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► **To cite this version:**

M. Jaquet, Michèle Bengue, Karen Lambert, Gilles Carnac, Dorothée Missé, et al.. Human muscle cells sensitivity to chikungunya virus infection relies on their glycolysis activity and differentiation stage. *Biochimie*, 2024, 218, pp.85-95. 10.1016/j.biochi.2023.09.005 . hal-04209327

HAL Id: hal-04209327

<https://hal.science/hal-04209327>

Submitted on 18 Jan 2024

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Human muscle cells sensitivity to chikungunya virus infection relies on their glycolysis activity and differentiation stage

M. Jaquet [a, b](#), M. Bengue [b](#), K. Lambert [a](#), G. Carnac [a](#), D. Missé [b, **](#), C. Bisbal [a, *](#)

[a](#) PhyMedExp, Univ. Montpellier, INSERM U1046, CNRS UMR 9214, 34295, Montpellier Cedex 5, France

[b](#) MIVEGEC, Univ. Montpellier, IRD, CNRS, 34394, Montpellier, France

Keywords:

Human skeletal muscle; Metabolism; Viral infection; Chikungunya virus; Viral reservoir
Stem cells.

* **Corresponding author.** PhyMedExp, INSERM U1046, Institut Universitaire de Recherche Clinique, 641 av du Doyen G. Giraud, 34093, Montpellier cedex 5, France.

** **Corresponding author.** MIVEGEC, UMR IRD 226 CNRS 5290, IRD 911 Avenue Agropolis, 34394, Montpellier cedex 5, France.

E-mail addresses:

dorothee.misse@ird.fr (D. Missé), catherine.bisbal@inserm.fr (C. Bisbal).

a b s t r a c t

Changes to our environment have led to the emergence of human pathogens such as chikungunya virus. Chikungunya virus infection is today a major public health concern. It is a debilitating chronic disease impeding patients' mobility, affecting millions of people. Disease development relies on skeletal muscle infection. The importance of skeletal muscle in chikungunya virus infection led to the hypothesis that it could serve as a viral reservoir and could participate to virus persistence. Here we questioned the interconnection between skeletal muscle cells metabolism, their differentiation stage and the infectivity of the chikungunya virus. We infected human skeletal muscle stem cells at different stages of differentiation with chikungunya virus to study the impact of their metabolism on virus production and inversely the impact of virus on cell metabolism. We observed that chikungunya virus infectivity is cell differentiation and metabolism-dependent. Chikungunya virus interferes with the cellular metabolism in quiescent undifferentiated and proliferative muscle cells. Moreover, activation of chikungunya infected quiescent muscle stem cells, induces their proliferation, increases glycolysis and amplifies virus production. Therefore, our results showed that Chikungunya virus infectivity and the antiviral response of skeletal muscle cells relies on their energetic metabolism and their differentiation stage. Then, muscle stem cells could serve as viral reservoir producing virus after their activation

1. Introduction

Changes to our environment, exacerbated by human activities, have led to the emergence of human pathogens such as chikungunya virus (CHIKV). CHIKV is an arbovirus of the genus Alphavirus of the Togaviridae family, transmitted to humans predominantly by *Aedes (Ae.) aegypti* and *Ae. albopictus* mosquitoes [1]. After decades of sporadic epidemics in sub-Saharan Africa, the global expansion of these mosquito species have led to an increasingly large geographical spread area of CHIKV to the tropical and subtropical regions. Moreover, several cases of local infections have now been identified in Europe, particularly in Italy and in the South of France [2,3]. CHIKV has a high pandemic potential [4] and there is currently no antiviral agents or treatments available. In addition, several candidate vaccines are still in the development stage and any vaccine is actually available [5]. The most common symptoms of CHIKV infection include high fever, headache, rash, arthralgia and myalgia. The last two clinical manifestations could be present over a period of months to years, making CHIKV infection a severely debilitating chronic disease that could lead to arthropathies and myopathies and impede patient mobility and activity [6,7]. Understanding the molecular mechanisms regulating infection of affected organs is thus crucial in the improvement of CHIKV disease prevention and treatment, to limit the development of chronic and severe forms of CHIKV infection. CHIKV displays broad cell and tissue tropism [8], however, several studies in human and mouse models indicate that CHIKV is preferentially detected in peripheral joints and muscle tissues [9]. Interestingly, Lentscher et al. showed recently that CHIKV-skeletal muscle (SM) infection and replication was required for the development of CHIKV pathogenicity [10], which strongly supports the findings of previous studies indicating that SM infection contributes to CHIKV pathogenicity [11e14]. The importance of SM in CHIKV infection led to the hypothesis that SM could serve as a viral reservoir and could, therefore, participate to CHIKV persistence [15]. One of the properties of adult SM is its remarkable capacity of effective regeneration to maintain its normal physiology and mass. This regenerative capacity is due to a population of SM resident stem cells localized under the muscle basal lamina, known as satellite cells (SC) [16]. SC are essential for normal turnover, growth, or repair of the SM [17]. In adult SM, SC are undifferentiated and unipotent. They are in a quiescent state, with little to no basal turnover. After activation, SC enter the cell cycle and undergo multiple rounds of proliferation (myoblast stage, MB), morphologically and biochemically differentiate (myotube stage, MT) to form mature myofibers and reconstitute SM during a sequential and highly coordinated process [17]. Quiescent undifferentiated (SC), proliferating (MB) and quiescent differentiated cells (MT) have different energetic metabolism due to their very different needs [18]. Energetic metabolism, via the production of ATP from lipids oxidation or glucose utilization, allows the production of the cellular energy and of the biological components necessary to all the anabolic processes. As such, energetic metabolism is essential to maintain cell homeostasis and survival. Moreover, this metabolism is an important regulator of SM cell fate [18,19]. The central sources of ATP are glycolysis in the cytoplasm [20] and the oxidative phosphorylation (OXPHOS) in the mitochondria [21]. Energetic metabolism has been identified as a master regulator of SC activation and proliferation [18]. SC in a quiescent state have low metabolic demands, however, when SC exit the quiescent state, they undergo a metabolic shift from lipids to glucose oxidation [18]. In addition, during infection, viruses use, interfere and reprogram host cell energetic metabolism to increase the supply of nutrients, energy, and metabolites necessary to their multiplication and spread [22]. This interference with cell metabolism can also allow viruses to evade anti-viral immune responses [23]. Moreover, the cell antiviral response and particularly the interferon (IFN) pathway could also regulates some aspects of the cell

