Human plasma metabolic environment favours HIV replication in primary CD4 T lymphocytes

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Abstract
Cellular metabolism provides all molecules essential for the cell's functions including nucleic acids, amino acids and fatty acids, but also energy via glycolysis and mitochondrial respiration (also known as OXPHOS). Viruses hijack these pathways to support the different steps of their replication, making the metabolic state of the infected cell a key factor influencing viral replication. In particular, Human Immunodeficiency virus (HIV) remains latent in resting, metabolically inactive CD4 T lymphocytes but actively replicates in activated CD4 T cells. In these cells, enhanced glycolysis and OXPHOS, and access to the major carbon sources such as glucose and glutamine are required for infection. Here, we took advantage of the recently developed Human Plasma-Like Medium (HPLM), that mimics physiological plasma concentration of metabolites, including carbon sources, to investigate how this physiologic environment would modulate HIV-1 infection of primary CD4 T cells. Using CD4 T cells activated in the conventional culture medium (RPMI) or HPLM, we show that HPLM enhanced HIV-1 infection despite similar levels of cell activation, proliferation and expression of viral receptor. Specifically, HPLM increases the reverse transcription step of the HIV-1 replication cycle. In contrast with previous studies performed in RPMI only, this increased infection in HPLM correlated with decreased glycolysis and mitochondrial metabolism, reflecting the low concentration of glucose and glutamine in this medium. Adjusting glucose and glutamine concentrations in RPMI or decreasing energy metabolism using chemical inhibitors did not increase infection to levels similar to HPLM suggesting that other metabolic pathways are involved. Taken together, this study shows that the metabolic state of activated CD4 T cells in a physiologically relevant environment is more permissive to HIV-1 infection than previously suggested by studies using conventional cell culture media. Our study demonstrates the importance of physiologic culture conditions to study viral infections while also highlighting the importance of non-energetic cell metabolism in HIV-1 infection.

Introduction

The Human Immunodeficiency Virus (HIV) is responsible for the worldwide pandemic of Acquired ImmunoDeficiency Syndrome (AIDS). Since the start of the pandemic, 40 years ago, HIV has infected an estimated 88.4 million patients and killed 42.3 million. In 2023, 39.9 million people were still living with HIV[1]. With the development of antiretroviral therapy, AIDS has become a lifelong infection that can be managed using a combination of drugs that need to be taken for life[2]. However, this treatment only prevents active virus replication and can lead to serious side effects. Moreover, no vaccine currently exists[3]. It is therefore still important to deepen our understanding of the interaction between HIV and its cellular targets in physiological conditions in order to propose better suited treatments.

Cellular metabolism encompasses all pathways involved in the generation or recycling of biomolecules used for energy production, nucleic acid or protein synthesis, regulation of gene expression, redox balance and many other cellular processes. All molecules involved are called metabolites and their homeostasis is tightly regulated. The two main pathways producing energy in the form of ATP are glycolysis and oxidative phosphorylation (OXPHOS). HIV infects cells of the immune system and its main target cells are CD4 T lymphocytes. These cells play a crucial role in establishing pathogen-specific adaptive immune responses. Upon recognition of pathogen-derived antigens, they are activated and shift from naïve cells to actively dividing cells producing large amounts of cytokines. Thus, while naïve T cells keep a low energetic supply using low levels of glycolysis and OXPHOS[4], activated CD4 T cells undergo a profound metabolic reprogramming to meet higher demands in energy but also in deoxynucleotide triphosphate (dNTPs), amino acids and lipids. This reprogramming involves increased glucose uptake leading to higher rates of glycolysis to produce energy. Additionally, intermediates in the glycolysis pathway are also diverted into other pathways such as the pentose phosphate pathway (PPP) to produce dNTPs and amino acids essential for the cells' immune functions.

HIV-1 is an obligatory intracellular pathogen that needs to subvert the cellular machinery to replicate. The HIV-1 replication cycle is initiated by the interaction of the viral particle with its receptor (CD4) and co-receptors (CXCR4 or CCR5), leading to the fusion of viral and cellular membranes. Following entry in the cytoplasm, the viral RNA genome is reverse transcribed into a proviral DNA that is imported in the nucleus and integrated in the host genome. From there, viral genes are transcribed and viral proteins are produced which leads to assembly of new viral particles and their budding from the plasma membrane. The different steps of the viral life cycle therefore require the availability of specific metabolites produced by various metabolic pathways. In HIV-1-infected individuals, the increased glycolysis and glucose uptake occurring during cell activation is associated with higher susceptibility of activated CD4 T cells to HIV-1 infection[5–9]. Recent studies on various subsets of CD4 T cells showed that HIV-1 preferentially infects cells with high glycolytic activity, irrespective of their activation status, and blocking glycolysis prevents infection of primary CD4 T cells[7,10]. Both glycolysis and glutaminolysis, which uses glutamine as a carbon source to feed OXPHOS, are essential for HIV-1 replication in these cells[11].

However, most of these studies have been performed *in vitro* using the standard, but not physiologic, RPMI-1640 cell culture medium that was formulated to sustain cell growth over extended periods of time[12]. Therefore, there is a growing need to explore more physiologically relevant cell culture conditions. Recent efforts led to the development of new cell culture media, such as Human Plasma-Like Medium (HPLM), formulated to closely mimic the metabolite composition of human plasma[13]. Unlike RPMI, HPLM contains 31 polar metabolites that are absent from commonly used media and carries other components such as glucose,

glutamine and amino acids at physiological concentrations. Recent studies revealed that the extracellular environment, and in particular the use of HPLM, can shape the immune response in CD4 T cells by influencing the metabolic state of the cells[14,15].

Here, we investigated how the human plasma metabolic environment affects HIV-1 replication in primary CD4 T cells. Comparing HPLM and RPMI, we found that this new physiologically relevant medium potentiated HIV-1 replication by increasing the efficiency of reverse transcription. In contrast with previous reports performed in RPMI, this increase was associated with a lower energy metabolism, and in particular a lower glycolysis. However, decreasing glycolysis and the overall energy metabolism by using chemical inhibitors or reducing glucose and glutamine concentrations in RPMI did not recapitulate the phenotype observed in HPLM. This finding confirms that, even in HPLM, glycolysis remains crucial for HIV-1 replication. Our results therefore demonstrate the importance of non-energetic cellular metabolic pathways that might be key to HIV-1 replication in more physiological conditions.

