technical replicates). A Ct value greater than 35 is considered undetectable (U.D.).

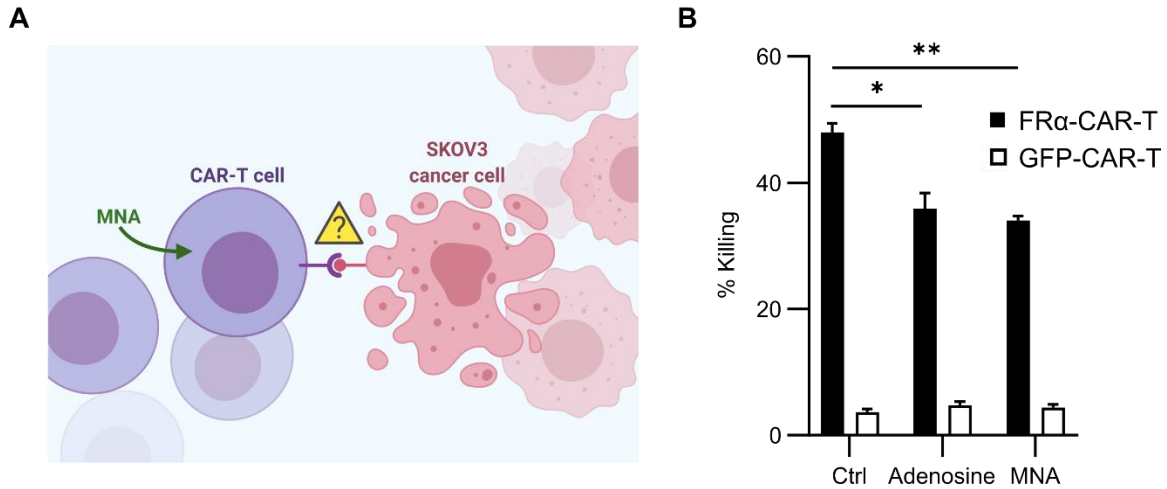


Figure 28. MNA decreases the cytolytic activity of CAR-T cells

(A) Schematic of CAR-T cell cytolytic assay. **(B)** Cytolytic assay. FRα-CAR-T or GFP-CAR-T cells were conditioned with adenosine (250 μ M) or MNA (10 mM) for 24 hours, or left untreated (Ctrl). % killing was measured against SK-OV-3 cells. P-value determined by Welch's t-test (*p<0.5, **p<0.01).