energetic metabolism as glycolysis [24,25]. During CHIKV infection, activation of intracellular pattern recognition receptors (PRR), melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene I-like receptors (RIGI) induces type I IFN [26]. IFN in turn, could induce the transcriptional up-regulation of several hundred of interferon-stimulated genes (ISG) with a broad spectrum of biological activities. Among these ISG, some are known to be highly induced during CHIKV infection and interfere with CHIKV production, such as 20-50-oligoadenylate synthetase 3 (OAS3) [27], interferon induced protein with tetratricopeptide repeats 1 (IFIT1) [28] and virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (viperin) [29]. Interestingly, viperin is known as an important regulator of metabolism during obesity [30], diet induced glucose intolerance [31] or cytomegalovirus (CMV) infection [32]. Therefore, a tri-directional relationship between host energetic metabolism, virus and IFN pathway could lead to different regulation of CHIKV production. This has led us to investigate, CHIKV infection of human SM cells at different stages of proliferation and differentiation SC, MB and MT. These cells are all components of adult SM and they possess an energetic metabolism corresponding to their needs. Importantly, these differentiation stages could be mimicked in vitro [25,33]. Our results show a differentiation-dependent susceptibility of human SM cells to CHIKV infection. In fact, CHIKV production relies on the energetic metabolism of SM cells and in particular on glycolysis activity and lactate levels. Moreover, CHIKV infection highly induces glycolysis in MB. We also observed that SC produce very low quantities of infectious viral particles, but their activation and proliferation that induce glycolysis also induce CHIKV production. These results suggests that, in vivo, SC could serve as a CHIKV reservoir in SM and that their activation could lead to viral production.

Abbreviations		
OAS3	(2'-5'-oligoadenylate synthetase 3)	MAVS (mitochondrial anti-viral signaling protein)
TNB	(5-thio-2-nitrobenzoate anion)	M-MLV (moloney murine leukemia virus)
DTNB	(5,5'-Dithiobis-2-nitrobenzoic acid)	MOI (multiplicity of infection)
CHIKV	(chikungunya virus)	MCMV (murine cytomegalovirus)
CS	(citrate synthase)	MB (myoblast)
HADH	(hydroxyacylCoA dehydrogenase)	MT (myotube)
CMV	(cytomegalovirus)	NAD (nicotinamide adenine dinucleotide)
DDR	(DNA damage response)	NADH (nicotinamide adenine dinucleotide oxidized)
DV	(dengue virus)	NI (non-infected)
DM	(differentiation medium)	OXPHOS (Oxidative phosphorylation)
DMEM	(Dulbecco's Modified Eagle Medium)	PRR (pattern recognition receptors)
FCS	(fetal calf serum)	PFK (phosphofructokinase)
FFAs	(free fatty acid)	PBS (phosphate buffer saline)
hpi	(hours post-infection)	PM (proliferation medium)
IAV	(influenza A virus)	FADH2 (reduced form of flavin adenine dinucleotide)
IFN	(interferon)	RIGI (retinoic acid-inducible gene I-like receptors)
IFIT1	(interferon induced protein with tetratricopeptide repeats 1)	RLR (Retinoic acid-inducible gene (RIG)-I-like receptor)
ISG	(interferon-stimulated genes)	SC (satellite cells)
LDH	(lactate dehydrogenase)	SLC (satellite like cells)
MACS	(magnetic-activated cell sorting technology)	SARS-CoV2 (severe acute respiratory syndrome coronavirus 2)
MDA5	(melanoma differentiation-associated protein 5)	SM (skeletal muscle)
		T2D (type 2 diabetes)
		viperin (virus inhibitory protein endoplasmic reticulum-associated IFN-inducible)

2. Materials and methods

2.1. Primary human cell isolation and culture

SM satellite cells (SC) were isolated from biopsies of three men (32 ± 4 years). SM biopsies were obtained from the left vastus lateralis [34] at rest and in fasting state, after local anesthesia with xylocaine 1%. The study protocol was approved by the French ministry of research (protocol number DC-2008-594), and, in accordance with the Declaration of Helsinki and ethical guidelines, written informed consent was obtained from each participant. SC were obtained from SM biopsies by immune-purification with anti-CD56 antibody (DB Biosciences, Le Pont-de-Claix, France) and magnetic-activated cell sorting technology (MACS) (Miltenyi Biotec, Paris, France). After purification, SC were separately pooled and grown as mass cultured cells as previously described [25,33]. Purified cells were plated at $3 \times 10^3/\text{cm}^2$ density in collagen coated 6 well plates (Corning, Dominique Dutscher, Brumath, France) in rich growth medium allowing cell proliferation: Proliferation medium (PM). PM: Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Thermo Fisher Scientific Illkirch Cedex - France) supplemented with fetal calf serum (FCS) 20% (V/V) (PAN BIOTECH, Dominique Dutscher, Brumath, France) and Ultrosor Serum Substitute 0.5% (V/V) (Pall Corporation, Saint-Germain-en-Laye France). These plated and proliferating cells are myoblasts (MB). After 4 days of culture, MB were infected with CHIKV (see paragraph 2.2). For myotubes (MT) infection, purified cells were plated at $7 \times 10^3/\text{cm}^2$ density in collagen coated 6 well plates in PM. After 3 days, cells were at 80% confluence and were switched to muscle differentiation medium (DM), which consisted of DMEM supplemented with 2% FCS. After 3 days of culture, cells have differentiated and fused to form multinucleated myotubes (MT) and are infected with CHIKV. Between differentiated MT, some cells do not proliferate and do not differentiate; these quiescent cells were designated as reserve cells [35] also known as satellite like cells (SLC) as they express a phenotype similar to quiescent cells [36]. MT are mechanically withdrawn to let only SLC cells. SLC cells are then infected with CHIKV. To avoid cell senescence, all experiments were performed on cell passages 4 to 5 after isolation of SLC [25].

The cytopathic effect of CHIKV was assessed by evaluating the activation of the cellular DNA damage response (DDR). The DDR was measured at various time points after infection by determining the ratio between the serine-phosphorylated H2A histone family member X (P-g-H2AX) and the non-phosphorylated H2AX, as previously observed during CHIKV infection [37,38].

2.2. Virus and cell infection

The low-passage-number LR2006_OPY1 strain (a kind gift from Dr Philippe Després, PIMIT, Inserm U1187, St Clotilde), was isolated from a viremic patient in La Réunion Island in 2006. CHIKV strain was grown in C6/36 cells (*Ae. albopictus* cells), at 28 °C in DMEM supplemented with 10% FCS. At the time of infection, the cell density was equivalent for the three types of cell: $1.22 \times 10^5 \pm 2.5 \times 10^3$ for SLC; $1.5 \times 10^5 \pm 2.1 \times 10^3$ for MB and $1.7 \times 10^5 \pm 1.25 \times 10^3$ for MT. Cells were rinsed twice with phosphate buffer saline (PBS) and incubated with CHIKV at a multiplicity of infection (MOI) of 2 in cell media for 2 h at 37 °C while gently agitating the plates. Control non-infected cells were incubated with culture supernatant from uninfected C6/36 cells. After removing the inoculum, cells were washed three times with PBS.