Results

Human plasma-like metabolic environment potentiates HIV-1 replication in primary activated CD4 T cells

We first wondered how the human plasma-like metabolic environment might affect HIV-1 replication in primary CD4 T cells. The original study that developed HPLM recommends to supplement it with dialysed Foetal Bovine Serum (dFBS) to remove all small molecules and therefore maintain the controlled metabolites concentrations in the medium[13]. Previous comparison of CD4 T cells activated in HPLM or RPMI supplemented with dFBS [14] revealed that cells were more activated in HPLM than RPMI. This effect was attributed to a lack of Ca^{2+} typically provided by non-dialysed FBS (ndFBS) in RPMI. Supplementing RPMI with ndFBS rescued CD4 T cells activation to levels similar to HPLM. Here, we wanted to assess the effects of HPLM independent of any influence on cell activation. Therefore, we used HPLM supplemented with dFBS and RPMI supplemented with ndFBS (hereafter HPLM and RPMI respectively). CD4 T cells were activated in these two conditions and their activation and cell proliferation were monitored by flow cytometry looking at the activation marker CD25 on day 4 post-activation and the percentage of divided cells by Cell Trace staining 5 days post-activation. As expected, CD4 T cells were activated to the same level in both conditions (Fig 1A and B). Activated cells were then infected with two doses of a replicating HIV-1 NL4-3 virus (25 and 100ng p24/mL) and virus replication was monitored by flow cytometry looking at expression of the viral Gag protein 4 days post-infection (dpi). Cells cultured in HPLM showed a significantly higher HIV-1 replication compared to those cultured in RPMI with a 2- to 5-fold increase depending on the viral dose used (Fig 1C). The absence of HIV-1 Gag detection upon treatment with the non-nucleoside reverse transcriptase inhibitor nevirapine (NVP) confirmed that the observed fluorescent signal correlated with HIV-1 replication (Fig 1C). Supplementing HPLM with ndFBS resulted in a similar increase in HIV-1 infection showing that this difference is not due to the use of different sera (Fig S1). We then tested if the increased HIV-1 replication could also be observed in a CD4 T cell line. Jurkat cells cultured in HPLM displayed slightly reduced cellular proliferation in HPLM compared to RPMI (Fig S2A) suggesting that some metabolite concentrations might be limiting their growth. We then infected these cells with a replicating HIV-1 NL4-3 virus and monitored replication by flow cytometry looking at Gag expression (Fig S2B). HIV-1 replication was not affected in this model of fast-dividing and metabolically highly active cells, suggesting that the enhanced replication observed in primary cells was due to differences in metabolism unique to primary CD4 T cells.

Therefore, HPLM potentiates HIV-1 replication in primary activated CD4 T cells.

HPLM enhances the reverse transcription step of the HIV-1 life cycle

To investigate the viral replication step affected by the medium, we first assessed the expression of the HIV-1 receptor (CD4) and co-receptors (CXCR4 and CCR5) on the surface of activated cells at the time of infection using flow cytometry. The percentage of cells expressing these receptors as well as the mean fluorescence intensity showed no significant difference between HPLM and RPMI (Fig 2A). We then tested if virus fusion at the plasma membrane was affected using the Vpr-Blam assay[16]. Briefly, Vpr is an HIV-1 protein that is incorporated into the nascent virus particles. In this assay, Vpr is fused with the beta-lactamase (Vpr-Blam) and viral particles containing Vpr-Blam are used to infect cells pre-loaded with CCF2-AM, a cell-permeable fluorescent substrate of beta-lactamase. Upon virus fusion with the target cell, beta-lactamase is delivered to the cytosol where it cleaves CCF2-AM, triggering a fluorescent switch that can be quantified by flow-cytometry. Using this method and two different doses of HIV-1 NL4-3 Vpr-Blam virus, we did not observe any difference in fusion between the two media conditions (Fig 2B). Following fusion, HIV-1 RNA is reverse transcribed into DNA, which can be quantified by qPCR using primers specific for the DNA products at different steps of the reverse transcription process (RT). CD4 T cells were infected for 20h in the presence or not of the reverse transcriptase inhibitor nevirapine (NVP). RT was quantified by qPCR on normalised amounts of cellular DNA. Using a plasmid standard, we calculated copy numbers of early and late RT products for 100ng of DNA. We observed a significant increase in both early and late RT products in cells cultured in HPLM (Fig 2C).

Next, to asses HPLM effects on the late phases of the HIV-1 life cycle, we first evaluated viral gene expression by quantifying the mean fluorescence intensity of the viral Gag protein in infected Gag+ cells by flow cytometry. HIV-1 Gag expression levels were similar in cells infected in both HPLM and RPMI (Fig 2D). We then quantified virus particle released in the supernatant of infected cells by ELISA for HIV-1 Gag p24. Concentration of virus particles were similar in the supernatant of cells infected in HPLM and RPMI, showing that HPLM did not affect viral particle release from the infected cells (Fig 2E).

Therefore, these results demonstrate that HPLM potentiates HIV-1 replication in activated CD4 T cells primarily by increasing the reverse transcription step of the viral replication cycle.

Primary CD4 T cells activated in HPLM exhibit a modified metabolism, including reduced energy metabolism

HPLM and RPMI formulations differ mainly in the concentrations of three groups of metabolites: carbon sources essential for energy production, amino acids, and other polar metabolites including those involved in the TCA cycle used in energy metabolism[13]. In particular, HPLM contains physiologic levels of the main carbon sources: glucose (5mM vs 11mM in RPMI) and glutamine (0.55mM vs 2mM in RPMI). To investigate how these differences affect cellular metabolism in CD4 T cells, we first analysed energy metabolism in cells activated in HPLM and RPMI for 4 days using the ATP rate assay with the Seahorse technology. This method allows to measure the oxygen consumption linked to mitochondrial respiration, as well as the medium acidification due to lactate export following glycolysis in live cells. It then translates these measures into overall ATP production rate and distinguishes ATP produced by glycolysis (glycoATP) and by mitochondrial respiration (mitoATP). Compared to RPMI, CD4 T cells activated in HPLM had a lower overall ATP production rate, primarily due to a significantly lower glycolytic activity (Fig 3A). We further analysed the metabolism of CD4

T cells activated in HPLM and RPMI for 5 days by metabolomics using high-resolution mass spectrometry (HRMS). In particular, we monitored central metabolism and amino acids by ion chromatography coupled HRMS (IC-HRMS) and liquid chromatography coupled HRMS (LC-HRMS) respectively. PCA analysis of the combined results indicated significant metabolic differences between the two conditions (Fig 3B). A volcano plot analysis revealed metabolites that are significantly different (both fold change >2 and p-value<0.1; Fig 3C). To visualise the specific metabolites variations, we generated heatmaps for each group of metabolites confidently identified, based on the average of 5 donors (Fig 3D). A small group of 7 metabolites were significantly more abundant in HPLM, including phosphorybosyl-pyrophosphate (PRPP), a key metabolite in the pentose phosphate pathway (PPP), and multiple pentose-5-phosphate metabolites. This suggests that the PPP is upregulated in HPLM-activated cells. Moreover, glycine, phenylalanine, threonine and tyrosine were also elevated in HPLM. Interestingly, this increase did not correspond to higher concentrations of phenylalanine, threonine and tyrosine in the culture media. In contrast, 20 metabolites were significantly more abundant in RPMI-activated cells, including (i) glutamine and glucose-6-phosphate, the main form of glucose present in cells, reflecting the higher concentrations of these carbon sources in RPMI; (ii) intermediates of glycolysis; (iii) intermediates of the TCA cycle; (iv) intermediates of the hexosamine pathway necessary for protein glycosylations; and (v) amino acids, mostly reflecting differences in the culture media. These results, summarised in Fig 3E, suggest that all energy metabolism pathways are down-regulated in cells activated in HPLM, while other metabolic processes such as amino acids metabolism, the pentose phosphate pathway (PPP) and the hexosamine pathway are altered. Of note, despite an increase in intermediates of the PPP in HPLM-activated cells, we did not observe any difference in nucleotide metabolism, one of the main pathways using PPP products.
Therefore, HPLM profoundly affects many crucial metabolic pathways in activated CD4 T cells, particularly their energy metabolism.