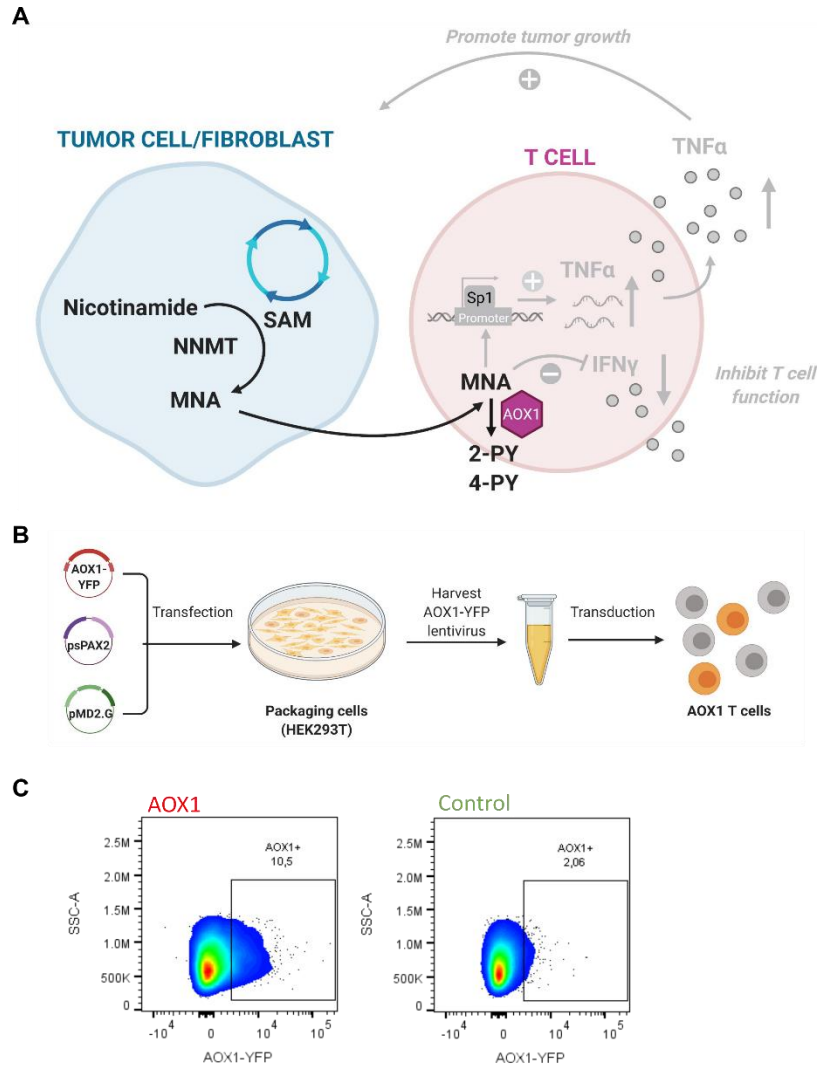


Figure 29. Overexpression of AOX1 in T cells.

(A) Proposed effect of AOX1 expression in T cells. Expression will lead production of 2-PY and 4-PY and the metabolism of MNA will overcome MNA mediated functional effects. (B) Lentiviral strategy to overexpress AOX1 in T cells. Transfection of AOX1-2A-YFP plasmids with 2nd generation lentiviral plasmids (psPAX2 and pMD2.G) in 293T cells to create virus. T cells are transduced with concentrated lentivirus and AOX1 is detected through YFP reporter expression. (C) Detection of AOX1 expression in transduced T cells through YFP reporter expression. AOX1 sample was transduced with AOX1 lentivirus and the control was untransduced.

4.6 Results

4.6.1 MNA is present in tumor infiltrating T cells

MNA had the most significant change in abundance across cell types and compartments among the ovarian cancer patients profiled by mass spectrometry (Figure 19. B). It was 10-100x more abundant in T cells from the tumor compared to T cells in the ascites. However, the impact of MNA on T cells is not well studied. Here, we look at the metabolism and transport of MNA in T cells, as well as its influence on cytokine production, and function (Figure 19. C).

4.6.2 Mechanisms of MNA synthesis, and degradation

To better understand the source of MNA in T cells in the TME, we used scRNA-seq to characterize *NNMT* expression across cell types in the ascites and tumor of three patients with HGSC (Table 5). Profiling ~6,500 cells revealed that *NNMT* expression was confined to presumptive fibroblast and tumor cell populations in both the ascites and tumor environments (Figure 20. A-B). Notably, there was no appreciable *NNMT* expression in any *PTPRC*-expressing (CD45+) populations (Figure 20. C), suggesting the MNA detected in the metabolite profiling is imported into T cells. The expression of *AOX1* was likewise restricted to *COL1A1*-expressing fibroblast populations (Figure 20. C), collectively suggesting that T cells lack the capacity for conventional MNA metabolism. The expression pattern of these MNA-related genes was validated using a second independent data set of cells from the ascites of HGSC patients (Figure 20. D; n=6) (162). These unexpected results suggest that MNA may be secreted from fibroblasts or tumors to neighboring T cells in the TME.

To confirm these results using a secondary method, we carried out a qPCR analysis of healthy donor T cells treated with MNA. The T cells showed little to no expression of either *NNMT* or *AOX1* relative to control SK-OV-3 ovarian tumor cells (Figure 21. A-B). This further supports the conclusion that MNA is not produced or degraded by T cells, and T cells likely take up MNA from their surrounding environment.