Then, the appropriate cell culture medium i. e PM or DM supplemented or not, as described below, was added to each well and the plates were incubated at 37 °C and 5% CO₂. Cell supernatants and cells were collected at 24 and 48 h post infection (hpi) to determine viral titers, viral RNA production, protein expression levels and enzymatic activity. Data are expressed as mean ± SEM of two independent experiments performed in triplicate.

2.3. Activation of SLC cells

At 24hpi, cells were kept in DM medium supplemented or not with 2-Deoxy-D-glucose (2DG) 5 mM (Sigma-Aldrich, ST Quentin Fallavier, France) or DM supplemented with 2DG (5 mM) and lactate (20mM) (Sigma-Aldrich) or were switch to PM supplemented or not with 2DG (5 mM) during 24 h (48hpi) and 48 h (72hpi).

2.4. Measure of cell viability

The cell viability of SLC and MB, treated with 5 mM 2 DG or 20 mM lactate, was evaluated at 24 h and 48 h using the Presto Blue Assay InVitrogen (Thermo Fisher Scientific), following the manufacturer's instructions.

2.5. Viral RNA quantification

Total RNA was extracted from SLC, MB and MT using Tri Reagent (Sigma-Aldrich). RNA was reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Charbonnières, France) according to the manufacturer's instructions. The reaction was carried out using 1 mg total RNA as template for the normalization of viral RNA to the amount of total RNA. The Maxima TM Probe/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific, Illkirch, France) was used in qPCR experiment. Each reaction of 25 mL contained 400 nM of each primer, 200 nM of specific probe and 1x Maxima Probe/ROX qPCR Master Mix. The following CHIKV Primer Sequence (5'->30) were used:

CHIKV-F 50AAGCT (CT)CGCGTCCTTTACCAAG3';

CHIKV-R 50CCAAATTGTCC(CT)GGTCTTCCT30';

CHIKV-P 50CCAATGTC (TC)TC (AC)GCCTGGACACCT3' [39].

The amplification conditions were 95 °C for 10 min followed by 45 amplification cycles of 95°C for 15 s, 60 °C for 20 s and 72 °C for 30 s. The reactions were performed in an Applied Biosystem 7300 system. Real time data were analyzed using the SDS software (Thermo Fischer Scientific). Viral RNA was quantified by comparing the sample's threshold cycle (Ct) values with each virus RNA standard curve that was obtained as previously described [39]. Data are expressed as mean ± SEM of two independent experiments performed in triplicate.

2.6. Plaque assay

Vero cells, grown to 70-80% confluence, were incubated with ten-fold dilutions of viral supernatant in DMEM at 37 °C for 2 h. A mix of nutriment solution with agar (Lonza, Levallois-Perret, France) was added and the cells were maintained at 37 °C for 6 days. For plaque counting, the cells were incubated with 3.7% formaldehyde (Sigma) and 0.5% Crystal

violet (Sigma) in 20% ethanol [39]. Data are expressed as mean \pm SEM of two independent experiments performed in triplicate.

2.7. Western blot analysis

Cell pellets were disrupted and homogenized in a hypotonic lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich) [40]. Total proteins (40 mg) were analyzed by Western blot. Nitrocellulose membranes were incubated with the specific antibodies: RIGI, MDA5, IFIT1, viperin, OAS3, phosphoserine 139-histone H2AX (P-g-H2AX) and H2AX (all from Cell Signaling Technology, Ozyme, Saint-Quentin-en-Yvelines), and species-directed secondary antibodies conjugated to IRDye800 or IRDye680 (LI-COR Biosciences, Eurobio). Membranes were analyzed with the Odyssey CLx Imaging System (LI-COR Biosciences, Bad Homburg, Germany). Protein expressions were quantified using ImageJ software (National Institutes of Health, USA, available online at <http://rsbweb.nih.gov/ij/index.html>). Data are expressed as mean \pm SEM of two independent experiments performed in triplicate.

2.8. Energetic metabolism-Enzymatic activity

2.8.1. Citrate synthase (CS) activity was monitored in 1 ml of total cell extract by following the formation of 5-thio-2-nitrobenzoate anion (TNB) generated from 5,5-Dithiobis-2-nitrobenzoic acid (DTNB) (Sigma-Aldrich) [41]. CS activity was normalized to protein quantity and expressed relatively to CS activity in control noninfected SLC at 24 h that level was set to 1.

2.8.2. HydroxyacylCoA dehydrogenase (HADH) activity: 2.5 ml of total cell extract were incubated at 25°C in 133 mM triethanolamine pH7.0, 10 mM aceto-acetyl-CoA, 3.4 mM NADH, 20 mM KCN and 100 mM EDTA (all from Sigma-Aldrich). The formation of NAD generated from NADH was evaluated by following the absorbance at 340 nm over 5 min as previously described [42]. HADH activity was normalized to protein quantity and expressed relatively to HADH activity in control non-infected SLC at 24 h that level was set to 1.

2.8.3. Phosphofructokinase (PFK) activity: 2.5 ml of total cell extract were incubated in 100 mM Tris pH 8.2, 120 mM MgCl₂, 2.5 M KCl, 20 mM KCN, 20 mM ATP, 40 mM AMP, 3.4 mM NADH, 60 mM Fructose 6 Phosphate, 10 units of glycerol 3 phosphate dehydrogenase, 0.9 unit of aldolase and 10 units of triose phosphate isomerase as previously described [42]. The disappearance of NADH was evaluated by following over 3 min the absorbance at 340 nm. PFK activity was normalized to protein quantity and expressed relatively to PFK activity in control non-infected SLC at 24 h that level was set to 1.

2.8.4. Lactate dehydrogenase (LDH) activity was measured in 1 ml of total cell extract with the LDH Assay Kit (Abcam, Paris, France) following manufacturer instructions. LDH activity was normalized to protein quantity and expressed relatively to LDH activity in control non-infected SC at 24 h that level was set to 1.

2.8.5. Lactate concentration was measured using the Lactate assay kit (Cell Biolabs, Clinisciences, Nanterre, France) in 10 ml of total cell extract following manufacturer instructions. Lactate concentration was normalized to protein quantity and expressed relatively to lactate concentration in control non-infected SLC at 24 h that level was set to 1.

Data are expressed as mean \pm SEM of two independent experiments performed in triplicate.