Altering energy metabolism does not enhance HIV-1 replication in RPMI-cultured CD4 T cells
Previous studies have demonstrated that glycolysis and glutaminolysis are crucial for HIV-1 replication in CD4 T cells when cultured in RPMI[10,17]. However, in our conditions, reduced concentrations of glucose and glutamine in the culture medium led to a lower energy metabolism, that was paradoxically associated with a higher virus replication. We therefore hypothesised that lower but physiological concentrations of glucose and glutamine in HPLM could be responsible for the enhanced HIV-1 replication in this medium. To test this hypothesis, we used RPMI without glucose and glutamine that was supplemented with 5mM glucose and 0.55mM glutamine to match the concentrations found in HPLM (RPMI+Carb). Cells were activated in RPMI, HPLM or RPMI+Carb and we first assessed the effect of these media on cellular energy metabolism by Seahorse analysis (Fig 4A). Cells activated in RPMI+Carb showed an intermediate metabolic phenotype that was not significantly different from either RPMI or HPLM. We then infected cells activated in these three media with HIV-1 NL4-3 and measured virus replication at day 4 post-infection by flow cytometry (Fig 4B). HIV-1 replication in cells activated in RPMI+Carb was similar to RPMI and significantly lower than in HPLM at the two viral doses tested. This suggests that the difference in energy metabolism of cells activated in RPMI and HPLM is not responsible for the enhanced HIV-1 replication observed in HPLM. To confirm that HIV-1 replication still relies on energy metabolism in HPLM, we used three chemical inhibitors targeting different steps in these pathways (summarised in Fig 4C): 2-deoxyglucose (2-DG) that targets hexokinase (HK) the first rate-limiting enzyme of

glycolysis; sodium fluoroacetate (NaFlAc) that targets Aconitase (ACO) and inhibits the TCA cycle shortly after entry of the products of glycolysis; and dimethyl-malonate (DMM) that inhibits succinate dehydrogenase (SDH), also known as complex II of the mitochondrial respiration chain and the key enzyme to use TCA metabolites in OXPHOS. CD4 T cells activated in RPMI or HPLM were infected with replicating HIV-1 NL4-3 (Fig 4D) in the presence of these inhibitors and virus infection was monitored at day 4 post-infection by flow cytometry. Regardless of the culture medium used, HIV-1 replication was blocked by treatment with 2-DG confirming previous reports and extending these findings to CD4 T cells cultured in HPLM. The other two inhibitors had no significant effect on viral replication neither in RPMI nor in HPLM.
Therefore, while the low glycolysis observed in CD4 T cells activated in HPLM is still crucial for HIV-1 infection, the differences in energy metabolism between RPMI and HPLM are not responsible for the increased HIV-1 replication seen in cells cultured in HPLM.

Discussion

In this study, we interrogated the impact of the human plasma metabolic environment on HIV-1 replication in primary activated CD4 T cells. Taking advantage of the recently formulated HPLM as a more physiologic medium, and comparing it to the standard RPMI medium, we show that CD4 T cells activated in HPLM are significantly more susceptible to HIV-1 infection. Surprisingly, and in contrast to previous studies performed in RPMI, this enhanced HIV-1 replication was associated with a lower energy metabolism, and in particular a lower glycolysis. We show that several metabolic pathways are affected by the use of HPLM but the change in energy metabolism is not responsible for the increased HIV-1 replication. We identified that HPLM affects the reverse transcription (RT) step of the viral replication cycle, providing further insight on the critical role of the metabolic context in shaping HIV-1 infection dynamics.
CD4 T cells activated in HPLM showed similar activation and proliferation profiles as cells activated in RPMI but increased replication of HIV-1. This is concordant with previous findings showing that CD4 T cells proliferation does not necessarily predict susceptibility to HIV-1 infection[7]. Instead, this study found that the metabolic state of the cells, and in particular their uptake of glucose, is important for HIV-1 reverse transcription. Similarly, we observed a 3-fold increase in HIV-1 RT at 20 hours post-infection in HPLM-activated CD4 T cells, which likely contributes to the increased viral replication observed at 4 days post-infection. The RT step of the viral life cycle is highly dependent on cellular metabolism, which conditions availability of the necessary dNTPs[18] and of the reverse transcriptase ion cofactor, Magnesium (Mg2+)[19]. The higher magnesium concentration in HPLM compared to RPMI could therefore enhance RT efficacy. Our metabolomic analyses also revealed an increase in metabolites from the pentose phosphate pathway (PPP) that is involved in nucleotide metabolism. While overall nucleotide pools were unchanged in HPLM-cultured cells, our study did not measure dNTPs specifically, leaving open the possibility of subtle shifts favouring RT. Previous investigations linked glucose uptake with a higher RT. However, CD4 T cells activated in HPLM exhibited a lower energy metabolism, characterised by a decrease in glycolysis and TCA cycle intermediates. Moreover, glucose concentration in HPLM is lower than in RPMI[13], and adjusting the concentrations of glucose and glutamine in RPMI to match HPLM did not reproduce a similar increase in HIV-1 replication. Therefore, other metabolic pathways and metabolites may account for the difference between the two conditions. We can also not exclude that another unexamined

step of the viral replication cycle, such as integration of the viral genome or infectivity of the virus particles, may contribute to the increased HIV-1 replication in HPLM conditions.