To test the possibility that T cells have the capacity to take up exogenous MNA, healthy donor T cells were cultured in the presence of varying concentrations of MNA for 72 hours. In the absence of exogenous MNA, the cellular content of MNA was undetectable (Figure 21. C).

However, activated T cells treated with exogenous MNA exhibited a dose-dependent increase in cellular MNA content up to 6 mM MNA (Figure 21. C). This result suggests that TIL could take up MNA despite a low level of transporter expression and lack of the major enzymes responsible for intracellular MNA metabolism.

4.6.3 MNA decreases intracellular SAM without changing histone methylation

It is not known if MNA can change T cell metabolism. To test this, T cells were cultured with MNA and the relative abundance of metabolites upstream and downstream of MNA were measured using mass spectrometry (Figure 22. A). The changes in metabolite abundance were consistent with the dose response to MNA (Figure 22. B-D). There were decreases in nicotinamide mononucleotide, SAM, nicotinamide, and tryptophan. Alternatively, there were increases in 4-PYR, nicotinamide N-oxidase, and anthranilic acid (Figure 22. C).

Strikingly, exposure to MNA led to 10x decrease in the relative abundance of SAM at the highest dose (Figure 22. D). Although the end result is similar, the mechanism doesn't align with what is currently known about MNA metabolism. In cancer cells, MNA is produced by NNMT and acts as a methyl sink by decreasing the methyl groups available from intracellular SAM. Adding exogenous MNA to T cells cannot be decreasing SAM through this mechanism, so it is currently unknown why SAM is decreasing upon MNA treatment.

In cancer cells, a decrease in SAM corresponds with histone hypomethylation, so we tested if exogenous MNA leads to global changes in histone methylation in T cells. Using flow cytometry, we measured the tri-methylation of H3K9, H3K27, and H3K4 (Figure 23. A-B). We did not find significant changes in histone methylation using flow cytometry.

4.6.4 MNA induces upregulation of TNF α in T cells and a decrease in IFN γ

The metabolite profiles in T cells from patients and the *in vitro* MNA uptake experiments raised the possibility that secretion of MNA by CAFs and tumor cells could modulate TIL phenotype and function. To determine the impact of MNA on T cells, healthy donor T cells were activated *in vitro* in the presence or absence of MNA and assessed for proliferation and cytokine production. Addition of MNA for 7 days led to a modest decrease in the number of population doublings at the highest dose, while viability was maintained across all doses (Figure 24. A).

Furthermore, treatment with exogenous MNA led to an increased proportion of CD4⁺ and CD8⁺ T cells that expressed tumor necrosis factor alpha (TNF α) (Figure 24. B). In contrast, there was a significant decrease in intracellular production of IFN γ in CD4⁺ T cells, but not CD8⁺ T cells, with no significant change in interleukin 2 (IL2) (Figure 24. C-D). Accordingly, ELISA of supernatants from these MNA treated T cell cultures showed a significant increase in TNF α , a decrease in IFN γ and no change in IL2 (Figure 24. E-G).

Since T cells do not have the capacity to produce or degrade MNA, they must be taking up MNA from the surrounding environment. One mechanism is through the organic cation transporter (OCT) family. Blocking these transporters using diphenhydramine (OCT1 (195)), verapamil (OCT2 (196)), or fluoxetine (OCT1 (195)) did not lead to changes in MNA induced TNF α secretion in CD4⁺ or CD8⁺ T cells (Figure 25). Although these inhibitors can also have other effects on the cell, this data suggests an alternative method for MNA uptake into T cells.

