

Impact of Proline and Glutamine on Glucose Handling and Immune Signaling in THP-1 Monocytic Cells

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Abstract

Type 1 diabetes (T1D) arises from immune-mediated destruction of pancreatic β -cells; rising incidence implicates environmental and metabolic contributors. Prospective metabolomic studies have identified altered circulating amino-acid profiles, notably elevated glutamine and proline, preceding the first islet autoantibody in children who later develop T1D. This thesis tested whether supraphysiological extracellular concentrations of L-proline and L-glutamine increase glucose consumption by innate immune cells and activate nutrient-sensing signaling pathways, particularly the mTOR axis. Human THP-1 monocytic cells were exposed to baseline (1 \times), 2 \times , and 5 \times concentrations of either L-glutamine or L-proline; glucose uptake/consumption was estimated from residual medium glucose at 1 hour (with an additional \pm FBS, \pm insulin comparison at 24 and 48 hours). Secondary outcomes were RPS6KB1 and NFE2L2 transcript levels by RT-qPCR (48 hours) and cell viability by MTS (48 hours). Contrary to the hypothesis, 5 \times proline and 5 \times glutamine was associated with higher residual glucose at 1 hour (reduced net glucose uptake/consumption), while RPS6KB1 and NFE2L2 transcript levels showed no statistically significant differences across treatments. These findings indicate that acute extracellular amino-acid elevation alters glucose utilization in THP-1 cells under the tested conditions and motivates follow-up studies using protein-level and metabolic-flux readouts.

Keywords: *Type 1 diabetes, glutamine, proline, THP-1, glucose uptake, mTOR, immunometabolism*

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Abbreviations

AKT	Protein kinase B
ANOVA	Analysis of variance
cDNA	Complementary DNA
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
HLA	Human leukocyte antigen
IA	Islet autoantibody
LPS	Lipopolysaccharide
mTOR	Mechanistic target of rapamycin
qPCR	Quantitative polymerase chain reaction
RPTOR	Regulatory associated protein of mTOR complex 1
THP-1	Human monocytic cell line
T1D	Type 1 diabetes mellitus
$\Delta\Delta Ct$	Double-delta cycle threshold

Popular Scientific Summary

Type 1 diabetes (T1D) is an autoimmune disorder in which immune-mediated destruction of pancreatic β -cells results in lifelong insulin deficiency. Although genetic susceptibility is a principal determinant of risk, longitudinal metabolomic studies of at-risk children have identified perturbations in circulating metabolites that preceded seroconversion, notably elevated concentrations of the amino acids glutamine and proline. Because amino acids serve both as protein building blocks and as metabolic substrates, altered extracellular amino-acid availability may plausibly influence immune-cell metabolism and signaling during the earliest stages of disease development.

This study evaluated whether increased extracellular L-glutamine or L-proline modifies glucose handling, cell viability, and selected nutrient- and stress-responsive transcriptional programs in an established human monocytic model. THP-1 cells were exposed to three concentrations of each amino acid corresponding to baseline (1 \times), two-fold (2 \times) and five-fold (5 \times) relative to standard culture medium. Short-term glucose consumption was estimated indirectly by measuring residual glucose in conditioned medium after one hour; additional measurements at 24 and 48 hours were used to assess the influence of serum. Cell viability was assessed at 48 hours by an MTS assay, and relative mRNA abundance of **RPS6KB1** and **NFE2L2** was quantified by RT-qPCR at 48 hours with normalization to multiple reference genes. Contrary to the initial hypothesis that supraphysiological amino-acid availability would increase cellular fuel use, exposure to the highest concentrations (5 \times) of both proline and glutamine was associated with significantly greater residual glucose in the medium after one hour, consistent with reduced net glucose uptake under these conditions.

The presence of fetal bovine serum altered glucose handling at later time points, with serum-containing control wells exhibiting reduced glucose consumption at 24 hours. Despite the observed acute change in glucose utilisation, cell viability at 48 hours remained comparable across treatment groups, and transcript levels of **RPS6KB1** and **NFE2L2** did not differ significantly between conditions. These findings indicate that very high extracellular concentrations of glutamine or proline can acutely reconfigure substrate utilisation in THP-1 monocytic cells without inducing overt cytotoxicity or detectable transcriptional activation of the selected nutrient- and stress-responsive markers.

The direction of the effect suggests a shift in metabolic routing or substrate preference rather than a uniform increase in metabolic throughput. However, interpretation is constrained by several methodological considerations: THP-1 cells are an immortalized cell line that may not recapitulate primary immune-cell behavior; the highest amino-acid doses exceed typical physiological ranges; and glucose consumption was inferred indirectly from residual medium glucose rather than quantified by direct flux measurements. To establish mechanistic relevance to early immune activation and T1D pathogenesis, subsequent studies should employ primary human immune cells, isotopic tracer approaches to quantify metabolic flux, and protein-level assays (for example, phosphorylation of mTOR targets and NRF2 subcellular localisation). Experimental paradigms that combine elevated amino acids with inflammatory co-stimulation will be necessary to determine whether the observed metabolic shifts modulate functional immune responses pertinent to the initiation of autoimmunity.

1 Introduction

1.1 Disease background and epidemiology

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by immune-mediated destruction of pancreatic β cells, leading to insulin deficiency and lifelong dependence on exogenous insulin (Atkinson et al., 2014). Susceptibility is strongly influenced by genetics, particularly HLA class II haplotypes yet disease onset is not fully determined by genotype alone (Atkinson et al., 2014; Steck & Rewers, 2011). Epidemiologic data show that T1D incidence has increased in many populations over recent decades, and incomplete concordance in monozygotic twins supports an important role for non-genetic factors (Maahs et al., 2010; Steck & Rewers, 2011). These observations motivate efforts to identify early biological changes and potential environmental or metabolic triggers that precede clinically apparent disease (Knip et al., 2012). The autoimmune process often begins years before diagnosis. A key early hallmark is the appearance of islet autoantibodies circulating antibodies directed against β -cell antigens such as insulin, GAD65, IA-2, and ZnT8 (Atkinson et al., 2014). The first detection of one or more islet autoantibodies is commonly termed seroconversion, and it marks the transition from genetic risk to measurable islet-directed autoimmunity (Atkinson et al., 2014; Pflueger et al., 2011). Understanding biological changes that occur before seroconversion is therefore central to clarifying the earliest stages of T1D pathogenesis and to identifying candidate mechanisms for prevention (Knip et al., 2012, Atkinson, Eisenbarth, & Michels, 2014; Maahs et al., 2010).

1.2 Pre-autoantibody metabolomic signals

Prospective cohort studies of genetically at-risk children have shown that circulating metabolic profiles can shift months to years before the emergence of islet autoantibodies (Orešič et al., 2008; Li et al., 2020). Across multiple cohorts, perturbations have been observed in lipid pathways and amino-acid metabolism during the pre-autoantibody period (Orešič et al., 2008; Li et al., 2020). Among these signals, changes in the amino acids glutamine and proline have been reported around the time of seroconversion or in children who later developed islet autoimmunity (Pflueger et al., 2011; Araujo de Pina Cabral et al., 2015). However, most cohort findings remain correlative: altered metabolite concentrations could reflect upstream drivers, compensatory responses, or unrelated developmental factors. Experimental models are therefore needed to test whether elevations in specific amino acids can directly influence immune-cell metabolic behavior and transcriptional programs in ways that are plausibly relevant to early autoimmunity (O'Neill et al., 2016).

