Analyzing macrophage metabolism with Met-Flow

Application note

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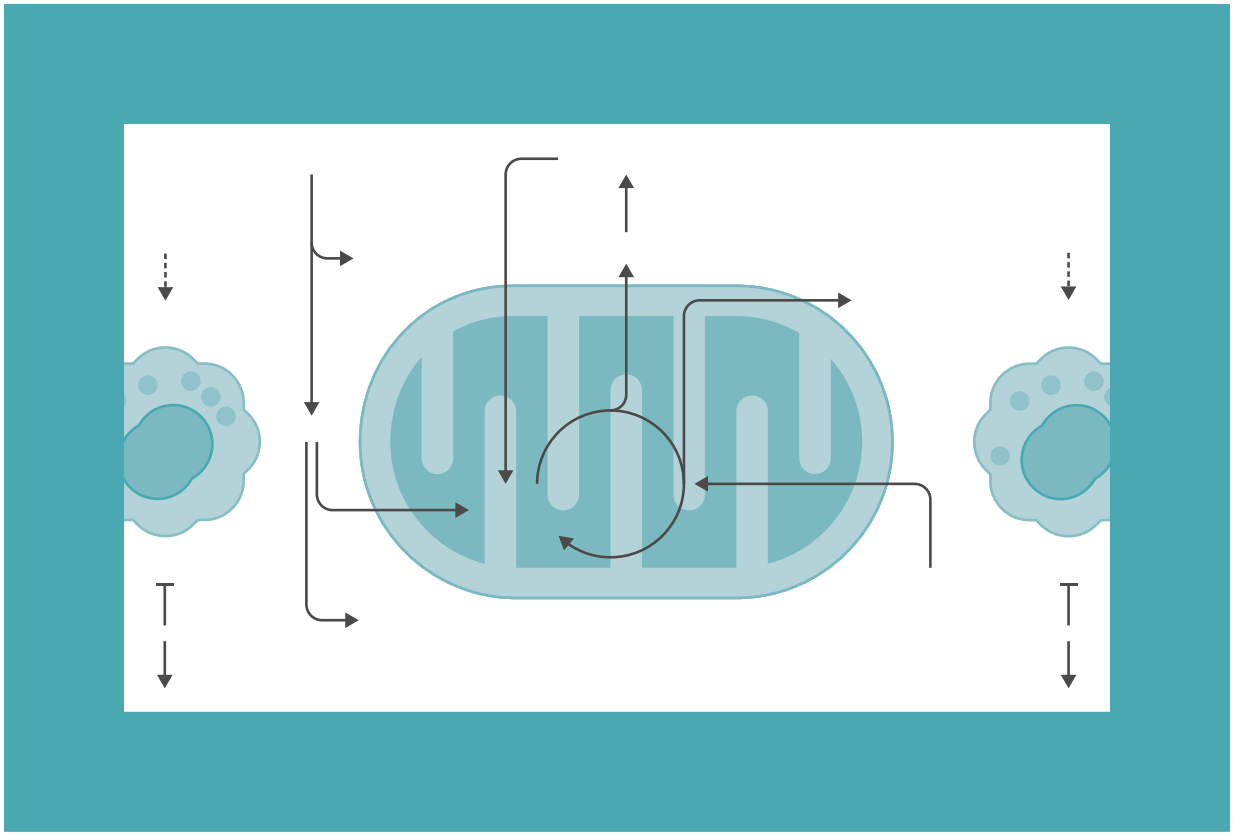

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Introduction

Cellular metabolism is central to the survival and function of any living cell. Yet, its contribution in controlling the behavior of immune cells has become widely appreciated much more recently. Immune cells must be able to respond rapidly and diversely to combat the wide array of infectious threats to the host. It is now clear that changes in intrinsic metabolic activity are an underlying facet of this functional flexibility[1,2]. As intense research in immunometabolism within the past decade has provided important insights into disease mechanisms, it has also yielded great therapeutic potential, particularly in regards to tumor immunotherapy[3]. Given the importance of metabolism in immunity, and its promise to develop new treatments, it is increasingly becoming an integrated part of immunological studies.