4.6.5 MNA increases TNF α mRNA expression through promoter occupancy

To gain mechanistic insight into MNA-dependent regulation of TNF α expression, MNA treated T cells were assessed for changes in TNF α mRNA (Figure 26. A). Healthy donor T cells treated with MNA showed a 2-fold increase in TNF α transcripts, suggesting MNA-dependent transcriptional regulation of TNF α . To investigate this possible mechanism of regulation, two transcription factors that are known to regulate TNF α , nuclear factor of activated T cells (NFAT) and specificity protein 1 (Sp1), were assessed for binding to the proximal TNF α promoter in response to MNA (197). The TNF α promoter contains six identified NFAT binding sites and two Sp1 binding sites, with overlap at one site (-55 base pairs from the 5' cap) (197). Chromatin immunoprecipitation revealed a 3-fold increase in Sp1 binding to the TNF α promoter when treated with MNA. Binding of NFAT also increased and approached significance (Figure 26. B). These data suggest that MNA transcriptionally regulates the expression of TNF α through Sp1 and to a lesser extent NFAT.

While TNF α has been reported to have context-dependent pro- and anti-tumor effects, it has a well-described role in promoting ovarian cancer growth and metastasis (198–200). Patients with ovarian cancer have been reported to have higher concentrations of TNF α within their ascites and tumor tissue than benign tissues (201–203). Mechanistically, TNF α can modulate activation,

function and proliferation of leukocytes, and change the phenotype of cancer cells (204, 205). Consistent with these findings, differential gene expression analysis revealed a significant up-regulation of *TNF* in T cells from tumor tissue compared to ascites (Figure 26. C). Increased *TNF* expression was only apparent in T cell populations with a non-cytotoxic phenotype (Figure 26. C). Taken together, these data support the notion of a dual immune suppressive and tumor promoting role for MNA in HGSC through the regulation of $TNF\alpha$ and $IFN\gamma$ (Figure 26. D).

4.6.6 Human and mouse ovarian and breast cancer cell lines produce and secrete MNA in vitro

Our proposed mechanism suggests that tumor cells (along with CAFs) can produce and secrete MNA. We tested this by measuring the concentration of MNA within various cancer cell lines, as well as the concentration of MNA excreted in the media (Figure 27). SKOV3 and CaOV3 cell lines had the greatest concentration of MNA within the cells and secreted into the media. Alternatively, the 293T cells did not contain detectable levels of MNA (Figure 27. A-B). SKOV3 and CaOV3 cell lines also had the greatest expression of NNMT and AOX1 by qPCR, while the 293T cells had undetectable expression (Figure 27. C). This further suggests that NNMT is responsible for MNA production in cancer cells. Furthermore, the SKOV3 and CaOV3 cell lines would provide an opportunity to study MNA.

4.6.7 CAR-T cell function is suppressed in the presence of MNA

The reduction in $IFN\gamma$ suggested that MNA may function to suppress T cell antitumor activity. To model the impact of MNA on T cell mediated cytotoxicity, folate-receptor alpha targeting chimeric antigen receptor T cells ($FR\alpha$ -CAR-T) and GFP-control CAR-T (GFP-CAR-T) cells were generated from healthy donor PBMCs. CAR-T cells were cultured in the presence of MNA for 24 hours and subsequently co-cultured with folate receptor alpha-expressing human SK-OV-3 ovarian tumor cells at a 10:1 effector-to-target ratio (Figure 28. A). MNA treatment resulted in a significant reduction in $FR\alpha$ -CAR-T cell killing activity that was similar to adenosine treated $FR\alpha$ -CAR-T cells (Figure 28. B).

4.6.8 Overcoming MNA mediated immunosuppression

T cells have no detectable expression of *AOX1* by qPCR and scRNA-seq, suggesting they are unable to degrade MNA. One method to overcome MNA mediated immunosuppression on T cells was to overexpress the enzyme *AOX1* (Figure 29. A). A 2nd generation lentiviral system was designed for the overexpression of *AOX1*. An *AOX1*-2A-YFP sequence was inserted into a pTwistLenti SSFV backbone (Twist Bioscience). The construct, along with the packaging plasmids psPAX2 and pMD2.G were transfected in to 293T cells. The T cells were activated and transduced with *AOX1*-2A-YFP lentivirus (Figure 29. B). Using this method, we were able to achieve expression of the YFP reporter gene in healthy donor T cells (Figure 29. C).