Our metabolomics analysis highlighted differential regulation of two metabolic pathways between HPLM and RPMI conditions: In HPLM, the hexosamine pathway is down-regulated, while the pentose phosphate pathway (PPP) is up-regulated. The hexosamine pathway produces UDP-N-acetyl-D-glucosamine (UDP-GlcNAc), the substrate for both N- and O-protein glycosylations, and is therefore important for this protein post-translational modification. In the context of HIV-1 infection, O-linked glycosylation of the Sp1 transcription factor was shown to inhibit HIV-1 transcription from the LTR both in T cell lines and in primary CD4 T cells[20]. In HPLM, the decreased hexosamine pathway and the resulting lower concentration of UDP-GlcNAc could decrease Sp1 O-linked glycosylation and concomitantly increase HIV-1 transcription. However, this appears unlikely as we observed no difference in Gag expression between CD4 T cells infected in HPLM versus RPMI. Similarly, the HIV-1 envelope gp120 can also be O- and N-glycosylated which has been shown to increase viral particle infectivity while decreasing recognition by neutralising antibodies[21]. In HPLM, the hexosamine pathway is downregulated and this mechanism would therefore decrease HIV-1 infectivity which is not consistent with our observed increase in HIV-1 replication. The pentose phosphate pathway (PPP) provides precursors for nucleotides, amino acids and fatty acids synthesis, and NADPH for oxidative stress management. The PPP was found to be crucial for the establishment and maintenance of HIV-1 latency [22]. Indeed, in this context, as activated cells were going back to a resting state, they decreased glycolysis and relied more on the PPP for pyrimidine metabolism and generation of the anti-oxidant NADPH which increased HIV-1 latency. This study did not observe these changes in productively infected cells which could be attributed to the use of cell lines (Jurkat T cells) and of RPMI. In contrast, our findings suggest that, in primary CD4 T cells activated in HPLM, glucose could be similarly diverted from glycolysis and into the PPP to support nucleotide metabolism necessary for reverse transcription. Interestingly, the metabolic phenotype we observe in HPLM – downregulated glycolysis with increased PPP activity - was shown as a hallmark of the establishment of HIV-1 latency. This new medium could therefore serve as a valuable model for studying HIV-1 latency and exploring therapeutic strategies such as “shock and kill”. Further investigations are needed to elucidate the specific roles of these pathways in HIV-1 replication and latency in primary CD4 T cells cultured in HPLM.

Here, we focussed our investigations on glycolysis and OXPHOS, key pathways previously shown to support HIV-1 replication in CD4 T cells activated in RPMI [10,17,23]. Inhibitors of these pathways were reported to significantly reduce HIV-1 infection. However, despite exhibiting lower energy metabolism, CD4 T cells activated in HPLM showed a higher susceptibility to HIV-1 infection. Our findings reveal that decreased energy metabolism is not directly responsible for the increased infection, as adjusting glucose and glutamine levels in RPMI to match HPLM did not produce the same effect. We also confirm that glycolysis remains crucial for HIV-1 infection in HPLM, as the glycolysis inhibitor 2-DG decreased HIV-1 replication, with an even more pronounced effect in HPLM than in RPMI. 2-DG is an analogue of glucose that blocks the very first steps of glucose metabolism in the cells, including glycolysis but also the PPP. We can speculate that the enhanced 2-DG efficacy observed in the HPLM context could be attributed to the limited glucose availability and to blocking both glycolysis and the PPP. In contrast, we did not observe a significant effect with TCA cycle or OXPHOS inhibitors such as DMM that generated a non-significant decrease in HIV-1 replication in RPMI and only a

modest effect in HPLM. This result contradicts recent studies[23] and suggests that HIV-1's reliance on OXPHOS might differ between the two media.
In conclusion, our study underscores the importance of using physiologically relevant models, which encompasses primary cells and more physiologic culture media, to investigate host-pathogen interactions, especially metabolic dependencies. This needs to be particularly considered for antiviral drugs development and for the study of latent HIV-1-infected CD4 T cells which account for HIV-1 persistence and incurable life-long infection in patients. While our model could be further refined, replacing FBS with human serum and using hypoxic conditions to mimic tissues, it already provides new information on metabolic pathways key for HIV-1 replication in near-physiologic conditions. It further highlights the critical role of metabolic pathways other than glycolysis and OXPHOS that will deserve attention in the future to identify new therapeutic targets.

Material and methods

Cells

Cells were kept in culture in either classical RPMI-1640 medium (Gibco), newly developed Human Plasma-Like Medium (HPLM, Gibco) or RPMI without glucose and glutamine (PAN Biotech) where glucose was added at 5mM and glutamine at 0.55 mM to match the concentrations in HPLM (RPMI+Carb). RPMI and RPMI+Carb were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% PenStrep (Gibco). HPLM was supplemented with 10% heat-inactivated dialysed FBS (Gibco) and 1% PenStrep (Gibco).
PBMCs were isolated from the blood of healthy donors by Ficoll centrifugation. The blood was provided by the EFS (Etablissement Francais du Sang, the official French blood bank). CD4+ T lymphocytes were isolated from PBMCs by positive selection using magnetic beads (EasySep Human CD4 positive selection kit II, StemCell Technologies) and activated using ImmunoCult human CD3/CD28 T cell activator cocktail (StemCell Technologies) then grown in presence of 100U/mL IL-2 (StemCell Technologies) for 4 days before being used in the various experiments. In all experiments, IL-2 was added in the culture medium at a final concentration of 100u/mL to support cell survival and growth.
The Jurkat T cell line was from ATCC (TIB-152) and cultured in RPMI.
HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1% PenStrep (Gibco).

Reagents

The reverse transcription inhibitor nevirapine (NVP) was from the NIH AIDS reagents program. The following metabolic inhibitors were used: 2-deoxy-glucose (2-DG, 5mM; Sigma); dimethyl malonate (DMM, 10mM; Sigma); sodium fluoroacetate (NaFlAc, 10µM; CliniSciences).

Viruses

Replicating HIV-1 NL4-3 was generated by transfection of pNL4-3 in HEK293T cells. Briefly, HEK293T cells were seeded in T175 flasks and transfected the next day using PEIpro (Polyplus). After overnight incubation, the medium was changed and the supernatant was collected 24h, 32h and 48h later. The three harvests were pooled and concentrated by ultracentrifugation through a 20% sucrose cushion at 100,000g for 1h30 then resuspended in PBS. The concentration of the viral stock was determined using p24 ELISA (Lenti-X™ p24 Rapid Titer Kit #631476 from Takara). And the doses used in all experiments were chosen by infecting primary CD4 T cells to reach appropriate levels of infection.

HIV-1 infection

150 000 primary CD4 T cells were seeded in round-bottom 96-well plates and infected with 25 or 100 ng p24/mL of HIV-1 NL4-3 WT in a final volume of 100uL. When indicated, the reverse transcriptase inhibitor NVP or the metabolic inhibitors were added in the wells at the time of infection.

For analysis of HIV-1 reverse transcription, the input virus was first treated with RQ1 DNase (Promega) for 1h at 37°C before adding to the cells. Two hours after infection, the cells were washed in PBS to remove input virus and kept in culture for an additional 18h.

For analysis of the virus particle release in the supernatant, the input virus was washed in PBS 2h after infection and cells were kept in culture for 3 days.

Flow cytometry

Cells were harvested at day 4 post-infection, stained with a viability dye (LIVE/DEAD fixable far red viability stain, ThermoFisher scientific) and fixed in 4% PFA for 15min. Intracellular Gag staining was performed in PBS+1%BSA+0.05%Saponine using the KC-57 anti-Gag antibody (Beckman-Coulter) for 30min at 4°C. After two washes in saponine buffer, the infection was analysed by flow cytometry looking at the percentage of cells expressing Gag. All samples were analysed with a NovoCyte Flow Cytometer (Agilent).