Metabolism is an extensive network of transporters and enzymes, but early work in immunometabolism focused on a handful of central core pathways used to generate energy for the cell (Fig. 1). These pathways were glycolysis, oxidative phosphorylation (OXPHOS), glutaminolysis, long-chained fatty acid oxidation and synthesis, and the pentose phosphate pathway (PPP)[1]. Depending on the activation, location or effector state of the cell, these pathways were found to be dynamically regulated, particularly at the branch point of aerobic glycolysis and mitochondrial oxidation. Glycolysis has been linked more strongly to cells in an inflammatory or acute effector state, such as recently activated T cells. In contrast, mitochondrial OXPHOS is associated more with cells possessing a regulatory phenotype or are long-lived, including memory and regulatory T cells[1,4].

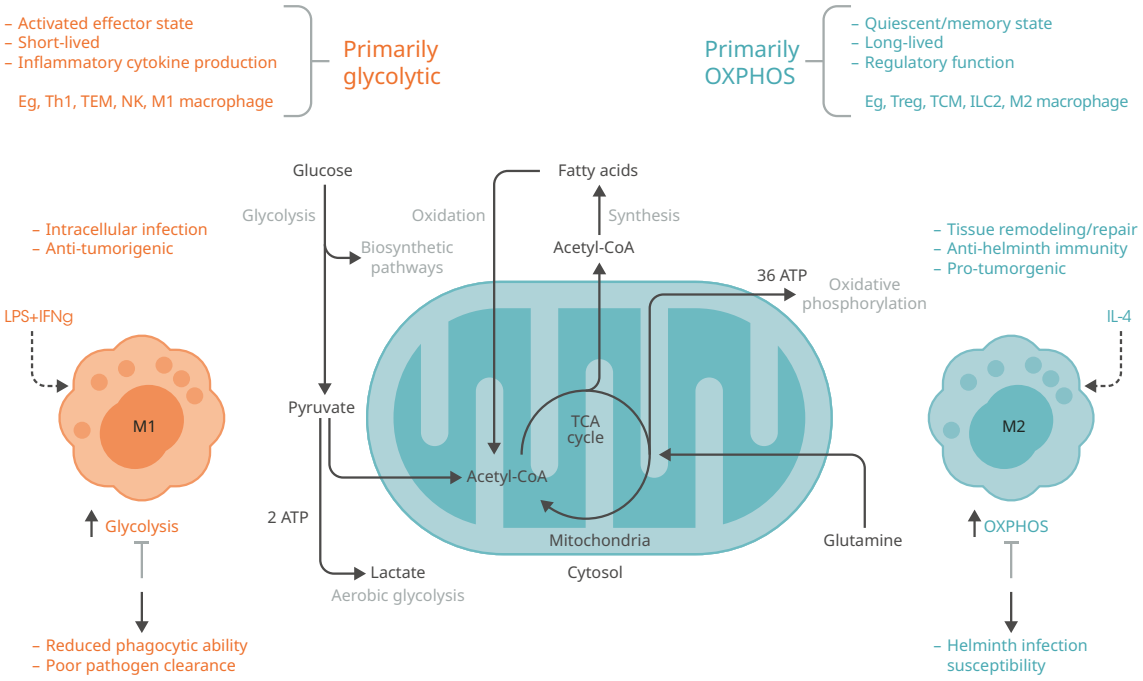

Figure 1. Metabolic divergence in immune cell populations. Core metabolism represents the key pathways known to contribute to ATP production and redox status of the cell, including glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), pentose phosphate pathway (PPP), amino acid (namely, glutamine) catabolism, and fatty acid synthesis/oxidation. Early discoveries in immunometabolism revealed dynamic regulation of metabolic pathways depending on immune activation or function. A pattern emerged of inflammatory/cytotoxic cells acquiring an anabolic metabolism defined by increased (aerobic) glycolysis at the expense of OXPHOS, in contrast to the catabolic breakdown of fatty acids and amino acids observed in quiescent or regulatory cell populations. This dichotomy is exemplified by the extremes of M1/M2 macrophage polarization. Inhibition of the respectively required pathways interferes with macrophage polarization, highlighting the critical contribution of metabolism to immune function.