4.7 Discussion

Expression of NNMT, the enzyme that generates MNA, was predominately restricted to CAFs and to a lesser extent tumor cells, yet detectable levels of MNA was observed in T cells from the tumor. The over-expression of NNMT in ovarian CAFs has a known pro-tumorigenic role in part by promoting CAF metabolism, tumor invasion and metastasis (178). Importantly, NNMT expression in CAFs is strongly associated with the TCGA mesenchymal subtype, which is associated with poor prognosis, despite moderate overall levels of TIL (178, 206, 207). Lastly, the expression of *AOX1*, the enzyme responsible for the degradation of MNA, was also restricted to CAF populations, suggesting that T cells lack the capacity to metabolize MNA. These results support the notion that high levels of MNA in T cells may be indicative of an immune suppressive CAF microenvironment although further work will be required to verify this finding.

The presence of MNA in T cells was unexpected given the low level of MNA transporter expression and undetectable levels of key proteins that are involved in MNA metabolism. Both *NNMT* and *AOX1* were undetectable by scRNA-seq analysis of two independent cohorts as well as by targeted qPCR. These results indicate that MNA is not synthesized by T cells but rather taken up from the surrounding TME. Indeed, *in vitro* assays showed that T cells readily accumulate exogenous MNA. However, by treating T cells with drugs that block uptake of metabolites through the proposed OCTs 1-3, we were unable to reverse the function effects

caused by exogenous MNA. This could be due to the drugs having alternative affects on the T cells, the functional effects of MNA being caused by extracellular rather than intracellular MNA, or uptake of MNA is a result of another mechanism.

Our *in vitro* studies demonstrate that exogenous MNA induces expression of TNF α and increased binding of Sp1 to the TNF α promoter in T cells. Although TNF α has both pro- and anti-tumor functions, in ovarian cancer TNF α is known to promote ovarian cancer growth (198–200). Neutralization of TNF α in ovarian tumor cell culture or abolishing TNF α signaling in murine models ameliorates TNF α -mediated production of inflammatory cytokines and suppresses tumor growth (199, 202). Thus, in this context TME-derived MNA may be acting as a pro-inflammatory metabolite through an autocrine loop via a TNF α -dependent mechanism to drive ovarian cancer tumorigenesis and dissemination (198). In accordance with this possibility, TNF α blockade is under investigation as a potential therapeutic for ovarian cancer (204, 208, 209). In addition, MNA impaired the cytotoxicity of CAR-T cells against ovarian tumor cells, providing further evidence of an MNA-mediated immune suppressive role. Overall, these results imply a model whereby tumor and CAF cells secrete MNA into the extracellular TME. This may have dual pro-tumor effects through (a) TNF-induced stimulation of ovarian cancer growth, and (B) MNA-induced suppression of T cell cytotoxic activity (Figure 26. D).

To overcome these MNA-mediated effects, we created T cells that express the enzyme AOX1 using a lentiviral approach. By creating AOX1 expressing T cells, we hypothesize that we will be able to degrade the MNA in the TME and reverse the MNA mediated effects, however, we have been unable to test the function and metabolism of these AOX1 expressing T cells yet. Further experiments will probe the feasibility of this strategy, specifically the efficacy of AOX1 expressing T cells, and eventually AOX1 expressing CAR-T cells in an ovarian TME. These experiments are currently underway.

Chapter 5: Concluding Remarks

5.1 Summary

To understand the immune component of the tumor and overcome challenges in most existing metabolomics studies conducted using bulk samples, we developed a method to study metabolomics within specific cell populations of the TME. In Chapter 2, we describe this novel approach in detail. We also included details for specimen collection and storage, as well as complementary high-dimensional flow cytometry.