1.3 Immunometabolism: linking nutrients to function

Immunometabolism describes the two-way relationship between cellular metabolism and immune function: metabolic pathways do not only supply energy and biosynthetic precursors, they also shape signaling, gene expression, and effector phenotypes of immune cells (O'Neill et al., 2016). For many innate immune cells, activation is accompanied by increased glycolytic flux and elevated glucose uptake, supported by upregulation of glucose transport and glycolytic enzymes (O'Neill et al.,

2016). This metabolic rewiring can influence downstream functional outputs, including inflammatory signaling and antigen-presenting capacity (O'Neill et al., 2016; Loftus & Finlay, 2016).

1.4 Why glutamine and proline?

Glutamine is the most abundant circulating amino acid and contributes carbon and nitrogen to biosynthesis while also feeding the tricarboxylic acid (TCA) cycle via conversion to glutamate and α -ketoglutarate (Nicklin et al., 2009). Beyond its role as a substrate, glutamine availability can regulate nutrient-sensing pathways, including mTOR signaling, partly through amino-acid transport and exchange processes that couple glutamine flux to mTORC1 activation (Nicklin et al., 2009; LaPlante & Sabatini, 2012). Proline participates in protein synthesis and can engage redox-linked metabolic cycling between proline and pyrroline-5-carboxylate, influencing mitochondrial function and reactive oxygen species (ROS) balance (Phang, 2019). Because redox state and mitochondrial ROS are closely connected to immune activation and stress-response pathways, proline availability could plausibly modulate immune-cell metabolic programmers in a context-dependent manner (Kasai et al., 2020; O'Neill et al., 2016).

1.5 Amino acids and the danger model of immunity

The danger model proposes that immune activation is driven not only by recognition of “non-self”, but also by endogenous signals released or displayed by stressed or damaged tissues (Matzinger, 1994). Although amino acids are not classical damage-associated molecular patterns, abnormal extracellular metabolite concentrations may contribute to a broader “danger” context by altering cellular stress responses, redox homeostasis (the balance between oxidant production and antioxidant defenses), and autophagic flux (the rate of cellular self-digestion and recycling that can influence antigen presentation) (Matzinger, 1994; Kasai et al., 2020). This study therefore treats elevated glutamine and proline as candidate metabolic cues that could modulate early innate immune cell behavior rather than as direct inflammatory stimuli.

1.6 mTOR and immune signaling

The mechanistic target of rapamycin (mTOR) is a conserved kinase that integrates nutrient availability, growth-factor signaling, and cellular energy status to regulate metabolism, growth, and survival (LaPlante & Sabatini, 2012). In immune cells, mTOR complexes (mTORC1 and mTORC2) help coordinate metabolic programmers with activation state and can influence glucose utilization and immune signaling pathways (Linke et al., 2017). Two transcriptional readouts are used in this study to probe nutrient- and stress-responsive pathways.

RPS6KB1 encodes p70S6 kinase 1 (S6K1), a canonical downstream effector in the mTORC1 axis, and changes in its expression can be interpreted as supportive (but indirect) evidence of altered nutrient-sensing programmers (LaPlante & Sabatini, 2012; Salmond et al., 2015). NFE2L2 encodes NRF2, a transcription factor that orchestrates antioxidant and cytoprotective responses and is activated by redox and mitochondrial ROS signals; its expression provides a window into oxidative-stress-linked adaptation that may accompany amino-acid driven metabolic changes (Kasai et al., 2020).

1.7 Gluten hypothesis and the danger model

Gluten-related inflammation and changes in the gut barrier may alter the nutrient environment in the body and influence immune-cell behavior. In simple terms, when tissues are stressed or the gut microbiome is disturbed, the body releases signals that can affect how immune cells respond. Changes in amino-acid availability (including glutamine and proline) may therefore occur as a downstream effect of inflammation and altered digestion, rather than being the original cause. Within the danger-model framework, endogenous molecules released or elevated during tissue stress or dysbiosis can function as danger cues that prime innate antigen-presenting cells. Accordingly, alterations in amino-acid patterns including changes in glutamine and proline availability may arise secondary to gut inflammation, malabsorption, or microbiome remodeling. Increased extracellular amino-acid availability could therefore act as a nutrient cue for innate immune cells and influence cellular metabolism. In this thesis, this nutrient-cue hypothesis is examined in a controlled THP-1 model by testing whether elevated glutamine or proline changes glucose uptake/consumption and selected transcriptional readouts.

1.8 Working model specific to this study

This thesis proposes the following working model: In cell culture, fetal bovine serum (FBS) represents a richer nutrient environment because it contains growth factors, hormones, lipids and baseline glucose. In cell culture, fetal bovine serum (FBS) constitutes a richer nutrient environment because it contains growth factors, hormones, lipids and baseline glucose; therefore whether the nutrient milieu modifies the magnitude or direction of proline- or glutamine-related effects. It is proposed that elevated extracellular L-glutamine and/or L-proline is sensed by THP-1 monocytic cells and activates nutrient-responsive signaling programmers, including the mTOR axis, together with redox-linked stress responses. Because of these signaling events, cellular net glucose uptake and consumption may change as part of a treatment-related metabolic shift, and transcriptional alterations in **RPS6KB1** and **NFE2L2** are expected to accompany such metabolic responses. Because phosphorylation-based pathway activation is not measured in the present experiments, any inference regarding pathway involvement is restricted to functional (glucose consumption) and transcriptional readouts.

Hypothesis: Elevated extracellular glutamine and/or proline will alter glucose uptake/consumption and modulate expression of nutrient-sensing (**RPS6KB1**) and oxidative-stress response (**NFE2L2**) genes in human THP-1 monocytic cells, with the magnitude of the response influenced by the nutrient environment (+FBS). Accordingly, this study examines whether glutamine or proline alters glucose uptake/consumption relative to baseline conditions, whether these amino acids are associated with changes in **RPS6KB1** and **NFE2L2** transcript abundance, and whether serum deprivation modifies the magnitude of these responses.

Research questions

The following primary research issues will be addressed to assess the suggested immune metabolic cascade in a regulated THP-1 monocytic system:

1. Does net glucose uptake and consumption, as reflected by glucose remaining in the culture medium, change at higher extracellular concentrations of L-proline or L-glutamine compared with baseline conditions?
2. Do elevated levels of these amino acids alter the abundance of mTOR-axis and immunometabolism-related transcripts, specifically **RPS6KB1** and **NFE2L2**, relative to baseline?
3. How do co-stimulatory factors such as serum availability and insulin influence the magnitude and temporal dynamics of the metabolic and transcriptional responses?
4. Do glucose uptake/consumption and gene-expression readouts vary systematically with amino-acid dose and exposure duration?

1.9 Aim and objectives

The aim of this study is to be elevated extracellular L-glutamine or L-proline alters glucose uptake/consumption and selected nutrient- and stress-responsive gene-expression programmers in human THP-1 monocytic cells, and to define how serum availability modifies these responses.

Objectives

- Does net glucose uptake and consumption, as reflected by glucose remaining in the culture medium, change at higher extracellular concentrations of L proline or L glutamine compared with baseline conditions?
- Do elevated levels of these amino acids alter the abundance of mTOR axis and immunometabolism related transcripts, specifically RPS6KB1 and NFE2L2, relative to baseline?
- How do co stimulatory factors such as serum availability and insulin influence the magnitude and temporal dynamics of the metabolic and transcriptional responses?
- Do glucose uptake/consumption and gene expression readouts vary systematically with amino acid dose and exposure duration?

2 Materials and Methods; THP-1 Cell Seeding

2.1 Cell Culture and Seeding Preparation

Complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 2.0 mM L-glutamine (Thermo Fisher Scientific; FBS product no. 12585014) was used to maintain THP-1 cells. All manipulations were performed using aseptic technique in a Class II biosafety cabinet. Media and reagents were pre-warmed to 37°C prior to use. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cell concentration and viability were determined by Trypan Blue exclusion: equal volumes (10 µL) of cell suspension and 0.4% Trypan Blue were mixed (1:1 dilution), 10 µL of the mixture was loaded onto a hemocytometer, and viable cells were counted using the average of the four corner squares (Chanput et al., 2014).