Several technologies have been applied to investigate immune cells' metabolic requirements; however, as studies progress deeper into our understanding of immunometabolism, the technologies become more limited. Commonly used Seahorse XF analysis provides detailed real-time measurements of metabolic activity but requires an abundance of cells that is often not achievable, especially in the single-cell era. A preliminary picture of cellular metabolism can be obtained by single-cell RNA-sequencing but faces the caveats of limited read depth and discordance between transcription and translation, which was previously shown for metabolic enzymes in immune cells[5].

Flow-cytometric methods so far have found a compromise for overcoming some of these limitations. Recent studies on T cells have used both standard flow cytometry[6] or mass cytometry[7,8] to validate the detection of enzymatic targets as a metabolic readout *in vitro* and *ex vivo*, matching changes in metabolism and protein expression to the cell functional status. Here, we further validate if a single-cell metabolic analysis strategy called Met-Flow[6] can distinguish the metabolic status of immune cells using *in vitro* differentiated macrophages.

Macrophages are seeded throughout all tissues in the body and carry out vital functions to maintain tissue homeostasis and protect against pathogens. The metabolism of macrophages has been well studied *in vitro*, using the classical "M1" or "M2" models that represent the extremes of macrophage activation. In this system, M1 inflammatory macrophages, associated with intracellular infection, adopt a highly glycolytic metabolism[9]. In contrast, alternatively activated M2 macrophages, normally associated with helminth infection or tissue wound-healing, acquire a dominantly mitochondrial metabolism that uses glutaminolysis and fatty acid oxidation to sustain OXPHOS[10,11] (Fig. 1). Here we assess macrophage metabolism using Met-Flow with a selection of abcam antibodies (Table 1) that target various core metabolic proteins (Fig. 2). We confirm that the selected antibodies elucidate metabolic differences between *in vitro* differentiated bone-marrow macrophages (Fig. 3) and can also distinguish murine immune populations directly *ex vivo* (Fig. 4). This method opens the door for further interrogation of metabolism using the vast array of metabolic targets available through abcam with flexible panel design and further analysis of physiological samples from rodents or humans.