We used the methods we developed in Chapter 2 to reveal large immune metabolome differences between cells within the tumor and ascites of matched patients with ovarian cancer, which we described in Chapter 3. This integrated analysis demonstrated differences in glucose uptake and mitochondrial activity between T cells and identified MNA as a non-cell autonomous immune regulatory metabolite. MNA was identified as a metabolite of interest due to its changes in abundance across T cells and tumor cells between the ascites and tumor.

In Chapter 4 we explored the immune modulatory role for MNA. Importantly, T cells treated with MNA had changes in cytokine expression and inhibition of function. To better understand these functional changes, we created T cells that overexpress the enzyme AOX1 to overcome MNA mediated effects. Future studies will test these T cells in *in vitro* and *in vivo* models to determine if AOX1 could be a potential target for cancer immunotherapy.

5.2 Perspective and future directions

It has been known since the 1920s that cancer cells have an altered metabolic state. However, in 2011, in the follow-up to their landmark article (26), Hanahan and Weinberg still defined dysregulated cellular metabolism as an emerging hallmark of cancer (after not being part of their original six hallmarks in their first article published in 2000 (210)). It wasn't until Hanahan's most recent article a decade later that dysregulated cellular metabolism became one of the key hallmarks of cancer (211). Even more recently, immunometabolism has become an emerging field with the rise in interest in immunotherapy that began in the 1980s (212). Thus, the cancer

immunometabolism field as a whole is still in its infancy and requires substantially more research to truly understand the metabolic relationship between metabolism, immunology, and cancer (213).

As the field of immunometabolism matures, so do the techniques available. Of note, metabolic dyes such as 2-NBDG were commonly used at the beginning of this project, during which time we used 2-NBDG to measure glucose uptake in both lymphocytes and tumor cells from patients with ovarian cancer. However, between the publication in *Science Advances* in 2021, and *Nature Protocols* in 2022, it became clear that 2-NBDG is not an accurate measure of glucose uptake in lymphocytes and it is no longer being used for this purpose (89, 90). I have included the 2-NBDG data in the thesis, but please note (as is the nature of scientific progress) other methods are currently being used, and should be used, to measure glucose uptake (89, 138).

More recent work in the cancer immunometabolism field has taken to trying to unravel the metabolic relationship between cancer and the immune system. Most researchers have their “favourite metabolite” that they discovered through a screening method (typically metabolomics) and subsequently demonstrate that it has immune regulatory function (136, 155). In our case, our “favourite metabolite” was 1-methylnicotinamide. Given the redundancy and flexibility of metabolic pathways, the likelihood of any one specific metabolite suddenly improving the survival of cancer patients is low. However, a combination of metabolic alterations and interventions in combination with other treatment strategies could improve the quality and quantity of life for patients with cancer (108).

Our contribution to the field was to develop a novel method and use that method to understand the metabolism of the ovarian TME. Yet, we remain with many unanswered questions. (1) We focused specifically on tumor cells and T cells; however, given the high metabolic activity and prevalence of macrophages in the ovarian TME, this population could be of interest for future studies. (2) While we have stated elucidating the functional effects and respective mechanism for MNA in ovarian cancer, there is still much unknown in this area. How the treatment of T cells with MNA led to decreases in SAM, the transport of MNA into T cells, and MNA metabolism within T cells remains unknown. (3) We have only just started attempting to overcome MNA-mediated effects in the ovarian TME. Pursuing AOX1 expressing T cells or branching off into using NNMT inhibitors (174) are potential avenues in this field. (4) Finally, although MNA had

the largest significant change in abundance, it was one metabolite in a dataset of 99 metabolites. How might these other metabolites be influencing the immune response to ovarian cancer?

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Appendix 1

Metabolomic Analysis Demonstration (created by P.T.H.)