Fusion assay

Viral fusion was assessed as previously described[16]. Briefly, ultracentrifuged HIV-1 NL4-3 virions containing the Blam-Vpr fusion protein (a kind gift from Caroline Goujon) were used to infect primary CD4 T cells. After 2h, cells were washed in CO_2-independent medium supplemented with 10% FBS then incubated in CCF2-AM loading solution (CCF2-AM kit, ThermoFisher scientific) containing 2.5mM Probenecid (Sigma) for 2h at room temperature. Cells were then washed in CO_2-independent medium supplemented with 10% FBS and 2.5mM Probenicid and further incubated overnight in the same medium. The next day, cells were washed with PBS, fixed in 4% PFA for 15min, resuspended in FACS Buffer and analysed with a Novocyte Flow Cytometer looking at CCF2-AM in the AmCyan channel and cleaved CCF2-AM in the Violet channel.

Reverse transcription assay

Infected cells DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). Between 50-100ng of total DNA were used with the LightCycler 480 SYBR Green I Master mix (Roche) and amplified on a BioRad CFX Opus 384 Real-time PCR system. Primers for HIV-1 NL4-3 ssDNA early products were: M667 (5'- GGCTAACTAGGGAACCCACTG -3') and AA55M (5'- GCTAGAGATTTTCCACACTGACTAA -3') and for strand transfer late products: M667 and M661 (5'- CCTGCGTCGAGAGAGCTCCTCTGG -3'). PCR conditions were as follows: 95°C for 10min then 40 cycles of 95°C 15s, 65°C 15s and 70°C 20s. Quantities of cellular DNA were calculated using a plasmid standard and normalised to the amount of DNA used.

Virus particle release assay

At days 2 and 3 post-infection, supernatant of infected cells was harvested after cell centrifugation and frozen at -80°C. p24 titration was performed using an anti-p24 ELISA kit according to the manufacturer's protocol (Lenti-X™ p24 Rapid Titer Kit #631476 from Takara).

Seahorse ATP rate assay

Seahorse analyses of the energy metabolism in activated CD4 T cells were performed using the ATP rate assay (Seahorse Biosciences, Agilent) following manufacturer's protocol. Briefly, cells were counted, resuspended in Seahorse RPMI culture medium and seeded in six replicates in XF96 plates pre-coated with 0.1mg/mL poly-D-lysine. Cells were then incubated for a minimum of 1h in a CO_2-free incubator at 37°C before the plate was loaded in the Seahorse XF96e analyser. The protocol for the ATP rate assay was used following manufacturer's instructions with the following injections: (1) oligomycin (1μM); and (2) rotenone and antimycin A (1μM each) in ports A and B respectively. The ATP rate assay analysis was performed using the Seahorse Analytics online software.

Metabolomics analyses by IC- and LC-HRMS

For metabolomics analyses, CD4 T cells were activated in RPMI or HPLM for 5 days then washed in cold PBS and cell pellets were snap-frozen in liquid nitrogen and stored at -80°C until metabolites extraction. Sampling buffer (acetonitrile:methanol:water at a ratio of 4:4:2 + 1.25mM formic acid) was prepared and cooled to -20°C 2h before extraction. Cell pellets were resuspended in 1mL sampling buffer with IDMS controls and incubated for 1h at -20°C. Cell debris were discarded by centrifugation at 2000g for 5min at 4°C. Supernatants containing metabolites were transferred into a new tube, dried using a Speedvac and stored at -80°C until mass spectrometry analyses.

Central metabolites were separated on an ionic chromatography column IonPac AS11 (250 × 2 mm i.d.; Dionex, CA, USA). Mobile phase used was a gradient of KOH at a flow rate of 380 µL/min. Mobile phase was varied as follows: 0 min: 7 mM, 1 min: 7 mM, 9.5 min: 15 mM, 20 min: 15mM, 30 min: 45 mM, 33 min: 70mM, 33.1 min: 100mM and 42 min: 100mM. The column was then equilibrated for 5 min at the initial conditions before the next sample was analyzed. Time of analysis is 50 minutes. The volume of injection was 15µL. High-resolution experiments were performed with an ICS5000+, ion chromatography system (Dionex, CA, USA) system coupled to an Orbitrap Qexactive+ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization probe. MS analyses were performed in negative FTMS mode at a resolution of 70 000 (at 400 m/z) in full-scan mode, with the following source parameters: the capillary temperature was 350 °C, the source heater temperature, 350 °C, the sheath gas flow rate, 50 a.u. (arbitrary unit), the auxiliary gas flow rate, 10 a.u., the S-Lens RF level, 65 %, and the source voltage, 2.75 kV. Metabolites were determined by extracting the exact mass with a tolerance of 5 ppm.

For amino acid analysis, amino acids were separated on a PFP column (150 x 2.1 mm i.d., particle size 5 μm; Supelco Bellefonte, PEN, USA). Solvent 1 was 0.1% formic acid in H2O and solvent B was 0.1% formic acid in acetonitrile at a flow rate of 250 mL/min. The gradient was adapted from the method used by Boudah et al. Solvent B was varied as follows: 0min: 2%, 2 min: 2%, 10 min: 5%, 16 min: 35%, 20 min: 100% and 24 min: 100%. The column was then equilibrated for 6 min at the initial conditions before the next sample was analysed. The volume of injection was 5µL. For LTQ-Orbitrap High-resolution experiments were performed with Vanquish HPLC system coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization probe. MS analyses were performed in positive FTMS mode at a resolution of 60 000 (at 400 m/z) in full-scan mode, with the following source parameters: the capillary temperature was 275 °C, the source heater temperature, 250 °C, the sheath gas flow rate, 45 a.u. (arbitrary unit), the auxiliary gas flow rate, 20 a.u., the S-Lens RF level, 40 %, and the source voltage, 5 kV. Metabolites were determined by extracting the exact mass with a tolerance of 5 ppm.

Combined analysis was performed using MetaboAnalyst v6.0.