2.9. Statistical analyses

Multiple groups comparisons were performed using 2-way ANOVA (two variable) or one-way ANOVA (one variable) followed by Tukey's post-hoc test. Two groups' comparisons were performed using student t-test. All analyses were performed with GraphPad PRISM 6 software. For all tests, we considered that significance was reached when $P \leq 0.05$ with * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

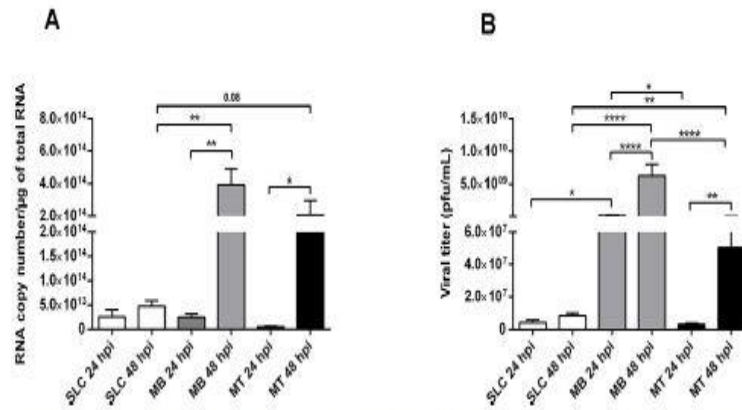


Fig. 1. Human skeletal muscle satellite cells (SLC), myoblasts (MB) and myotubes (MT) present different sensitivity to CHIKV infection. SLC, MB and MT were infected with CHIKV at multiplicity of infection (MOI) of 2. At 24 hpi and 48 hpi, RT-qPCR was performed to measure viral RNA level (A) and infected cell supernatants were analyzed using plaque assay to quantify infectious viral particles (B). Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way or 1-way ANOVA followed by Tukey's post-hoc test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

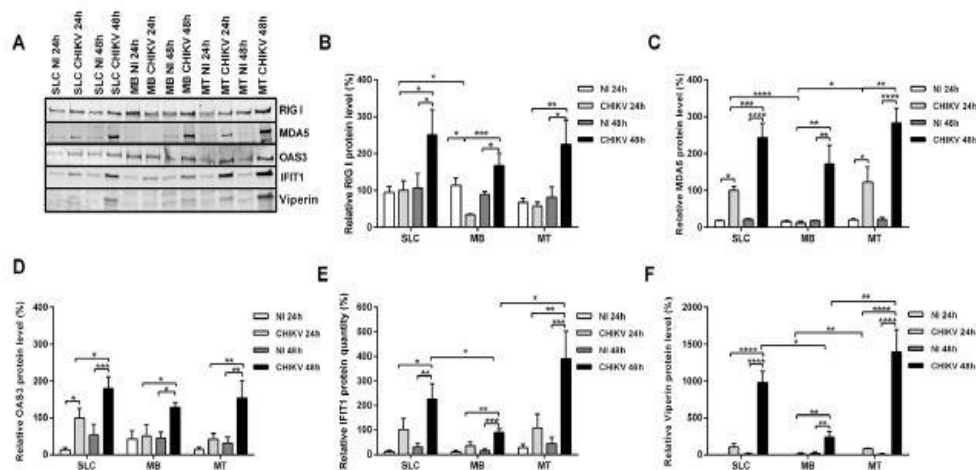


Fig. 2. Induction of ISG following CHIKV infection of human SM cells. SLC, MB and MT were infected with CHIKV at MOI of 2. Total proteins were extracted in non-infected and infected cells at 24 hpi and 48 hpi. Protein expression levels of RIG I, MDA5, IFT1, viperin, OAS3, were quantified by immunoblotting. A) Representative blots of the different ISG are shown. B) RIG I, C) MDA5, D) OAS3, E) IFT1 and F) Viperin protein levels were expressed relatively to their level in CHIKV-infected SLC cells at 24 hpi that was set at 100%. Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way or 1-way ANOVA followed by Tukey's post-hoc test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

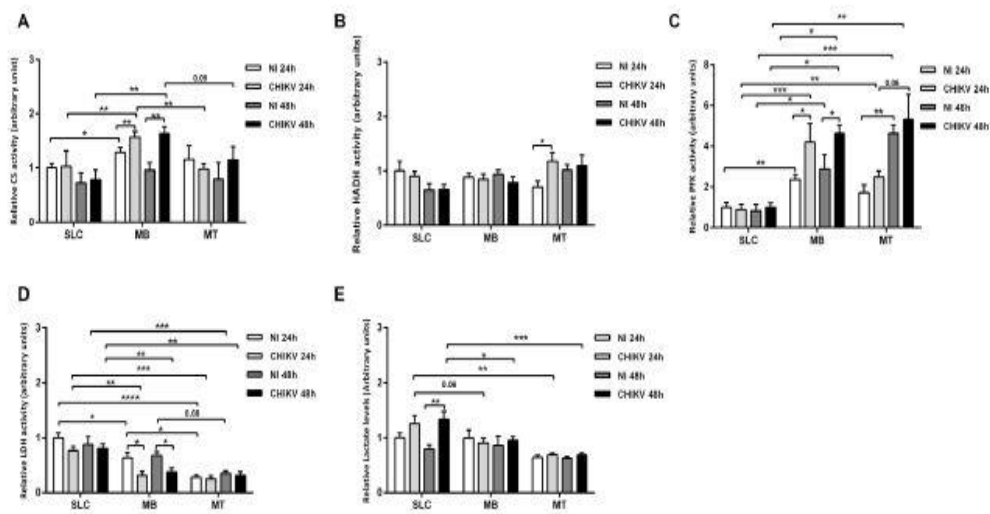


Fig. 3. Energetic metabolism in human SM cells before and during CHIKV infection. SLC, MB and MT were infected with CHIKV at MOI of 2. Total proteins were extracted in non-infected and infected cells at 24 hpi and 48 hpi. Cell extracts were analyzed for A) Citrate synthase activity (CS). B) 3-hydroxyacyl-CoA dehydrogenase (HADH) activity. C) Phosphofruktokinase (PK) activity. D) lactate dehydrogenase (LDH) activity. E) lactate level. Data are expressed relatively to their level in SLC non-infected cells at 24 hpi that was set at 1. Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way or 1-way ANOVA followed by Tukey's post-hoc test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3.1. Human SM cells have different sensitivity and antiviral response to CHIKV infection