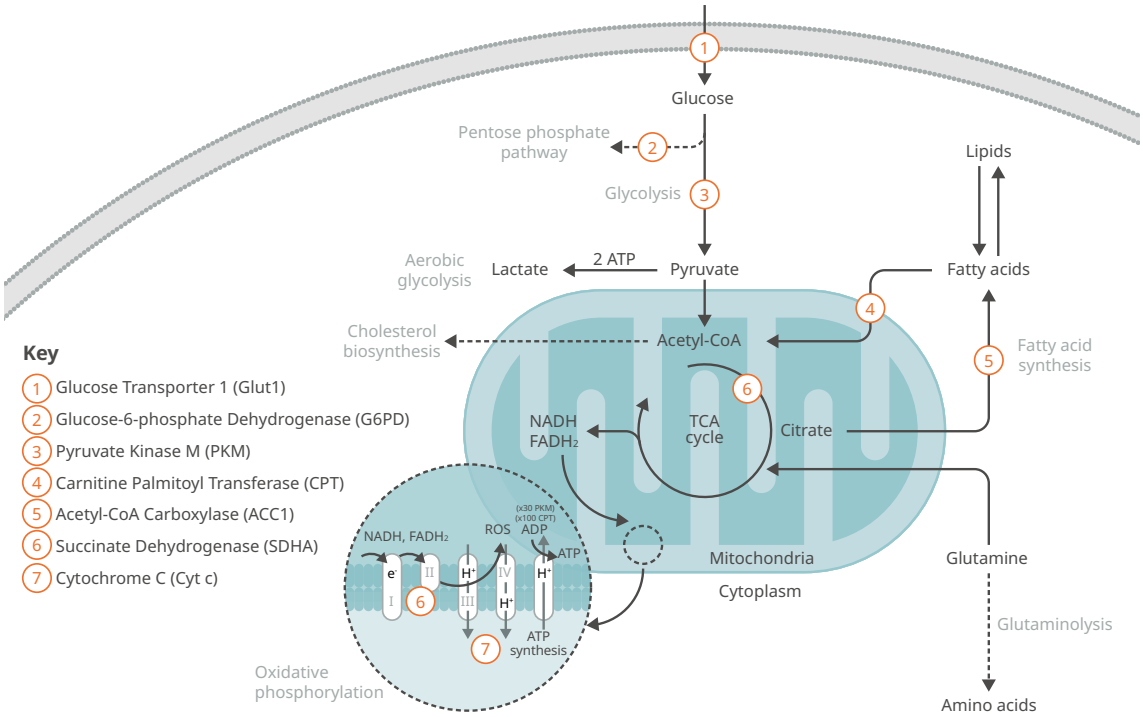

Figure 2. Diagram of selected targets for flow cytometric analysis of macrophage metabolism. This graphical representation illustrates corresponding metabolic pathways for selected targets highlighted in Table 1.

Methods

Macrophages were generated from murine bone marrow over seven days of culture with M-CSF (15% L929 supernatant), then differentiated into M1 or M2 with either LPS (100 ng/mL) and IFNγ (50 ng/mL) or IL-4 (20 ng/mL), respectively. Cells were analyzed 24 hours post-stimulation (Figure 3A). Peritoneal cells were collected by injection and subsequent extraction of 5mL cold buffer (PBS, 2% FCS, 2mM EDTA) into the peritoneal cavity.

Seahorse analysis was done using the mitochondrial-stress test (2 µM oligomycin, 1.5 µM FCCP, 1.5 µM Rotenone/Antimycin A) on an XFe96 analyzer. $1x10^5$ cells were plated in an XF assay plate for overnight stimulation and measured after 20 hours.

To determine the metabolic phenotype of differentiated macrophages, several antibodies from abcam were selected according to previously published literature regarding macrophage metabolism or enzyme detection by flow cytometry[6,8] (Figure 2, Table 1). Antibodies were ordered BSA- and azide-free and conjugated to a fluorochrome using abcam Lightning-Link® kits. Where possible, bright fluorochromes were paired with targets of low expression according to published data[6,8]. Conjugations were done according to the provided kit instructions (Table 1).

Cells were fixed using the eBioscience™ Foxp3 fixation/permeabilization kit and stained for 30 minutes at room temperature in 1x perm/wash buffer. Data acquisition was performed with a 5-laser Cytek® Aurora instrument and analyzed with FlowJo™ v10 Software (BD Life Sciences).

Results

Target resolution for selected antibodies

An obvious but critical requirement for an antibody used in flow cytometry is a sufficient increase in the fluorescent intensity between the stained and unstained samples. The antibodies tested here provide excellent separation between the fully stained sample and corresponding fluorescence minus one (FMO) control (Fig. 3- 4). Generally, detection of targets is still achievable using similar dilutions for bone marrow-derived macrophages (BMDM) or peritoneal exudate cells (PEC), but with reduced intensities, and hence resolution, of some markers. Therefore, in some cases, a more concentrated dilution is recommended for staining *ex vivo* samples compared to *in vitro* (Table 1). The data shown here were generated using a spectral flow cytometer possessing greater sensitivity than standard flow cytometry instruments. Therefore, using standard instruments may need a higher final concentration for staining.

Table 1. Antibodies and corresponding conjugation kits used for flow cytometry. 100 µg of azide- and BSA-free antibody was conjugated according to the respective Lightning-Link protocols. Dilutions are based on the final conjugated stock. *Dilutions are shown for staining the total *in vitro* BMDM cultured in a 24-well plate or for 1x10^6 cells from the peritoneal cavity. Antibodies should be titrated independently before use.

Target	ID	Clone	Reactivity	Dilution*		Modification	Fluorochrome
				in vitro	*ex vivo*		
Glut1	ab252403	EPR3915	Hu/Ms/Rat	1/1000	1/400	Lightning-Link	Dylight 405
PKM	ab206129	EPR10138(B)	Hu/Ms/Rat	1/1000	1/1000	Lightning-Link	PE
SDHA	ab240098	EPR9043(B)	Hu/Ms/Rat	1/1000	1/1000	Lightning-Link	AF647
CPT1A	ab235841	EPR21843-71 -2F	Hu/Ms/Rat	1/1000	1/400	Lightning-Link	PE-Cy5
ACC1	ab272704	EPR23235 -147	Hu/Ms/Rat	1/1000	1/400	Lightning-Link	AF488
CytC	ab237966	7H8.2C12	Hu/Ms (Rat pr.)	1/1000	1/1000	Lightning-Link	PE-Cy7
G6PD	ab231828	EPR20668	Hu/Ms/Rat	1/5000	1/1000	Lightning-Link	APC-Cy7

Protein expression parallels metabolic activity in macrophages

Our flow cytometry analysis of core metabolic pathways, such as glycolysis, OXPHOS, PPP, and fatty acid synthesis, demonstrated metabolic differences between M1 and M2 macrophages (Fig. 3).