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Figures and Figures Legends

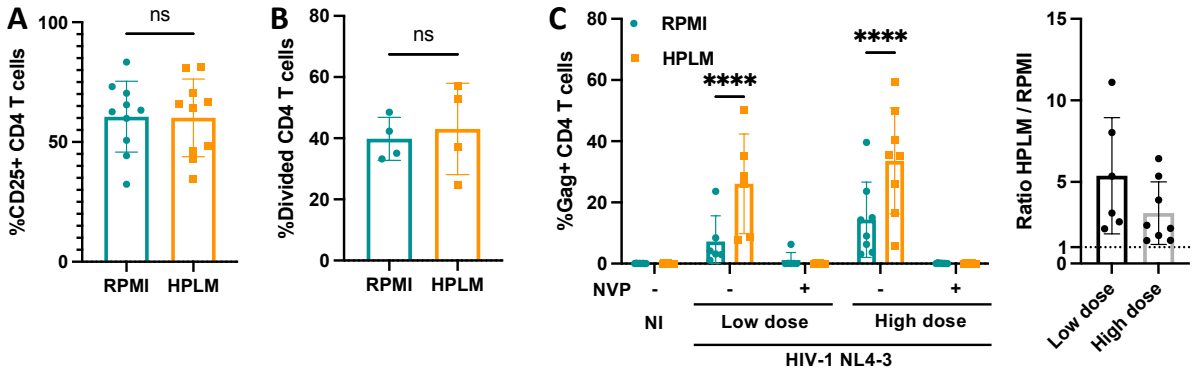

Figure 1: Human plasma-like metabolic environment potentiates HIV-1 replication in primary activated CD4 T cells. Primary CD4 T cells were activated in RPMI or in Human Plasma-Like Medium (HPLM). **A.** Expression of the CD25 activation marker was measured by flow cytometry at day 4 post-activation. Data show mean±SD of n=10 donors. **B.** Cell proliferation was monitored at day 5 post-activation by flow cytometry using CellTrace. Data show mean±SD of n=4 donors. **C.** Cells activated in the indicated medium for 4 days were infected with 25 or 100ng p24/mL of HIV-1 NL4-3 virus in the presence or absence of the RT inhibitor nevirapine (NVP) as a control. Virus replication was assessed at day 4 post-infection looking at HIV-1 Gag expression by flow cytometry. NI: non-infected. Data show mean±SD of n= at least 6 donors (left) and the ratio between infection rates in HPLM vs RPMI (right).
Statistical analyses were performed using 2-way ANOVA followed by Sidak's multiple comparison test. ns: non-significant; ****: $p<0.0001$

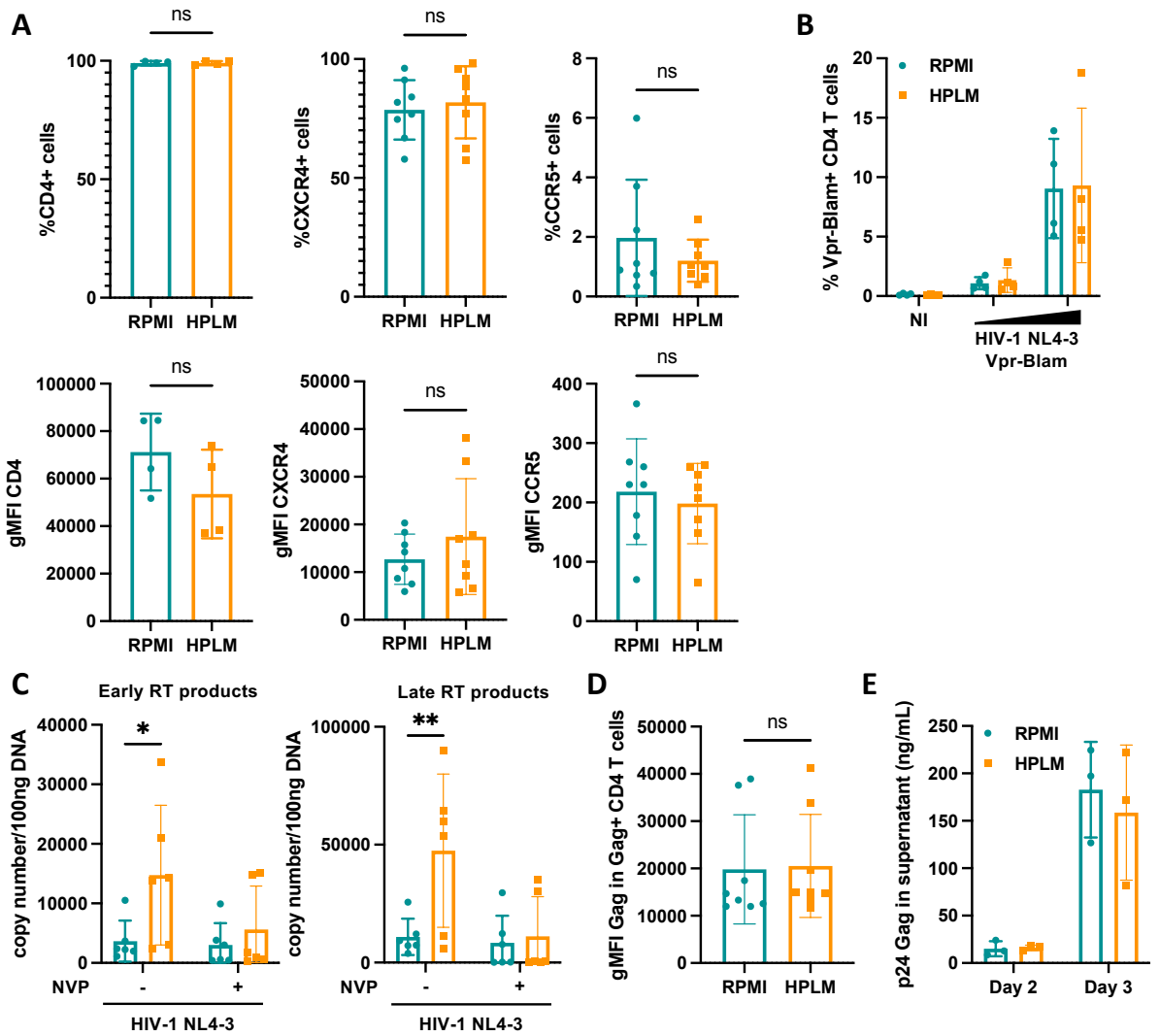

Figure 2: HPLM enhances the reverse transcription step of the HIV-1 life cycle. Primary CD4 T cells were activated in HPLM or RPMI. **A.** Expression of the HIV-1 receptor CD4 and co-receptors CXCR4 and CCR5 were monitored by flow cytometry at day 4 post-activation looking at percentage of positive cells (upper panels) and mean fluorescence intensity of the staining (lower panels). Data show mean±SD of n=at least 4 donors. **B.** Activated CD4 T cells were infected for 2h with 2 doses of an HIV-1 NL4-3 virus containing the Vpr-Blam fusion protein. Viral fusion with the cells was measured by flow cytometry, monitoring the cleavage of the beta-lactamase substrate CCF2-AM that results in a fluorescence shift. NI: non-infected. Data show the percentage of cells in which fusion has occurred as mean±SD of n=4 donors. **C.** Activated CD4 T cells were infected for 2h with DNase treated HIV-1 NL4-3 in the presence or absence of the RT inhibitor nevirapine (NVP) and incubated for 18h before DNA extraction. qPCR for the early and late RT products was performed on normalised quantities of cellular DNA. Data show mean±SD of 4 donors. **D.** Activated CD4 T cells were infected as described in Figure 1C. Expression levels of the HIV-1 Gag protein were measured by flow cytometry at day 4 pi quantifying the mean fluorescence intensity (gMFI) in Gag+ cells. Data show mean±SD of 8 donors. **E.** Activated cells were infected with HIV-1 NL4-3 for 2h, extensively washed and incubated for 3 days. At days 2 and 3 post-infection, supernatants were collected and the amount of released virus particles was quantified using anti-p24 ELISA. Data show mean±SD of 3 donors.