We first characterized SLC, MB and MT sensitivity to CHIKV infection by measuring CHIKV viral RNA levels and infectious viral particles production in the three human SM cells after CHIKV exposure. As shown in Fig. 1A, CHIKV RNA levels were equivalent in the three types of cells at 24hpi. CHIKV RNA levels highly and specifically increased at 48hpi in MB and MT compared to 24 hpi, while CHIKV RNA level remained low in SLC. At 24hpi, virus titer was higher in MB compared to MT and SLC. Similar to viral RNA, virus titers increased in MB and MT at 48 hpi, while it remained constant in SLC (Fig. 1B). While SLC, MB, and MT were infected with CHIKV at the same cell density, proliferative MB cells produced a higher quantity of viral particles compared to quiescent undifferentiated SLC and differentiated MT cells (Fig. S1). Furthermore, we assessed whether CHIKV could induce a cytopathic effect in SLC, MB, and MT cells. One such effect observed during CHIKV infection is DNA damage, followed by the activation of the cellular DNA damage response (DDR) [37,38,43]. The DDR was measured at different time points post-infection by quantifying the ratio between the serine phosphorylated H2A histone family member X (Pg-H2AX) and the non-phosphorylated H2AX, as previously described during CHIKV infection [38]. As depicted in Fig. S2, we did not observe any activation of the DDR in the three types of human muscle cells during CHIKV infection, neither at 24 hpi nor at 48 hpi. We then analyzed the protein expression levels of some ISG well known to regulate CHIKV infection: RIGI/MDA5 [26], OAS3 [27], IFIT1 [28] and viperin [29].

We observed a comparable induction of RIGI, MDA5 and OAS3 proteins in SLC, MB and MT (Fig. 2A, 2B, 2C and 2D). However, there were some differences in the kinetic of induction of RIGI and MDA5 (Fig. 2A, 2B and 2C) in MB. IFIT1 and viperin proteins expressions were also lower in MB compared to SLC and MT (Fig. 2A, 2E and 2F).

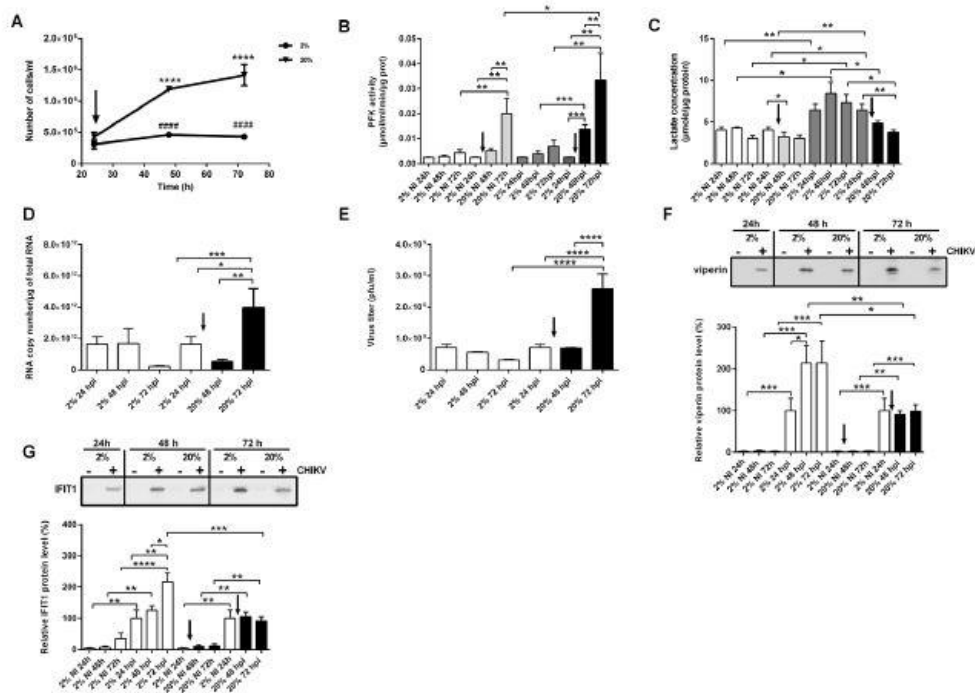


Fig. 4. Cell growth, energetic metabolism, viral production and ISG induction in activated SLC cells. SLC were infected or not with CHIKV at MOI of 2. At 24 hpi, cells were kept in cell differentiation medium (2%) or switched to cell proliferation medium (20%). A) Cell growth curve of non-infected SLC cells at 24 h, 48 h and 72 h in (2%) or (20%) medium. B) PFK activity expressed in $\mu\text{mol}/\text{min}/\mu\text{g}$ protein evaluated in non-infected (NI) and infected cells (hpi) growing in differentiation medium (2%) or after medium change to proliferation medium (20%) as indicated by an arrow. C) Lactate level, expressed in $\mu\text{mol}/\mu\text{g}$ protein measured in non-infected (NI) and infected cells (hpi) growing in differentiation medium (2%) or after medium change to proliferation medium (20%) as indicated by an arrow. D) Viral RNA level, expressed in copy number/ μg of total RNA measured in infected cells growing in differentiation medium (2%) or after medium change to proliferation medium (20%) as indicated by an arrow. E) Infectious viral particles (pfu/ml) measured in infected cells growing in differentiation medium (2%) or after medium change to proliferation medium (20%) as indicated by an arrow. F) Viperin and G) IFIT1 protein levels. Upper panels: representative blots of viperin and IFIT1 proteins expression in infected and non-infected cells growing in differentiation medium (2%) or after medium change to proliferation medium (20%) as indicated. Lower panels: Quantification of the Viperin and IFIT1 proteins levels measured in infected and non-infected (NI) cells growing in differentiation medium (2%) or after medium change to proliferation medium (20%) as indicated by an arrow. Viperin and IFIT1 proteins levels are expressed relatively to their level in SLC infected cells at 24 hpi that was set at 100%. Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way or 1-way ANOVA followed by Tukey's post-hoc test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2. Sensitivity of human SM cells to CHIKV infection relies on their growth stage and energetic metabolism and CHIKV infection increased glycolysis activity only in proliferating MB