Glycolysis (Glut1, PKM): Seahorse is a commonly used assay to determine the metabolic phenotype of macrophages *in vitro*. We compared Met-Flow staining with the glycolytic rate (ECAR) of BMDM determined from the mitochondrial stress test. As already established, lipopolysaccharide (LPS) and interferon-(IFN)γ stimulated macrophages maintained a high basal, in contrast to the low basal ECAR observed for IL-4 treated BMDM (Fig. 3B). BMDM from the same culture showed a significant increase in glucose transporter 1 (Glut1) and pyruvate kinase M (PKM) expression, with little to no increase in M2 over unstimulated macrophages (Fig. 3C). Both targets have previously been linked to positive roles in M1 polarization[9,12].

Mitochondrial OXPHOS (SDHA, Cyt c): This divergence in mitochondrial metabolism between M1 and M2 macrophages is also easily observed by Seahorse, where the oxygen consumption rate (OCR) demonstrates an absence of mitochondrial OXPHOS in M1 macrophages compared to the high OCR seen in M2 (Fig. 3B). Our Seahorse data parallels increased Cytochrome c (Cyt *c*) staining following IL-4 stimulation, the terminal electron transporter of OXPHOS. IL-4 has previously been shown to drive increased Cyt *c* expression[13]. Conversely, succinate dehydrogenase A (SDHA) is required to oxidate succinate and produce reactive oxygen species in M1 macrophages[14], which agrees with increased SDHA expression according to flow cytometry data shown here (Fig 3C).