2.2 Preparation of Seeding Stock

Cells were seeded at an intermediate concentration of 1.20×10^6 cells/mL to achieve a final density of 4.0×10^5 cells/mL in each well (2,500 µL total volume). Including pipetting loss, 23 mL of seeding stock was prepared for 24 wells. The required number of cells (27.6×10^6) was collected in 23 mL, centrifuged at $300 \times g$ for 5 min, the supernatant was removed, and the pellet was resuspended in 23 mL of fresh complete RPMI 1640 (RPMI + 10% FBS).

2.3 Preparation of Amino Acids

L-proline (Merck P5607-25G) and L-glutamine (Merck G8540-25G) were prepared as sterile concentrated stocks and diluted into final media immediately before use. The experimental design and the results report final (1×) concentrations of L-glutamine 2.0 mM and L-proline 0.4 mM; treatment levels are 1× (baseline), 2× and 5×. To produce 10× stocks consistent with these final concentrations, prepare 10× stock solutions of 20.0 mM L-glutamine and 4.0 mM L-proline and dilute 1:10 into medium to obtain the stated 1×, 2× and 5× conditions.

Masses for 40.0 mL 10× stocks (for reproducibility) show;

- L-glutamine (MW = 146.15 g·mol⁻¹): 20.0 mM in 0.04 L → $0.020 \times 0.04 = 0.0008$ mol → $0.0008 \times 146.15 = 0.1169$ g → 116.9 mg.
- L-proline (MW = 115.13 g·mol⁻¹): 4.0 mM in 0.04 L → $0.004 \times 0.04 = 0.00016$ mol → $0.00016 \times 115.13 = 0.0184$ g → 18.4 mg.

Dissolve the calculated mass of each amino acid in sterile RPMI to 40.0 mL, filter-sterilize through a 0.22 µm syringe filter, aliquot and store at -20°C; thawed aliquots were used once. Prepare treatment media by diluting the 10× stocks 1:10 into RPMI to obtain final concentrations of 1× (L-glutamine 2.0 mM; L-proline 0.4 mM), 2× (4.0 mM; 0.8 mM) and 5× (10.0 mM; 2.0 mM). Standard culture conditions used 10% FBS were used. Osmolarity and pH of supplemented media were checked where indicated.

Net glucose uptake/consumption was estimated indirectly by measuring residual glucose in conditioned medium at 1, 24 and 48 hours. For each measurement, 1 μ L of culture medium was sampled from each well and analyzed in technical triplicate; readings were background-corrected using time-matched cell-free control wells containing the same medium formulation.

2.4 Plate Seeding

Cells were seeded into 24-well deep-well plates (10 mL well capacity) to allow a final per-well volume of 1000 μ L. Treatment groups comprised baseline (1 \times), 2 \times , and 5 \times supplementation with either L-proline or L-glutamine under the indicated serum and insulin conditions. Ten-fold amino-acid stock solutions were prepared in RPMI 1640 and added to wells as indicated. The final cell density in each well was 4.0×10^5 cells/mL; to achieve this, 250 μ L of cell suspension was added to each well as the final step. Insulin (when used) was added at 10.8 μ L per well. Each well received a total volume of 1 mL, as shown in Table 1.

Table 1: proline and glutamine concentration in different treatments.

	Insulin	10xAmino Acid (RPMI+ 10%FBS)	DMEM	THP-1 CELLS (DMEM+ FBS)
Baseline+Insulin +Fbs	10.8 μ l	100 μ l	639 μ l	250 μ l
Baseline-Insulin +Fbs	0 μ l	100 μ l	574.8 μ l	250 μ l
2x +Insulin +Fbs	10.8 μ l	200 μ l	539 μ l	250 μ l
2x -Insulin +Fbs	0 μ l	200 μ l	474.8 μ l	250 μ l
5x+Insulin +Fbs	10.8 μ l	500 μ l	239 μ l	250 μ l
5x -Insulin +Fbs	0 μ l	500 μ l	174.8 μ l	250 μ l
starvation +Insulin +Fbs	10.8 μ l	0 μ l	739 μ l	250 μ l
starvation - Insulin +Fbs	0 μ l	0 μ l	674.8 μ l	250 μ l
Negative +Insulin +Fbs	10.8 μ l	100 μ l	639 μ l	250 μ l
Negative -Insulin +Fbs	0 μ l	100 μ l	574.8 μ l	250 μ l

Note: “Baseline (1×)” is the reference condition used for comparison; “2×” and “5×” indicate higher amino-acid dosing levels relative to baseline. Treatments reported in the Results section: the amino-acid dose-response comparisons (Baseline/1×, 2×, 5×).

2.5 MTS Assay

Cell viability was assessed using the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, product no. G3582) 48 hours after cell seeding. For each condition, 100 µL of cell suspension (culture medium) was transferred to a 96-well plate and 20 µL of MTS reagent was added. Plates were incubated at 37°C for 1 hour (protected from light) until colour development, and absorbance was measured using a FLUOstar Omega plate reader (BMG Labtech) at 490 nm following the manufacturer’s instructions.

2.6 RNA Extraction

Cells from each condition (approximately 1×10^7 cells, corresponding to ~1 mL culture suspension) were harvested from the wells by collecting the contents and pelleting the cells by centrifugation prior to RNA extraction. Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, cat. no. 74106) following the manufacturer’s protocol. RNA concentration and purity were assessed using a DeNovix DS-11 spectrophotometer (1 µL measurement volume), which provides rapid quantification and A260/A280 purity ratios suitable for downstream RT-qPCR applications. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA was eluted in 30 µL RNase-free water. RNA concentration and purity were assessed using a DeNovix DS-11 spectrophotometer (1 µL measurement volume).

2.7 Reverse Transcription and Quantitative PCR (RT-qPCR)

Complementary DNA (cDNA) was synthesized from 500 ng using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, product no. 4368814) in a 20 µL reaction volume, according to the manufacturer’s protocol. RNA input was kept consistent between samples by using the same RNA extracted volume per reaction. Reverse transcription was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min, followed by a hold at 4°C. cDNA samples were stored at –20°C until qPCR. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s protocol. RT-qPCR was performed following cDNA synthesis. One microliter of cDNA was diluted 1:40 by adding it to 40 µL nuclease-free water. Each well received 2.25 µL of this dilution and 2.75 µL of master mix (2.5 µL TaqMan® Gene Expression Master Mix, Thermo Fisher Scientific, product no. 4369016 + 0.25 µL TaqMan Gene Expression Assay), giving a total reaction volume of 5 µL per well. All qPCR reactions were run in technical triplicate for each biological replicate. TaqMan Gene Expression Assays (Applied Biosystems, Thermo Fisher Scientific) were used for RPS6KB1 (Hs00356369_m1), NFE2L2 (Hs00232352_m1), and the reference genes GUSB (Hs99999908_m1), GAPDH (Hs02786624_g1), and PPIA (Hs99999904_m1). A PikoReal 96 Real-Time PCR System was used to run the plate according to the manufacturer’s cycling protocol and was performed in triplicate.

2.8 Statistical Analysis

Ct values from qPCR runs were exported and processed in Microsoft Excel (Microsoft 365). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). TaqMan assay IDs used were: RPS6KB1 (Hs00356369_m1), NFE2L2 (Hs00232352_m1), GUSB (Hs99999908_m1), GAPDH (Hs02786624_g1), and PPIA (Hs99999904_m1). For normalization, reference-gene Ct values were converted to relative quantities $Q=2^{-Ct}$; the geometric mean of the three reference-gene Q values was calculated for each sample and used as the normalization factor. Target-gene relative expression was then calculated by the $2^{-\Delta\Delta Ct}$ method using that geometric mean for normalization. Reference-gene Ct values were inspected for stability across experimental conditions prior to analysis.