Background

Here, we use the R package `limma` to conduct tests for differential abundance of metabolites across sampled conditions. `limma` is flexible enough that we can specify complex models if needed, including accounting for the fact that we have different sample types that were taken from the same case (i.e., they are not independent).

We require the R packages `MetaboAnalystR` (for data pre-processing and normalization; install instructions [here](#), including the dependency `OptiLCMS`.), and `limma` (for hypothesis testing). `dplyr` and `stringr` are also used for data manipulation.

```
# analysis library(MetaboAnalystR)
library(limma) library(dplyr)
library(stringr)
```

Reading and normalizing data

We begin by normalizing metabolite abundances using `MetaboAnalystR`. This requires that metabolite data be in a specific format; please see their [website](#) for formatting instructions, or look at the example data we have included. Alternatively, normalization can be conducted by dividing each ion count by the total ion count for a sample, log transforming these values, then (optionally) scaling across each metabolite.

In this dataset we've previously imputed all missing values with an ion count of 1000. However, we include a minimum imputation step by `MetaboAnalyst` (which has no effect here). Alternatively, missing data could be imputed using other methods that attempt to supply a more informative value.

We normalize raw ion counts using a sum normalization for each sample ('SumNorm'), then a log transformation ('LogNorm'). In our manuscript we autoscaled each metabolite ('AutoNorm') afterwards, but here we illustrate skipping this step in order to use `limma-trend`. The results are more-or-less the same, in this case.

```
# initialize object and normalize
mSet <- MetaboAnalystR::InitDataObjects("pktable", "stat", FALSE) mSet <-
MetaboAnalystR::Read.TextData(mSet,
    '~/Documents/R/mna_ovarian/data/20190707_Cell_Screening_1213_metabo_2_clean.csv',
    format='colu', 'disc')
mSet <- MetaboAnalystR::SanityCheckData(mSet)
mSet <- MetaboAnalystR::ReplaceMin(mSet) # set at 1000 - has no effect mSet <-
MetaboAnalystR::PreparePrenormData(mSet) # omit scaling by setting scaleNorm = ""
mSet <- MetaboAnalystR::Normalization(mSet, rowNorm = "SumNorm", transNorm =
"LogNorm", scaleNorm = ""
)
```

Analysis of differential abundance

Because we wish to use a more complex statistical framework than `MetaboAnalystR` provides (or provided at the time of our study), we extract the matrix of normalized metabolite abundances from the `MetaboAnalyst` for downstream analysis.

```
metabo_norm <- mSet$dataSet$norm
```

Next, we need the sample metadata to construct a design matrix for statistical modelling. In this case, all of the data we need is embedded in the sample names that were formatted by MetaboAnalyst. We'll abstract these into a new data.frame.

We'll also make a vector corresponding to our 'condition' grouping. Although we have a two-way (factorial) design (i.e., 3 types of cells from each of 2 types of tumor compartment (ascites and tumor)), it can be difficult to interpret such models in multivariate analyses. Instead, we'll create a single grouping factor that combines cell type and tissue compartment, and test for differences between each group (see [limma user's guide](#)).

```
design_df <- metabo_norm %>%
  rownames() %>% str_replace('-', 'n')
  %>% str_replace("\\+", " ") %>%
  str_split_fixed('_', 4) %>% data.frame()
  %>%
  `colnames<-`(c('patient', 'compartment',
                 'cell_type', 'replicate')) %>%
  mutate(group=paste(compartment, cell_type, sep='_'), merge_col=paste(patient,
                              compartment, cell_type, sep='_'))
```