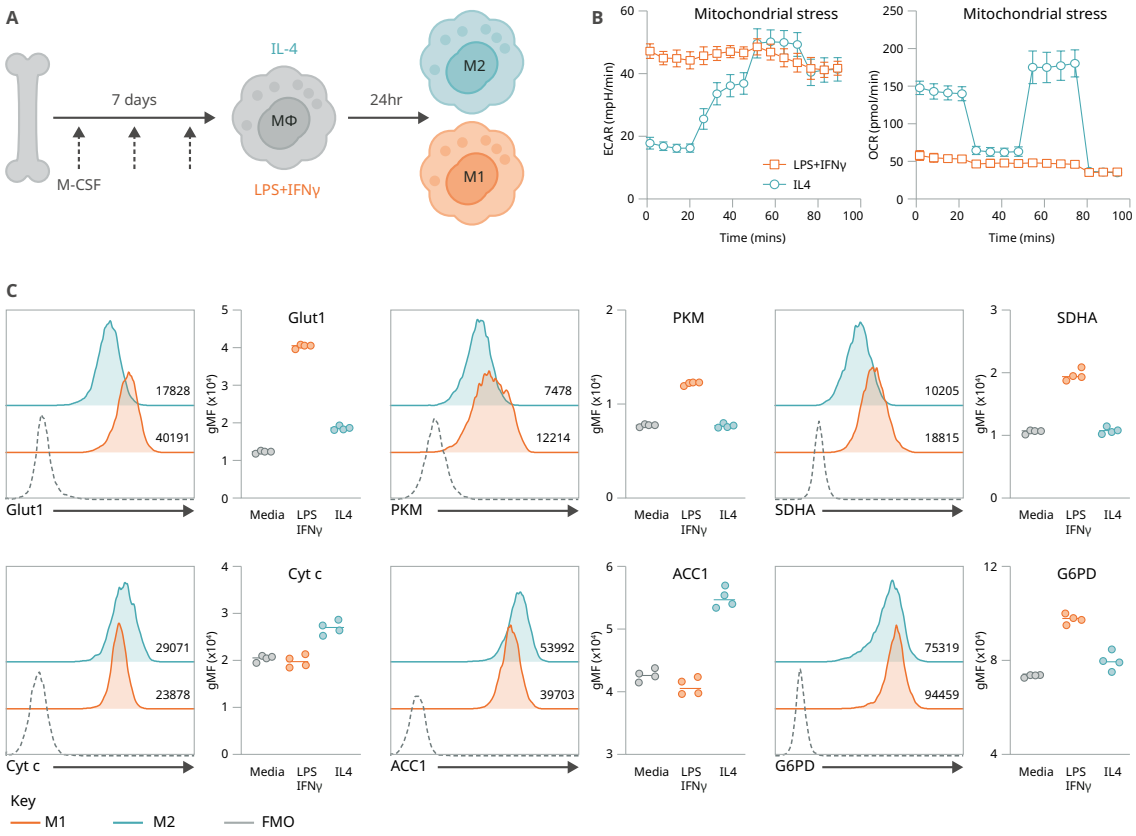

Figure 3. Flow cytometry reveals metabolic differences between M1 and M2 macrophages. (A) Illustration of bone-marrow macrophage differentiation protocol and subsequent polarization to M1 or M2. (B) Seahorse extracellular flux analysis of polarized BMDM using the mitochondrial stress test. ECAR, extracellular acidification rate; OCR, oxygen consumption rate. (C) Histograms and corresponding geometric mean fluorescent intensities (gMFI) of unstimulated (media) or stimulated macrophages for corresponding metabolic targets. Numbers on histogram refer to gMFI. Data points represent technical replicates. FMO, fluorescence minus one.

Pentose phosphate pathway (G6PD): G6PD controls glucose entry into the PPP, a pathway required for nucleotide synthesis, but it is also a critical hallmark of inflammatory macrophages[15]. Accordingly, G6PD staining is increased in M1 vs M2 macrophages (Fig. 3C).

Fatty acid synthesis (ACC1): Lipid droplet formation is a hallmark of M1 macrophages[16], and fatty acid oxidation is a facet of M2. Interestingly, Acetyl-carboxylase 1 (ACC1), a rate-limiting enzyme for fatty acid synthesis, was found to be increased following IL-4-mediated activation. However, lipid droplets may be formed from environmental sources of lipids in M1 macrophages[17], while increased ACC1 is consistent with a PPARγ-driven metabolic program in M2 macrophages[13].

Metabolic target expression varies between in *vivo cell* populations

A strength of having a flow-based readout for metabolism is that it can be readily applied to *in vivo* experiments. We validated the selected antibodies for *ex vivo* analysis of PEC. Previous use of Met-Flow identified major differences in metabolic phenotypes between human peripheral blood lymphocytes and circulating monocytes[6,8]. Therefore, we determined if the murine PEC's macrophages and total T cells display similar differences. A clear distinction can be made between these cellular populations, with macrophages expressing more markers related to fatty acid, mitochondrial and pentose-phosphate metabolism (Fig. 4). Therefore, analysis of *ex vivo* samples can be used to identify metabolic protein expression as a physiological readout for cellular immunometabolism.