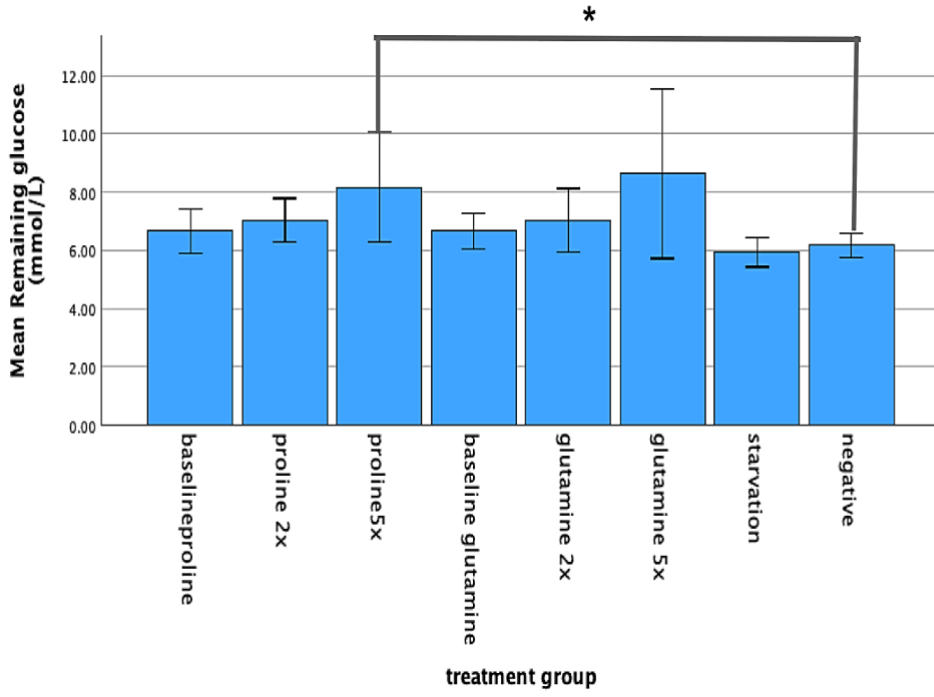
Statistical analysis was performed in IBM SPSS. Group differences were assessed using one-way ANOVA followed by Tukey's HSD post-hoc test for pairwise comparisons. Data are reported as mean \pm SD for glucose measurements, which reflect biological variability across replicates, and as mean \pm SEM for qPCR and MTS assays, where technical precision across triplicates is emphasized. This distinction aligns with standard reporting practices for biological versus technical replicates. Technical triplicates, no-template controls (NTC), and no-reverse-transcriptase (no-RT) controls were included for qPCR quality control, and $p < 0.05$ was considered statistically significant.

3 Results

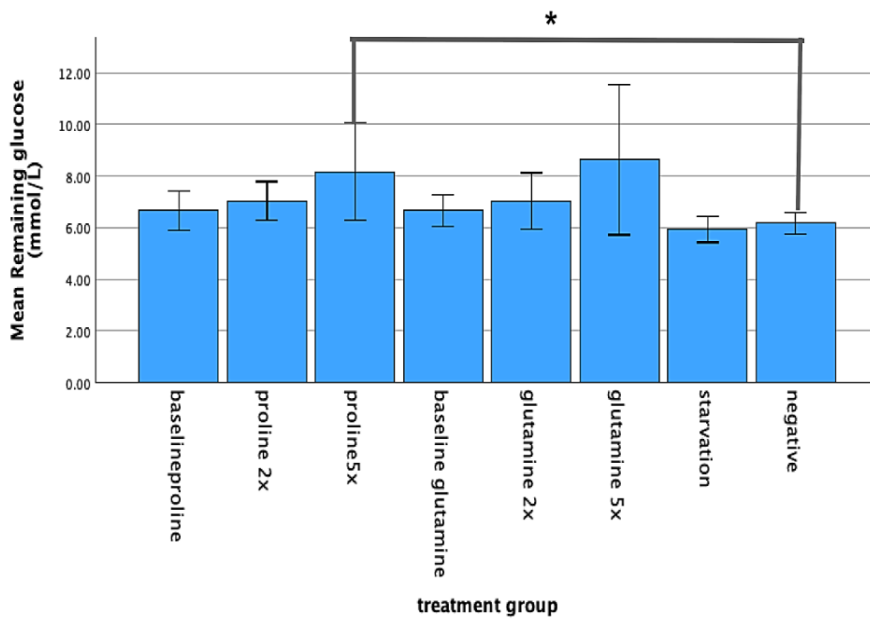
3.1 Effect of glutamine and proline on residual glucose (indirect measure of net glucose uptake/consumption)

Net glucose uptake/consumption was assessed indirectly by measuring the residual glucose remaining in the culture medium at the time points reported for each experiment. Residual glucose levels were significantly higher in cells treated with 5 \times proline than in the negative control ($p = 0.042$), indicating reduced net glucose uptake/consumption at 1 hour for the amino-acid treatments (Figure 1a). 5 \times glutamine results in higher residual glucose compared to the negative control ($p = 0.009$; Figure 1b). Higher glutamine concentrations were associated with increased remaining glucose under + insulin conditions, although the differences were not statistically significant ($p = 0.053$; Figure 1c).

A



B



C

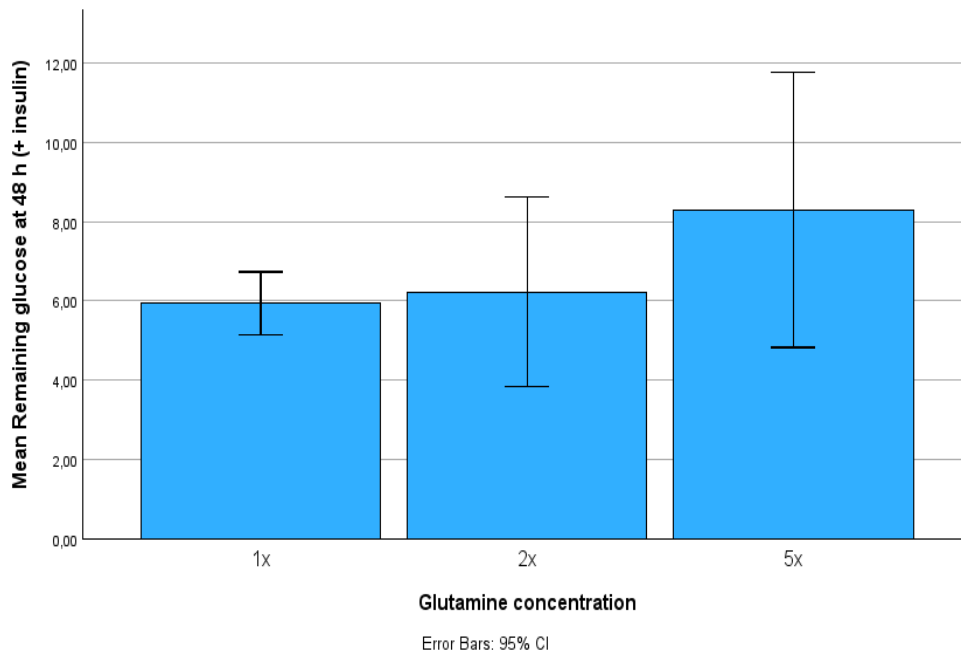


Figure1. Remaining glucose concentration was measured under different treatment conditions. **(a)** After 1 h of proline treatment (10% FBS), cells treated with 5× proline showed significantly higher residual glucose compared with the negative control. **(b)** After 1 h of glutamine treatment (10% FBS), 5× glutamine resulted in higher residual glucose than the negative control. **(c)** Remaining glucose concentration at 48 h under insulin-treated conditions across increasing glutamine concentrations showed no statistically significant differences between groups. Data are presented as mean ± SD from three independent experiments (n = 3), with each experiment performed in triplicate wells. Statistical analysis was performed using one-way ANOVA followed by Tukey’s HSD post hoc test. Significance levels are indicated as $p < 0.05$ and $p < 0.01$; exact p values are shown where applicable.