Statistical analyses were performed using Wilcoxon tests or 2-way ANOVA followed by Sidak's multiple comparison test. ns: non-significant, *: p<0.05; **: p<0.01.

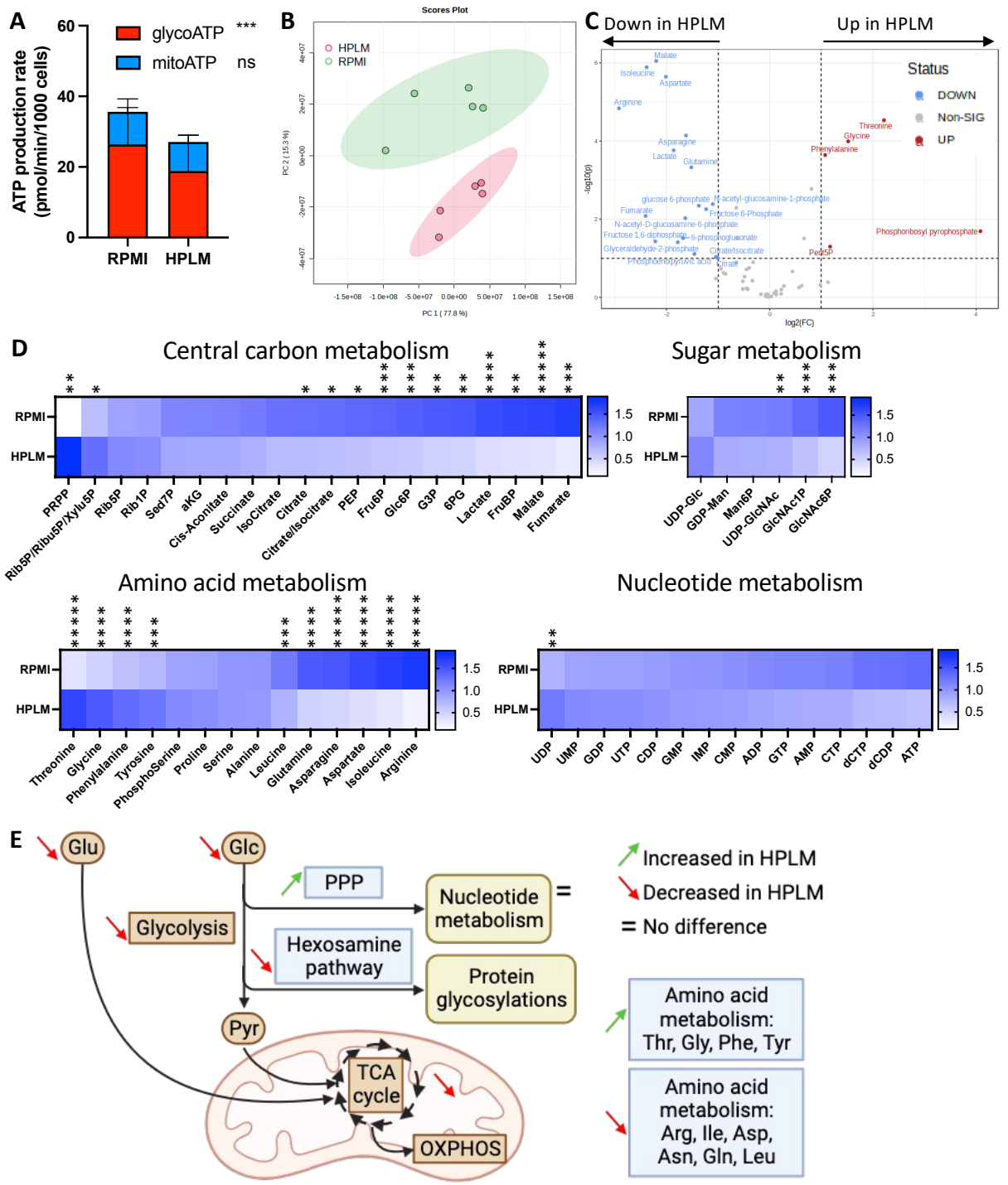

Figure 3: Primary CD4 T cells activated in HPLM exhibit a modified metabolism, including reduced energy metabolism. General metabolism of primary CD4 T cells activated in HPLM or RPMI was assessed at day 4 or 5 post-activation. **A**. Seahorse analysis showing the ATP production rate in live cells at day 4 post-activation and the individual contribution of glycolysis (GlycoATP) and mitochondrial respiration (mitoATP). Data show mean±SD of 6 donors. Statistical analysis was performed using 2-way ANOVA followed with Sidak's multiple comparisons test. ns: non-significant; ***: p<0.001. **B-D.** Activated cells were then analysed by metabolomics using mass spectrometry at day 5 post-activation. Comparison of the two

conditions is shown as a PCA plot (**B**) and a volcano plot highlighting metabolites with both fold change >2 and p-value <0.1 (**C**). Heatmaps of the mean values for all metabolites grouped by the main metabolic pathways (**D**). Analyses were performed on n=5 donors using MetaboAnalyst. *: p<0.1; **: p<0.5; ***: p<0.01; ****: p<0.001; *****: p<0.0001 **E**. Schematic representation of the metabolic pathways most affected by HPLM (Created in BioRender).