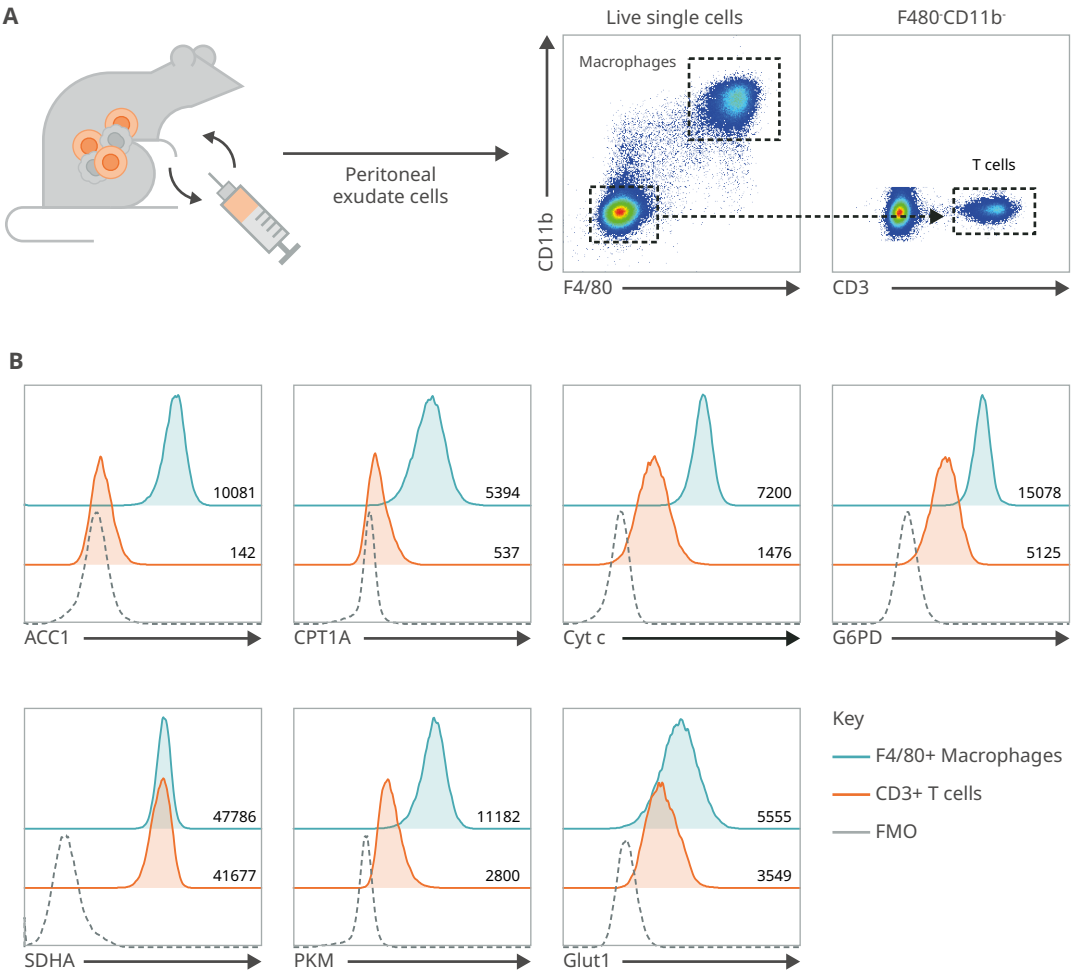

Figure 4. Metabolic targets reveal distinct metabolic phenotypes between immune populations. (A) Representative staining for macrophages and T cells of the murine PEC. (B) Histograms show fluorescent intensity of individual metabolic targets analyzed by flow cytometry for F4/80^{Hi} macrophages and CD3^{+} T cells isolated *ex vivo*. Numbers on histogram plots refer to gMFI. FMO gated on total CD45^{+} cells.

Conclusions

Recently published work has established the value of determining enzyme expression via flow/mass cytometry as a readout for metabolic phenotyping in immune cells. The availability and ease of using high-dimensional analyzers, such as the Cytek Aurora, has made this an achievable and desirable approach to understanding cellular metabolism at the single-cell resolution.

As shown here, abcam antibodies enable robust detection of key metabolic targets in flow-based methods. Combined with an extensive selection of conjugation kits suitable for in-house use, these antibodies can provide an added dimension of flexibility in designing large-scale panels without the cost of custom conjugations. Furthermore, we provide data showing that the single-cell flow cytometry analysis can be applied to macrophage biology.

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Meet the author

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Graham's research aims to understand how cellular metabolism in immune cells can contribute to health or disease, primarily in the context of parasitic infection.

This contribution was made during his time in Bart Everts' group.

His work there focuses on defining tissue macrophage immunometabolism and how it can be manipulated to control innate immune responses. Graham received his PhD from the University of British Columbia in Vancouver, Canada, in partnership with the University of Glasgow, UK.

To find out more about Dr Heieis research on macrophage metabolism, check out his publication: Heieis, G.A., Patente, T.A., Almeida, L. *et al*. Metabolic heterogeneity of tissue-resident macrophages in homeostasis and during helminth infection. *Nat Commun* **14**, 5627 (2023).

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