3.2 Cell viability varied between treatments after

Cell viability was assessed using the MTS assay following 48 hours of treatment. Mean viability varied between groups, but statistical analysis revealed no significant differences between treatment groups ($p = 0.515$; n = 3 independent experiments; Figure3).

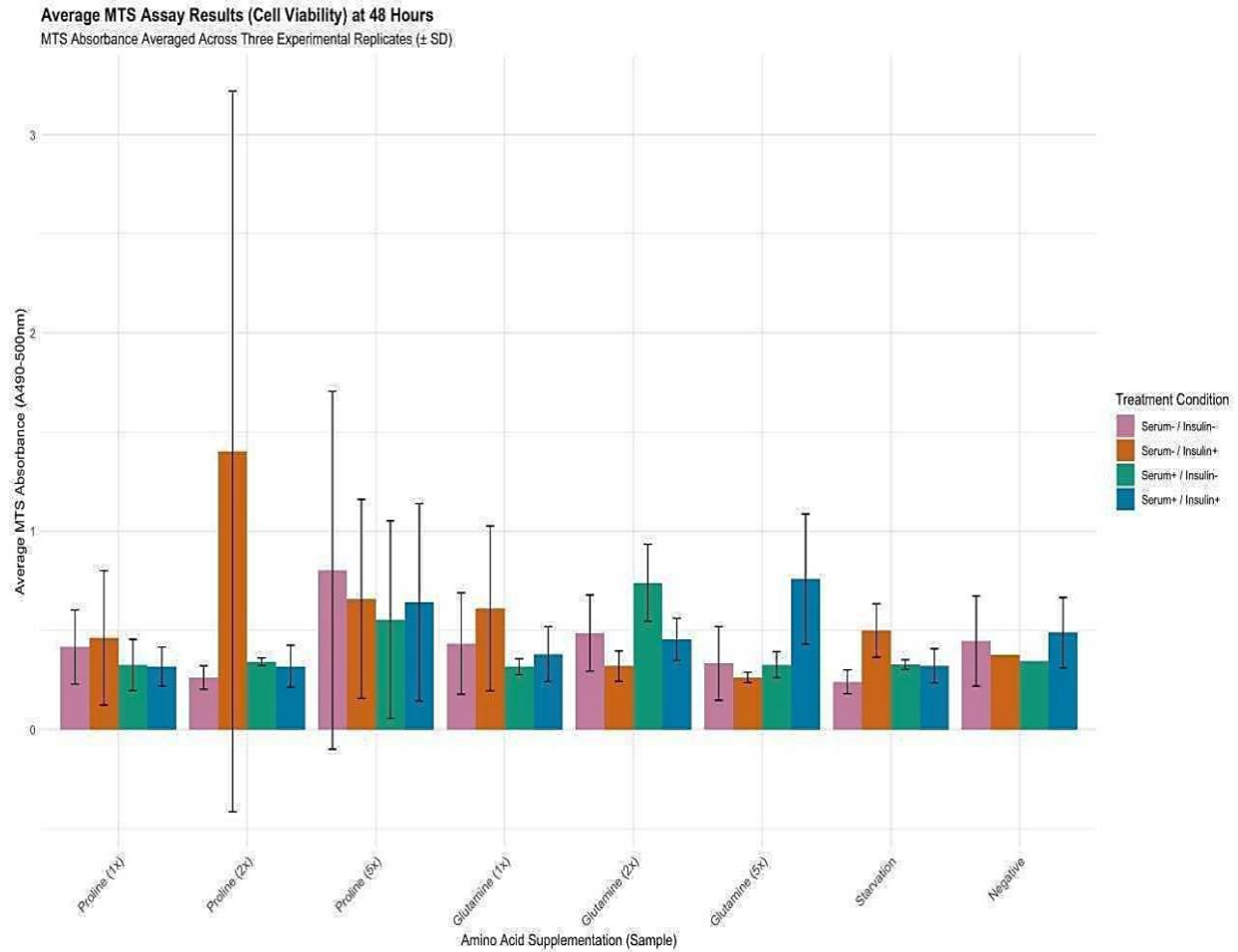


Figure 2. Cell viability (MTS) after 48 hours in the amino-acid dose-response experiment (baseline/1 \times , 2 \times , 5 \times) with the included control groups were analyzed together and normalized to the negative control. Data are mean \pm SEM. Data are mean \pm SEM of three independent experiments ($n = 3$). No significant differences observed among groups ($p = 0.515$).

RNA quality was assessed in each independent experiment (biological replicate) across the following treatment groups: baseline proline (1 \times), proline (2 \times), proline (5 \times), baseline glutamine (1 \times), glutamine (2 \times), glutamine (5 \times), starvation control, and negative control. All samples were seeded with equal cell numbers and processed in parallel to ensure consistency. RNA quality metrics were broadly comparable across groups, supporting downstream qPCR analysis. NanoDrop measurements across three independent experiments yielded A260/280 ratios of 1.95–2.05 and A260/230 ratios of 1.60–2.20. RNA concentrations ranged from 45–210 ng/ μ L. Samples with A260/230 < 1.8 were re-assessed and included only if cDNA synthesis QC metrics met acceptance criteria.

3.3 Gene expression

RPS6KB1 gene expression after 48 hours was evaluated under various amino acid treatments. There was no significant effect on fold change ($p = 0.657$). Proline and glutamine treatments did not significantly alter *RPS6KB1* expression compared to the negative control or each other ($p > 0.05$).

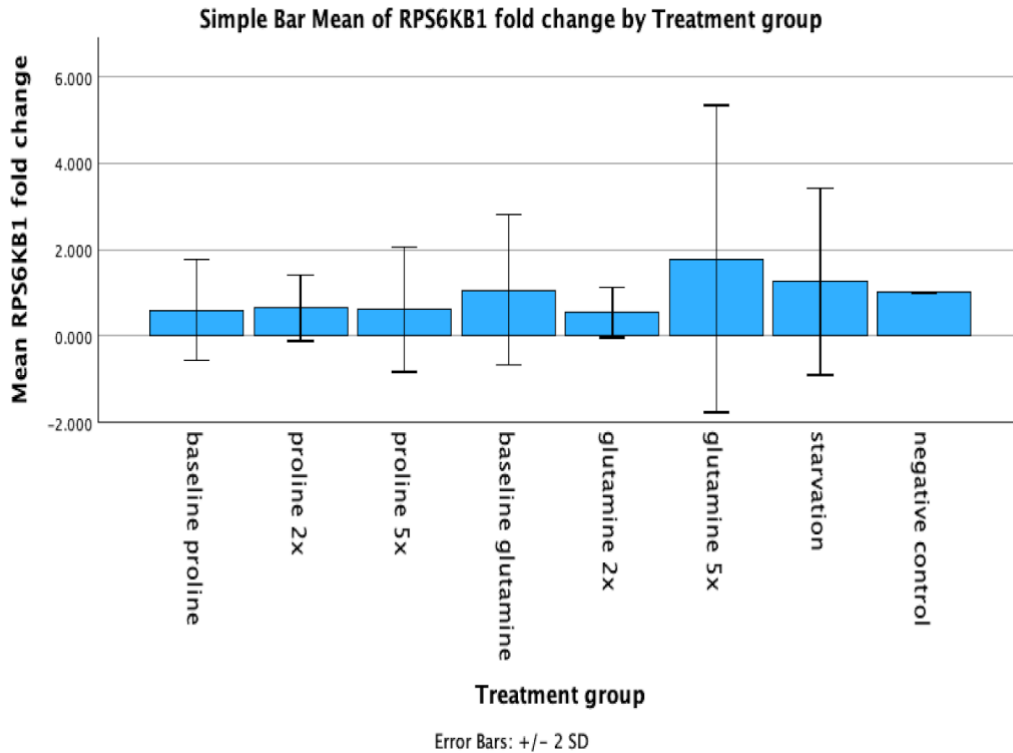


Figure 3: *RPS6KB1* relative expression calculated by $2^{-\Delta\Delta Ct}$ normalized to the geometric mean of *GUSB*, *GAPDH*, and *PPIA*. Data are the mean \pm SEM of three independent experiments ($n = 3$). Statistical test: one-way ANOVA with Tukey HSD. No significant differences observed among groups ($p = 0.657$).