Because our data contain technical replicates for each patient by condition grouping, we'll use the average metabolite value for each grouping per patient. 'Limma can average these for us as part of the analysis. We first create an expression set for convenience.

```
# Create expression set
metabo_e <- new("EList", list(E = t(metabo_norm), targets = design_df))

# Average values by patient and treatment metabo_ave <-
limma::avearrays(x = metabo_e,
  ID = metabo_e$targets$merge_col)

# Construct matrix of dummy variables (design matrix) from treatment. # We will also
simplify the cumbersome column names of the matrix design_matrix <- model.matrix(~ 0 +
metabo_ave$targets$group) colnames(design_matrix) <-
gsub('metabo_ave\\$targets\\$group', "", colnames(design_matrix))

# Make a 'block' of patient to account for correlation
# among samples from the same patient
corfit <- limma::duplicateCorrelation(object = metabo_ave,
  design = design_matrix,
  block = metabo_ave$targets$patient)
```

```

# Fit model using empirical Bayes
fit_lm <- limma::eBayes(limma::lmFit(object = metabo_ave,
                                   design = design_matrix,
                                   block = metabo_ave$targets$patient,
                                   correlation=corfit$consensus), trend = TRUE)

# We can specify the contrasts that we are interested in. # In this case we
will do all single pairwise comparisons contr <- makeContrasts('T_8-A_8',
                    'T_4-A_4',
                    'T_45n-A_45n',
                    'T_8-T_4',
                    'T_8-T_45n',
                    'T_4-T_45n',
                    'A_8-A_4',
                    'A_8-A_45n', 'A_4-A_45n',
                    levels=design_matrix
)

# run model
fit_contrast <- limma::contrasts.fit(fit_lm, contrasts = contr)

# here we use limma trend to better model mean-variance relationships
eb_contrasts <- limma::eBayes(fit_contrast, trend = TRUE)

```

Now that we have fit the core model to identify differential abundant metabolites, we only need to summarize the outputs.

We can use the decideTests function to identify which contrasts (i.e. differences between groups) are significant, and get the most overall significantly changing metabolites using topTable. Here we just show a few of these for space.

```

# identify significant contrasts with decideTests knitr::kable(limma::decideTests(eb_contrasts)[1:3, 1:5])

```

	T_8-A_8	T_4-A_4	T_45n-A_45n	T_8-T_4	T_8-T_45n
1-Methyladenosine	0	0	0	0	0
1-Methylhistidine	0	0	0	0	0
1-Methylnicotinamide	0	1	0	0	0

```

# look at most differential metabolites - just report P-values and F-stat for simplicity
knitr::kable(limma::topTable(eb_contrasts, n=5, coef=1:9)[, 11:13])

```

	F	P.Value	Adj.P.Val
1-Methylnicotinamide	23.970368	0.0000000	0.0000003
Xanthine	8.085605	0.0000813	0.0040242
Xanthosine	6.944117	0.0002518	0.0065067
L-Alanine	6.902235	0.0002629	0.0065067
Deoxycytidine	6.217229	0.0005422	0.0107364

##Plotting

Custom plot or further analyses (e.g. PCA, etc) can be conducted from the normalized data matrix and/or the limma outputs. Keep in mind that the metabolites have not yet been scaled, so scaling may be desirable for some outputs.

Below we show a simple volcano plot for differentially abundant metabolites between the tumor and ascites compartments for CD8 and CD4 t cells.

```
# we can make volcano plots based on the topTable output
# we use ggplot here library(ggplot2)
library(ggrepel) library(cowplot)

# we need to call topTable for the specific contrast we are interested
# in to get the appropriate P values
# add a column of significant names for labeling tt <-
limma::topTable(eb_contrasts, n=Inf, coef=2) tt$rn <-
ifelse(tt$adj.P.Val < 0.05, rownames(tt), "")

# plots - here

ggplot(tt, aes(x=logFC, y=-log10(adj.P.Val), color=ifelse(adj.P.Val < 0.05, 's', 'n.s'))) +
  geom_point() + xlim(c(-2.5, 2.5)) + guides(color='none') +
  scale_color_manual("", values=c('black', 'red')) + geom_text_repel(label=tt$rn) +
  ylab(expression(-log10 Padj)) +
  xlab(expression(log10 fold change (CD4 tumor - CD4 ascites))) + theme_cowplot()
```