To evaluate energetic metabolism, we first measured CS activity that reflects Krebs cycle and mitochondria activity. In non-infected cells (NI), quiescent SLC had the lower CS activity (Fig. 3A). During CHIKV infection, CS activity increased only in MB (Fig. 3A). Since β -oxidation and glycolysis can provide acetyl-CoA to fuel the Krebs cycle, we measured the activity of these pathways via respectively the activity of the Hydroxyacyl-CoA Dehydrogenase (HADH) and of the Phosphofruktokinase (PFK) [44,45]. Activity of the enzyme HADH indicated that mitochondrial β -oxidation levels were comparable between the three cell types and thus independent of the level of SM cells differentiation (Fig. 3B). During infection, HADH activity increased at 24 hpi only in MT but this low increase was transient. HADH activity at 48 hpi was again equivalent in the three cell types (Fig. 3B). Glycolysis was evaluated by measuring the activity of PFK, which catalyses the conversion of fructose-6-phosphate and ATP to fructose 1,6-bisphosphate and ADP, a rate limiting steps of glycolysis, making PFK a key glycolysis enzyme [45]. CHIKV infection increased PFK activity only in MB (Fig. 3C). PFK activity was increased in MT during their maturation between 24 and 48 h; however, CHIKV infection did not modify PFK activity in MT (Fig. 3C). Glycolysis gives rise to pyruvate, which could be transformed to acetyl-CoA incorporated in the Krebs cycle in mitochondria or to lactate thanks to the activity of the LDH. LDH activity was higher in NI SLC and MB cells compared to MT cells (Fig. 3D). However, lactate levels were comparable between the three types of cells (Fig. 3E). Surprisingly, CHIKV infection decreased LDH

activity in MB, but without consequence on lactate concentration and at the opposite, lactate levels increased in SLC without modification of LDH activity (Fig. 3D and E).

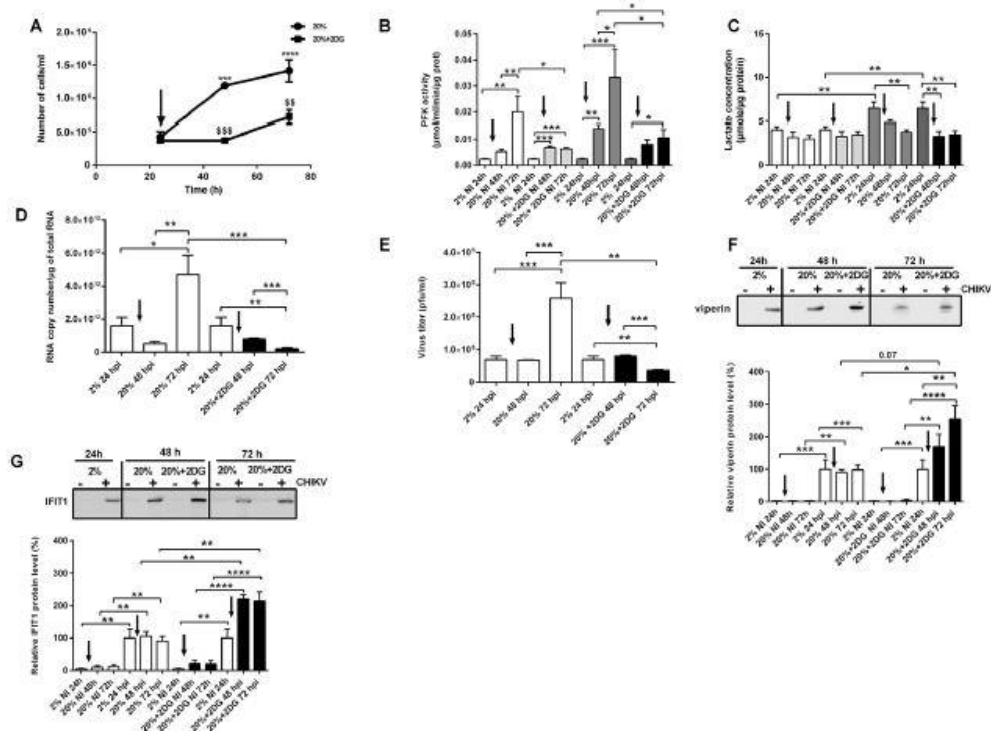


Fig. 5. Cell growth, energetic metabolism, viral production and ISG induction in activated SLC cells treated with 2DG. SLC were infected or not with CHIKV at MOI of 2. At 24 hpi, cells were switched to proliferation medium (20%) or proliferation medium supplemented with 2DG (20%+2DG). A) Cell growth curve of non-infected SLC cells at 24 h, 48 h and 72 h growing in (20%) or (20%+2DG) medium. B) PFK activity expressed in $\mu\text{mol}/\text{min}/\mu\text{g}$ protein evaluated in non-infected (NI) and infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. C) Lactate level, expressed in $\mu\text{mol}/\mu\text{g}$ protein, evaluated in non-infected (NI) and infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. D) Viral RNA level, expressed in copy number/ μg of total RNA, measured in infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. E) Infectious viral particles (pfu/ml) measured in infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. F) Viperin and G) IFIT1 protein levels. Upper panels: representative blots of viperin and IFIT1 proteins expression, evaluated in infected and non-infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated. Lower panels: Quantification of the Viperin and IFIT1 proteins levels in infected and non-infected (NI) cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. Viperin and IFIT1 proteins levels are expressed relatively to their level in SLC infected cells at 24 hpi that was set at 100%. Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way ANOVA followed by Tukey's post-hoc test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3. Activation of SLC and glycolysis induced virus production

In vivo, to re-constitute SM, activated resident SC enter the cell cycle and concomitantly glycolysis increases. Cells undergo multiple rounds of proliferation (MB stage). To mimic this activation in vitro, we induced the proliferation of SLC and their differentiation in MB by switching SLC from DM (2% FCS) to PM (20% FCS). We then compared CHIKV production in MB (activated SLC in 20% FCS) and in non-activated SLC (in 2% FCS).

As expected, this switch induced cell growth (Fig. 4A) and glycolysis, illustrated by increased PFK activity (Fig. 4B). In infected cells, as observed previously (Fig. 3C), CHIKV induced glycolysis only in proliferative MB (Fig. 4B). Lactate concentration was equivalent in NI cells: SLC and MB (Fig. 4C). CHIKV infection increased lactate concentration in SLC cells compared to MB cells (Fig. 4C) as previously observed (Fig. 3E). In these experimental conditions, we clearly observed a high induction of the viral transcription (Fig. 4D) and of the production of viral particles (Fig. 4E) in MB (activated SLC in 20% FCS). Interestingly, as observed in MB cells (Fig. 2E and F), virus production was associated with a concomitant decrease of viperin and IFIT1 proteins levels (Fig. 4F and G).