Although the overall effect was not significant, the proline-treated groups appeared to show lower mean *RPS6KB1* expression than the negative control in the plotted data; this should be interpreted as a non-significant trend given the variability and sample size

The effect of amino acid treatment on *NFE2L2* relative expression after 48 hours and the expression was evaluated by measuring fold change levels under proline, glutamine, starvation, and negative control conditions. No significant changes in *NFE2L2* expression were observed following amino acid treatment in any of the tested conditions (Figure 4).

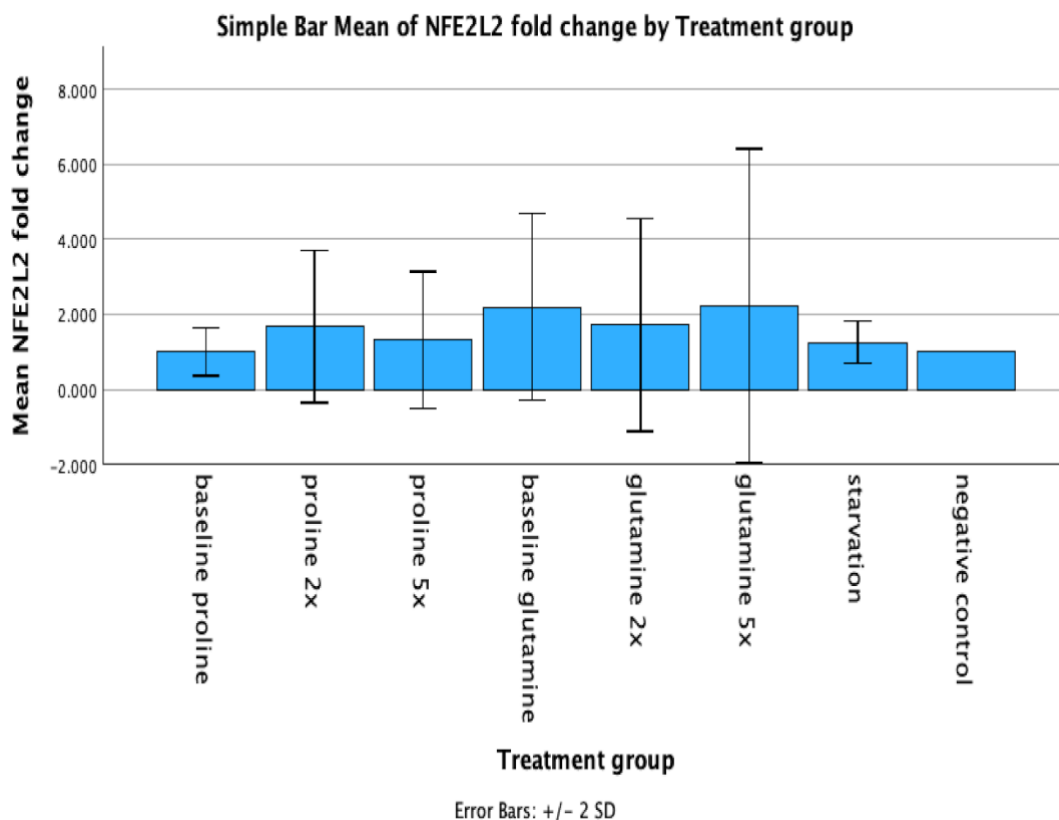


Figure 4: *NFE2L2* relative expression calculated by $2^{-\Delta\Delta Ct}$ normalized to the geometric mean of *GUSB*, *GAPDH*, and *PPIA*. Data are the mean \pm SEM of three independent experiments ($n = 3$). Statistical test: one-way ANOVA with Tukey HSD. No significant differences observed among groups ($p = 0.767$). Glutamine-treated groups showed a higher mean *NFE2L2* fold-change in the bar plot; however, this effect was not statistically significant and may reflect variability across biological replicates.

Individual replicate points are not shown here because the available Results dataset provides summary values; plotting replicate-level points would help assess whether a single replicate drives the mean.

4 Discussion

4.1 Metabolic Reprogramming & Contradictions to the Working Model

The primary objective of this study was to determine whether a short, high-dose increase in extracellular L-glutamine or L-proline alters glucose handling and activates nutrient-sensing pathways in THP-1 monocytic cells, with a focus on signalling via the mechanistic target of rapamycin (mTOR). The most consistent experimental finding was that, at the one-hour time point, both 5× glutamine and 5× proline treatments were associated with a significant increase in residual glucose in conditioned medium compared with baseline controls (glutamine $*p^* = 0.009$; proline $*p^* = 0.042$), indicating an acute reduction in net glucose removal rather than the expected rise in glycolytic consumption.

Put differently, the highest amino-acid concentrations produced an acute reduction in net glucose removal from the medium rather than the hypothesised enhancement of glycolytic glucose consumption. This outcome necessitates a careful reconsideration of the assumption that increased extracellular amino-acid availability will, by itself, drive a Warburg-like glycolytic programme in innate immune cells in the absence of inflammatory co-stimulation.

Two conceptual clarifications follow from these data. First, nutrient abundance and inflammatory activation are separable determinants of immunometabolic phenotype. The glycolytic reprogramming commonly reported for activated innate cells is most reliably induced by pattern-recognition receptor engagement or pro-inflammatory cytokines (for example, lipopolysaccharide or interferon- γ) rather than by nutrient signals alone (O'Neill et al., 2016; Loftus & Finlay, 2016).

Second, monocytic cells retain metabolic plasticity and can redistribute carbon flux away from glycolysis toward oxidative or anaplerotic pathways when alternative substrates are plentiful (Nicklin et al., 2009; Phang, 2019). The present findings are consistent with a substrate-substitution model in which abundant extracellular glutamine or proline reduces immediate glucose demand by supplying tricarboxylic acid (TCA) cycle intermediates or by supporting mitochondrial oxidative metabolism.

Several, non-exclusive mechanistic explanations can account for the observed reduction in net glucose consumption. Glutamine can be rapidly metabolised to glutamate and subsequently to α -ketoglutarate, thereby feeding the TCA cycle and diminishing the requirement for glycolytic carbon (Nicklin et al., 2009; LaPlante & Sabatini, 2012). Proline catabolism is integrated with the proline–pyrroline-5-carboxylate cycle and mitochondrial electron transport; through these pathways proline can influence cellular NAD(P)H pools and reactive oxygen species (ROS) signalling while providing anaplerotic input to the TCA cycle (Phang, 2019; Kasai et al., 2020).