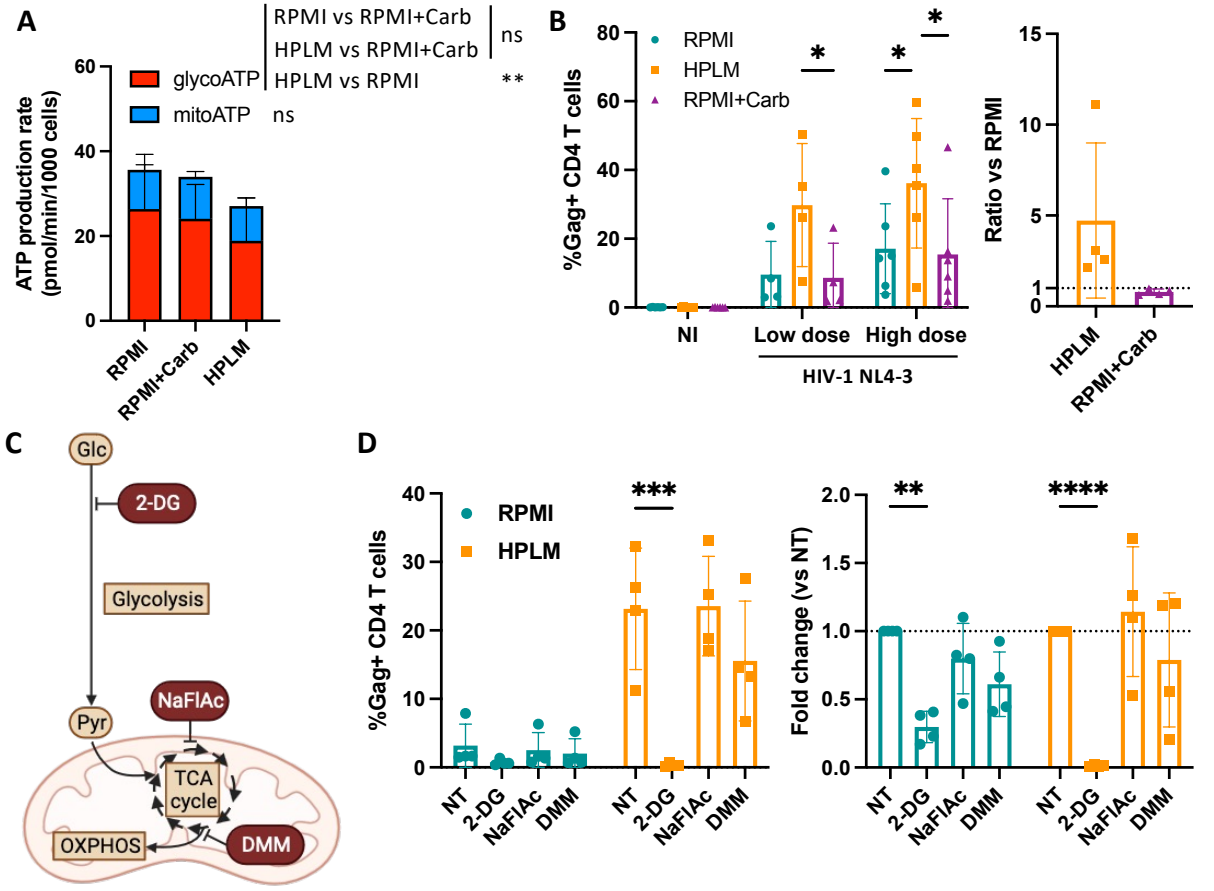

Figure 4: Altered energy metabolism does not enhance HIV-1 replication in RPMI-cultured CD4 T cells. Primary CD4 T cells were activated in HPLM, RPMI or RPMI medium where the concentrations of carbon sources were adjusted to match the ones in HPLM (RPMI+Carb). **A**. Seahorse analysis showing the ATP production rate in live cells at day 4 post-activation and the individual contribution of glycolysis (GlycoATP) and mitochondrial respiration (mitoATP). Data show mean±SD of 6 donors. **B**. Cells activated in the indicated media were infected at day 4 post-activation as in Fig 1C. Virus replication was assessed at day 4 post-infection by measuring HIV-1 Gag expression using flow cytometry. NI: non-infected. Data show mean±SD of n= at least 4 donors (left) and the ratio between infection rates in HPLM or RPMI+Carb vs RPMI (right). **C-D**. Schematic representation of the energy metabolic pathways targeted by the inhibitors used (**C**; Created in BioRender). CD4 T cells activated in HPLM or RPMI were infected with HIV-1 NL4-3 in the presence of the indicated drugs. Infection was monitored by flow cytometry looking at Gag at day 4 post-infection (**D**). Data show mean±SD of raw data (left) or fold change compared to the non-treated condition (NT) (right) for n=4 donors.
Statistical analysis was performed using 2-way ANOVA followed with Tukey's multiple comparisons test (B) or Dunnett's multiple comparisons test (D-E). ns: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

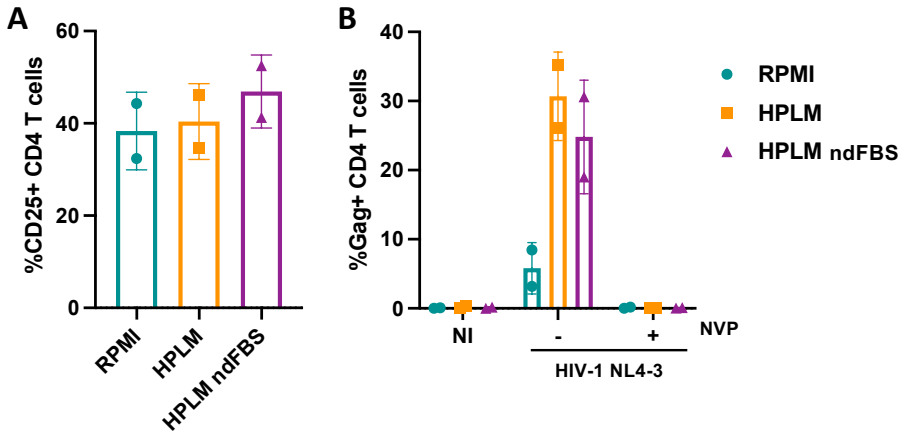

Figure S1: FBS does not impact the difference between RPMI and HPLM. Primary CD4 T cells were activated in RPMI, HPLM or HPLM supplemented with non-dialysed FBS (ndFBS). 4 days after activation, expression of the activation marker CD25 was measured by flow cytometry (**A**) and cells were infected with HIV-1 NL4-3. **B**. HIV-1 replication was monitored at day 4 post-infection by flow-cytometry looking at the expression of HIV-1 Gag. Data show mean±SD of 2 donors.

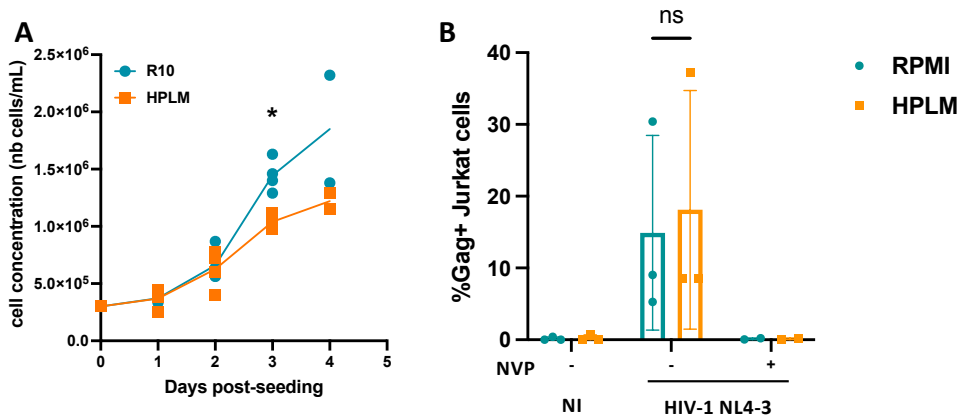

Figure S2: HPLM does not affect HIV-1 replication in the Jurkat T cell line. A. Jurkat T cells were kept in culture in HPLM or RPMI and their growth was measured by counting cells every day. Data show mean and individual values for n=at least 2 experiments. **B.** Jurkat T cells were infected with a replicating HIV-1 NL4-3 in the indicated medium. Infection was monitored at day 4 post-infection looking at HIV-1 Gag expression by flow cytometry. Data show mean±SD of n=3 independent experiments.
Statistical analyses were performed using 2-way ANOVA followed by Sidak's multiple comparisons test. ns: non-significant; *: $p<0.05$.

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