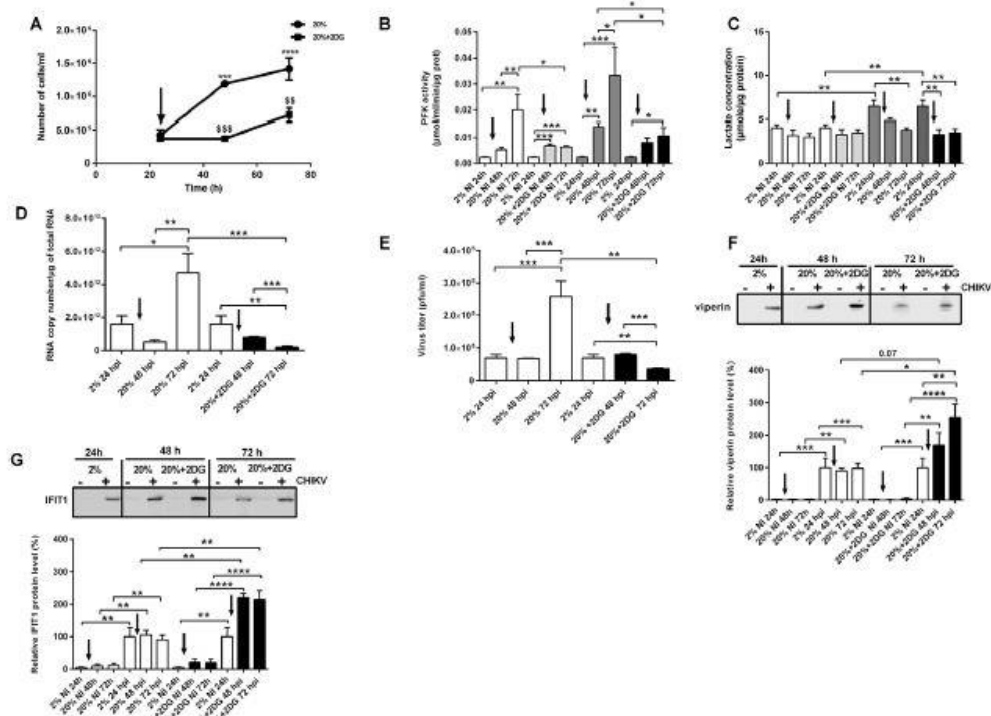


Fig. 5. Cell growth, energetic metabolism, viral production and ISG induction in activated SLC cells treated with 2DG. SLC were infected or not with CHIKV at MOI of 2. At 24 hpi, cells were switched to proliferation medium (20%) or proliferation medium supplemented with 2DG (20%+2DG). A) Cell growth curve of non-infected SLC cells at 24 h, 48 h and 72 h growing in (20%) or (20%+2DG) medium. B) PRK activity expressed in $\mu\text{mol}/\text{min}/\mu\text{g}$ protein, evaluated in non-infected (NI) and infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. C) Lactate level, expressed in $\mu\text{mol}/\mu\text{g}$ protein, evaluated in non-infected (NI) and infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. D) Viral RNA level, expressed in copy number/ μg of total RNA, measured in infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. E) Infectious viral particles (pfu/ml) measured in infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. F) Viperin and G) IFIT1 protein levels. Upper panels: representative blots of viperin and IFIT1 proteins expression, evaluated in infected and non-infected cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated. Lower panels: Quantification of the Viperin and IFIT1 proteins levels in infected and non-infected (NI) cells growing in proliferation medium (20%) or in (20%+2DG) medium as indicated by an arrow. Viperin and IFIT1 proteins levels are expressed relatively to their level in SLC infected cells at 24 hpi that was set at 100%. Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way ANOVA followed by Tukey's post-hoc test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Inhibition of cell growth and glycolysis in proliferating cellshampered virus production

In activated SLC, induction of proliferation was associated with increased virus production and decreased viperin and IFIT1 proteins expression concomitantly to activation of glycolysis. We therefore induced proliferation by switching SLC to the high serum PM (20% FCS) in presence or absence of the glucose analogue 2- Deoxy-D-glucose (2DG). 2DG inhibits glycolysis by impeding the synthesis of glucose-6-phosphate from glucose leading to depletion of energy and inhibition of cell growth [46].

In fact, 2DG blocked proliferation of cells induced to proliferate in PM (Fig. 5A), without affecting SCL or MB viability (Fig. S3). 2DG also blocked the induction of glycolysis in CHIKV infected cells (Fig. 5B). It has been previously shown that 2DG, by restricting glycolysis, decreases lactate production [47]. Surprisingly, despite decreased glycolysis, lactate concentration was not different between infected cells growing in PM and cells growing in PM+2DG (Fig. 5C). However, addition of 2DG blocked viral RNA transcription (Fig. 5D) and production of infectious viral particles (Fig. 5E). This decrease of virus production was associated with higher expression of viperin (Fig. 5F) and IFIT1 (Fig. 5G) protein expression.