In addition, elevated extracellular amino acids can elicit rapid post-translational regulatory events—such as altered transporter trafficking or phosphorylation of metabolic enzymes—that modulate glucose transporter activity or hexokinase function on a timescale of minutes to hours (LaPlante & Sabatini, 2012; Salmond et al., 2015). Finally, because the principal metabolic endpoint in this study was residual glucose in conditioned medium, extracellular factors (for example, changes in osmolarity, protein binding interactions, or assay interference) could in principle influence measured

concentrations; the inclusion of time-matched cell-free controls reduces but does not entirely remove this possibility. The reproducibility of the 5× effect across independent replicates, however, supports a biological contribution to the observation rather than a pure measurement artefact.

Taken together, these results prompt a refinement of the initial working model: acute increases in extracellular amino acids can rapidly reconfigure substrate utilisation in monocytic cells, but this reconfiguration does not necessarily present as an increase in glycolytic glucose uptake. Instead, abundant amino acids may favour utilisation of amino-acid carbon and engage rapid, post-translational mechanisms that reduce immediate glucose flux.

This interpretation is consistent with prior work emphasising that nutrient cues and inflammatory signals elicit distinct metabolic programmes in immune cells (O'Neill et al., 2016; Loftus & Finlay, 2016) and highlights the importance of considering cellular context and stimulus history when interpreting metabolomic associations.

4.2 Sustained Viability Despite Reduced Glucose Uptake: The Role of Proline

THP-1 viability measured at 48 hours did not differ between treatment groups (MTS *p* = 0.515), even though glucose consumption was reduced at the 1-hour time point. This separation of an early change in glucose handling from later cell survival argues against a general toxic effect on metabolism and instead indicates that cells compensated metabolically (Chanput et al., 2014). In other words, THP-1 cells appear able to maintain viability despite lower glucose uptake by using alternative substrates or metabolic pathways to meet their energy and biosynthetic needs.

Proline metabolism provides a plausible mechanistic explanation for this resilience. Proline dehydrogenase catalyses the oxidation of proline to pyrroline-5-carboxylate, thereby linking proline catabolism to mitochondrial electron transport and redox cycling; through this route proline can supply reducing equivalents to the electron transport chain and contribute anaplerotic carbon to the TCA cycle, supporting ATP production independently of glycolytic flux (Phang, 2019; Kasai et al., 2020).

In circumstances where glucose uptake is transiently reduced, proline oxidation could therefore sustain cellular energy production and buffer redox perturbations, preserving viability. Glutamine similarly supports survival via anaplerosis and biosynthetic pathways: glutaminolysis yields α -ketoglutarate for the TCA cycle, supports nucleotide and amino-acid synthesis, and contributes to NADPH generation through malic enzyme and related reactions (Nicklin et al., 2009). The preserved viability observed in glutamine-treated groups is therefore consistent with metabolic substitution rather than metabolic collapse.

The combined viability and metabolic-routing observations strengthen a mechanistic narrative in which amino acids shift substrate handling rather than causing generalized metabolic failure. This metabolic plasticity is likely relevant to immune cells that operate in fluctuating nutrient environments *in vivo* and suggests that amino-acid elevations reported in prospective human cohorts could alter immune-cell metabolic set points without necessarily provoking overt cytotoxicity or immediate inflammatory activation (O'Neill et al., 2016).

Therefore, the metabolomic changes observed before seroconversion in Type 1 diabetes may indicate broader shifts in systemic metabolism that indirectly influence

immune-cell behaviour, rather than serving as direct pro-inflammatory stimuli (Orešič et al., 2008; Pflueger et al., 2011).

4.3 Transcriptional Responses and the Lack of Upregulation in Key Markers

The study measured two transcriptional markers—RPS6KB1 (p70S6K1) and NFE2L2 (NRF2)—to assess nutrient-sensing and oxidative-stress responses. Quantitative RT-PCR at 48 hours showed no significant differences between treatment groups for either gene (RPS6KB1 $p = 0.657$; NFE2L2 $p = 0.767$).

This lack of transcriptional change can be explained by methodological and biological factors: both mTOR and NRF2 pathways are primarily regulated at the protein and post-translational level (for example, by phosphorylation or protein stabilisation), transient signalling events may have occurred earlier than the 48-hour sampling point, and single-gene mRNA measurements have limited sensitivity to detect pathway engagement. Consequently, mRNA readouts alone are insufficient to conclude that these nutrient-sensing or redox pathways were inactive; protein-level assays and time-resolved measurements would be required to determine whether pathway activation occurred.

First, mTOR signalling is principally regulated through protein-level mechanisms and phosphorylation events. Activation of mTORC1 is detected at the lysosomal surface and propagated via phosphorylation of downstream effectors such as S6K1 and 4E-BP1; therefore, steady-state changes in RPS6KB1 transcript abundance are an insensitive proxy for acute pathway activity (LaPlante & Sabatini, 2012; Salmond et al., 2015).

NRF2 is mainly regulated after translation via Keap1-dependent ubiquitination and proteasomal degradation. Functional activation is therefore best detected by increases in NRF2 protein stability, its translocation to the nucleus, and the subsequent upregulation of established target genes (for example, HMOX1, NQO1), rather than by measuring changes in NFE2L2 transcript levels (Kasai et al., 2020).

Third, temporal mismatch likely contributed to the null transcriptional result: the metabolic perturbation was evident at one hour, whereas transcriptional sampling occurred at 48 hours. Transient early signalling events may have been resolved or compensated by the later time point, leaving no persistent transcriptional footprint in the two genes measured. Fourth, the modest number of biological replicates reduces sensitivity to detect small or variable transcriptional effects and increases the risk of type II error (Chanput et al., 2014).

Beyond these technical factors, it is important to recognise that transcriptional programmes downstream of mTOR and NRF2 frequently involve secondary waves of gene expression and context-dependent target selection. mTORC1 activation can rapidly alter translation and enzyme activity without immediate changes in transcript levels, and NRF2 activation commonly manifests as coordinated induction of antioxidant and detoxification genes rather than changes in NFE2L2 transcript itself (LaPlante & Sabatini, 2012; Kasai et al., 2020).

A wider transcriptomic survey or a focused panel of established downstream targets would be more effective than single-gene assays for detecting subtle or pathway-specific responses. The lack of changes in RPS6KB1 and NFE2L2 transcripts in this study therefore cannot be taken as definitive evidence that nutrient-sensing or redox pathways

were inactive; instead, it underscores the importance of assessing protein-level events and sampling at multiple, earlier time points to capture rapid, flux-driven signalling.

4.4 Synthesis of Findings and Future Directions

Collectively, the data support a refined conceptualisation in which acute elevation of Overall, the results indicate that a short, high-dose increase in extracellular glutamine or proline can shift substrate use in monocytic cells, lowering immediate glucose uptake while leaving cell viability intact. The pattern observed is most consistent with rapid changes in metabolic flux and post-translational control of metabolic enzymes and transporters, rather than with durable changes in transcription of **RPS6KB1** or **NFE2L2**. To confirm and extend this interpretation, follow-up work should focus on direct, time-resolved measures of substrate handling and on protein-level readouts of nutrient-sensing pathways, and should validate key findings in primary human cells.

Concretely, experiments that measure glucose uptake directly—using fluorescent probes such as 2-NBDG or radiolabelled glucose—and that quantify extracellular lactate would determine whether higher residual medium glucose reflects reduced uptake or altered intracellular glycolysis (Chanput et al., 2014). Complementary stable-isotope tracing with uniformly labelled (¹³C)-glucose, (¹³C)-glutamine and (¹³C)-proline followed by mass spectrometry would map carbon flow into glycolysis, the TCA cycle and biosynthetic pathways and thus provide a direct test of the substrate-substitution hypothesis (Nicklin et al., 2009; Phang, 2019).

Real-time metabolic profiling using extracellular flux analysis to measure oxygen consumption rate and extracellular acidification rate would determine whether cells shift from glycolysis toward oxidative phosphorylation in response to amino-acid elevation (Loftus & Finlay, 2016). Protein and phosphorylation assays—Western blotting or phospho-flow cytometry for phosphorylated S6K1, 4E-BP1 and AKT—are essential to detect mTOR pathway engagement, while assessment of NRF2 protein stabilisation and nuclear localisation together with quantification of canonical NRF2 target genes would provide a functional readout of redox signalling (LaPlante & Sabatini, 2012; Kasai et al., 2020).

A denser temporal sampling scheme, capturing minutes to hours after amino-acid exposure, will be necessary to align molecular readouts with metabolic flux changes. Incorporating physiologically relevant inflammatory co-stimulation (for example, LPS or IFN- γ) will determine whether amino-acid context modulates the magnitude or direction of immunometabolic reprogramming and downstream functional outputs such as cytokine secretion and antigen presentation (O'Neill et al., 2016; Linke et al., 2017).

Replication of the principal findings in primary human monocytes, macrophages or dendritic cells is necessary to establish translational relevance and to account for differences in transporter expression and metabolic responsiveness between immortalised cell lines and primary cells (Chanput et al., 2014). Future experiments should use amino-acid concentrations that reflect physiological ranges reported in cohort studies and include explicit controls for osmolarity and pH; these measures will reduce artefacts from supraphysiological dosing and increase the likelihood that in vitro observations map onto in vivo biology (Orešič et al., 2008; Pflueger et al., 2011; Araujo de Pina Cabral et al., 2015).

In summary, the data reported here show that a short, high-dose exposure to extracellular glutamine or proline reduces immediate glucose uptake by THP-1 monocytic cells while leaving cell viability intact, and that this effect occurs without

detectable changes in RPS6KB1 or NFE2L2 transcript abundance at a late sampling point. These results point to metabolic flexibility in monocytic cells and indicate that regulation of substrate use is driven primarily by changes in metabolic flux and protein-level control rather than by sustained transcriptional reprogramming. For researchers interpreting metabolomic signals from prospective human cohorts—where altered glutamine and proline have been observed before seroconversion—our Findings suggest that elevated circulating amino acids may act as context-setting metabolic cues rather than as direct pro-inflammatory stimuli. Definitive assessment of whether such cues alter immune function *in vivo* will require flux-based measurements, protein-level signalling assays and functional tests in primary cells, ideally performed in the presence of relevant inflammatory co-stimulation. These steps will help determine whether amino-acid perturbations play a causal role in early autoimmune processes or instead serve as biomarkers of broader metabolic states.

Limitations to the study

Several limitations of the present work constrain the strength of mechanistic inference and should be acknowledged. The principal metabolic endpoint—residual medium glucose measured from conditioned medium—provides an indirect estimate of net glucose change and cannot resolve uptake kinetics, intracellular metabolism or lactate production; direct uptake and flux assays are therefore necessary to confirm the mechanism (Chanput et al., 2014). The transcriptional analysis was limited to two genes at a single late time point, an approach that is insufficient to capture transient or protein-level signalling events central to mTOR and NRF2 biology (LaPlante & Sabatini, 2012; Kasai et al., 2020).

The THP-1 cell line, while experimentally tractable, may not fully recapitulate primary human monocyte behaviour, particularly with respect to nutrient transport and insulin responsiveness; validation in primary cells is therefore required (Chanput et al., 2014). The supraphysiological 5× concentrations used here exceed typical plasma ranges reported in prospective metabolomic studies; although useful for probing mechanistic extremes, such dosing limits direct extrapolation to *in vivo* physiology and may introduce osmotic or transporter saturation effects (Orešič et al., 2008; Araujo de Pina Cabral et al., 2015; Li et al., 2020). Finally, the modest number of biological replicates reduces statistical power to detect small or variable effects, particularly at the transcriptional level. These limitations frame the present findings as hypothesis-generating and identify the most informative next experiments.

5 Ethical Considerations

The THP-1 cell line model used in this study raises particular ethical issues that are common to *in vitro* investigations that use biological materials originated from humans. The THP-1 line is a well-known, commercially accessible acute monocytic leukemia cell line that is accessible through reputable cell repositories (such as ATCC) and has undergone comprehensive characterization. The origin and continued use of this human cell line are the main ethical issues:

Informed Consent and Anonymity:

In the 1980s, a 1-year-old boy with acute monocytic leukemia was the original source of THP-1 cells. Despite the widespread usage of existing cell lines, best standards require the anonymization of related data and certification of the original donor's informed consent status. The source repository must verify that appropriate ethical approval was received at the time of derivation, according to current researchers. As long as suitable Material Transfer Agreements (MTAs) are in place, the current study's use of an established, anonymous line from a reliable source means that direct patient consent is not a continuous need for the experimental work itself.

Minimizing Injury and Resource Management:

While in vitro studies using well-established cell lines do not directly injure human subjects, it is morally necessary to make sure that research is carried out thoroughly and effectively to prevent squandering important resources (time, reagents). By guaranteeing data reliability, the use of a thoroughly defined, repeatable system such as THP-1 cells is consistent with these objectives.

Data Integrity and Reproducibility:

Honesty in data reporting is necessary to uphold ethical norms. All results, including non-significant findings for *RPS6KB1* and *NFE2L2* expression, should be reported transparently. The integrity of the scientific record depends on this openness and helps prevent publication bias, enabling other researchers to learn from unexpected outcomes.

Possibility of Societal Impact:

Understanding autoimmunity and chronic inflammatory illnesses is affected by research on immunometabolism and danger signals. Researchers must be aware of the possible long-term societal ramifications of their study, making sure that results are appropriately disseminated and serve the public interest rather than being misused.

From a societal perspective, understanding how nutrients shape immune responses could inform early-detection strategies and public-health nutrition guidance for autoimmune diseases. Because this work uses a human cell line instead of animals, it also supports the 3Rs principles (Replacement, Reduction, Refinement) in biomedical research.

6 Conclusion

Unexpected departures from the original working model were discovered when the effects of increased L-proline and L-glutamine on THP-1 monocytic cell immunometabolism were examined. The experimental results showed the contrary in the short term, despite the central theory predicting an instantaneous increase in glucose uptake/consumption and transcriptional activation of growth-related genes (*RPS6KB1*, *NFE2L2*). Important lessons learned include the following. High (5×) proline and glutamine were associated with higher residual glucose remaining after 1 hour, which is consistent with reduced net glucose uptake/consumption during the early phase under the tested conditions. Despite this short-term metabolic effect, cell viability at 48 hours did not differ significantly between groups. In addition, *RPS6KB1* and *NFE2L2* mRNA expression did not show significant changes at the measured timepoint, suggesting that any regulatory effects of these amino acids may occur through mechanisms not captured by transcript levels alone (for example protein-level regulation or transient earlier responses), which were not measured in this study.

In conclusion, although L-proline and L-glutamine appear to influence immune-cell metabolism, they were not sufficient on their own to reproduce a classical inflammatory “Warburg-like” shift or to induce strong transcriptional changes in RPS6KB1 and NFE2L2 in this in vitro system. The results highlight the context-dependent character of immunometabolism and the importance of experimental conditions (for example inflammatory co-stimulation) in shaping the metabolic response.

Future Perspectives

Future work could include inflammatory co-stimulation (e.g., LPS) to test how inflammatory cues interact with amino-acid exposure. Measuring phosphorylated S6K1, 4EBP1, and nuclear Nrf2 will help confirm pathway activation at the protein level. Validating these findings in primary human monocytes will clarify their relevance to early Type 1 diabetes and to potential dietary interventions.

Declaration of AI and AI-assisted technologies

Language and formatting suggestions were assisted by generative AI tools; all content and interpretations are the author’s own. No AI tools were used to generate experimental data, alter test-derived results, or perform statistical analyses.

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