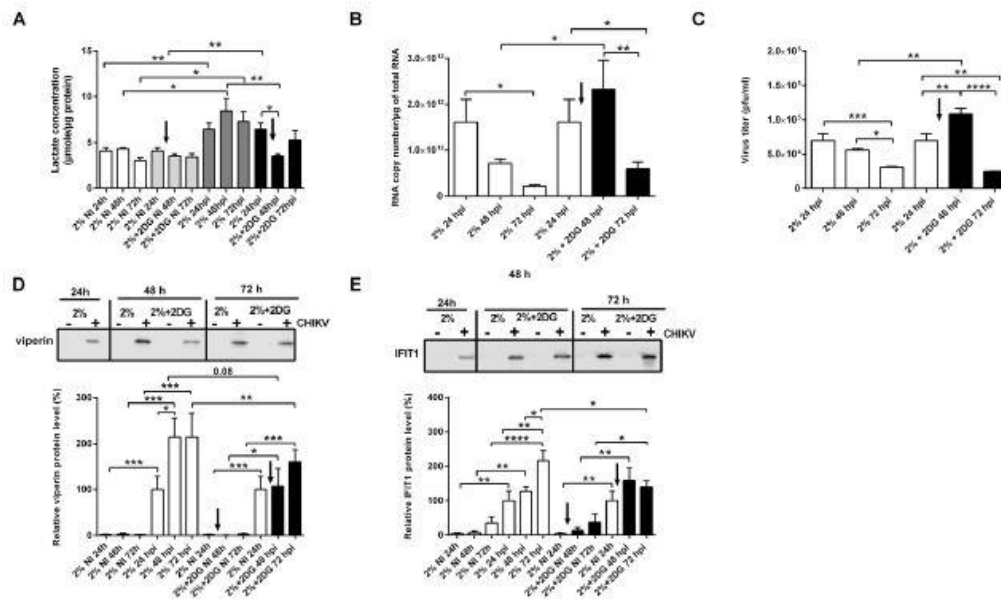


Fig. 6. Lactate concentration, viral production and ISG induction in SLC cells treated with 2DG. SLC were infected or not with CHIKV at MOI of 2. At 24 hpi, cells were kept in differentiation medium (2%) or switched to differentiation medium supplemented with 2DG (2%+2DG). A) Lactate level, expressed in $\mu\text{mol}/\mu\text{g}$ protein, evaluated in non-infected (NI) and infected cells growing in differentiation medium (2%) or in (2%+2DG) medium as indicated by an arrow. B) Viral RNA level, expressed in copy number/ μg of total RNA, measured in infected cells growing in differentiation medium (2%) or in (2%+2DG) medium as indicated by an arrow. C) Infectious viral particles (pfu/ml) evaluated in infected cells growing in differentiation medium (2%) or in (2%+2DG) medium as indicated by an arrow. D) Viperin and E) IFIT1 protein levels. Upper panels: representative blots of viperin and IFIT1 proteins expression in infected and non-infected cells, growing in differentiation medium (2%) or in (2%+2DG) medium as indicated. Lower panels: Quantification of the Viperin and IFIT1 proteins levels in infected and non-infected (NI) cells growing in differentiation medium (2%) or in (2%+2DG) medium as indicated by an arrow. Viperin and IFIT1 proteins levels are expressed relatively to their level in SLC infected cells at 24 hpi that was set at 100%. Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way ANOVA followed by Tukey's post-hoc test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5. Inhibition of glycolysis and lactate production in quiescent cells boosted virus production

Contrarily to the results observed above (Fig. 5C), in SLC infected with CHIKV and treated with DM (2% FCS) supplemented with 2DG, lactate concentration decreased following infection as expected (Fig. 6A). Interestingly in such conditions, viral RNA and virus titer were increased (Fig. 6B and C), and, viperin and IFIT1 protein induction was restricted (Fig. 6D and E).

Addition of 20 mM lactate which did not affect cell viability (Fig. S3), in DM (2% FCS) +2DG following SLC infection with CHIKV, limited the decrease of lactate (Fig. 7A) observed during infection at 48 hpi in DM (2% FCS)+2DG. In this condition, we observed restricted viral RNA amplification at 48hpi (Fig. 7B) and lower viperin and IFIT1 protein levels at 72hpi (Fig. 7D and E). However, virus titers were comparable between CHIKV-infected SLC in DM (2% FCS)+2DG and in DM (2% FCS)+2DG β lactate (Fig. 7C).

4. Discussion

To perpetuate their life cycle, viruses use and interfere with the host cell metabolism and machinery. This interaction with cell metabolic pathways also allow viruses to escape the cell immune response [22,23]. CHIKV displays broad cell and tissue tropism [8], but CHIKV is preferentially detected in peripheral joints and muscle tissues [9] and SM infection is central for the development of CHIKV pathology [10]. On the other side, SM is the major regulator of glucose metabolism. In fact, SM represent the largest organ in the body and is responsible of 80% of glucose uptake in post-prandial state [48]. SM glucose metabolism deficiency is the primary defect leading to type 2 diabetes (T2D) [49,50]. Moreover, some epidemiological

studies showed that diabetic subjects have higher risk of mortality during CHIKV infection [51, 52]. More generally, it is known now that some virus modifies glucose metabolism and that glucose metabolism could regulate viral infection [53e55]. So improving our understanding of the interaction between CHIKV and SM cells is crucial to fight and control CHIKV, a virus now in global expansion [4]. In this work, we show that the susceptibility of SM cells to CHIKV infection depends on their energy metabolism and that in turn the modifications of cellular metabolism by CHIKV relies on the muscle cellular metabolism. In MB, glycolysis activity is high and augments following infection. CHIKV production is high and significantly increases between 24hpi and 48hpi. Concomitantly to increased glycolysis, LDH activity decreases in MB indicating that glycolysis is mainly focused on the production of ATP via the Krebs cycle in the mitochondria as also attested by enhanced CS activity in infected MB. SLC have lower glycolysis activity than MB. CHIKV production is lower in SLC than in MB and CHIKV does not affect SLC glycolysis activity. Moreover, our results demonstrate a correlation between CHIKV RNA and the production of infectious CHIKV particles with glycolysis, as estimated by PFK activity ($P = 0.02$ and $P = 0.009$, respectively). In SLC, CHIKV infection leads to heightened lactate level. However nor glycolysis nor LDH activities are increased. Thus, this accumulation of lactate in SLC could be due to a decline in lactate export during CHIKV infection. Since CHIKV infection modulates glycolysis or lactate levels only in SLC and MB, we then studied the effect of inhibition of glycolysis in SLC and MB. Diminishing glycolysis in proliferating MB with 2DG treatment impedes their proliferation and restrains CHIKV production. As expected, we do not observe any modification in lactate concentration. In fact, as stated above, in MB, pyruvate, the product of glycolysis serves essentially to produce ATP via the Krebs cycle in mitochondria. However, when glycolysis is oriented to lactate production, blocking glycolysis impedes pyruvate production and consequently lactate production [47]. Glycolysis inhibition in SLC stops the increase of lactate level observed during CHIKV infection. At the same time, viral RNA and infectious viral particles production in SLC significantly increase despite glycolysis inhibition and no effect on cell growth. Lactate addition on SLC following glycolysis inhibition limits lactate level decrease, consequently, level of viral RNA decreases.

In SLC, our results indicate that lactate limits virus production, at least at the RNA level. Previous studies have demonstrated that lactate could negatively regulate innate immunity by inhibiting the production of IFN through the Retinoic acid-inducible gene (RIG)-I-like receptor (RLR) pathway, consequently affecting ISG induction [56, 57]. Thus, in these studies, lactate was suggested to promote virus production [56, 57]. However, lactate could have opposite effects on viral infection. In fact, Thyrted et al. showed that in human airway epithelium, lactate is pro-viral for influenza A virus (IAV) and instead limit replication of severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) [57]. Interestingly, here in SLC, we observed a limitation of CHIKV production associated with lactate production and we did not observe a decreased of ISG expression as previously described [56, 57]. On the contrary, the induction of ISG is highly pronounced in SLC. Our findings validate the notion that lactate's influence on infection is extremely intricate and varies depending on the specific virus and infected cell type. Additionally, in our cellular model, the regulation of glycolysis has divergent effects on CHIKV production depending on the stage of cell differentiation. Inhibiting glycolysis with 2 DG in MB cells resulted in a decrease in CHIKV production, while lactate levels remained unaffected, as these cells do not produce lactate during infection. Conversely, in SLC where lactate is produced during infection, 2DG treatment led to a reduction in lactate levels and an increase in CHIKV production. Notably, in both SLC and MB cells, the production of infectious viral particles correlated with lactate levels ($P = 0.03$). However, under our experimental conditions, these lactate levels do not appear to

regulate the expression of ISG. These latter factors are highly induced in SLC during CHIKV infection compared to MB cells, and this heightened expression may be responsible for the lower viral production in SLC. Conversely, in MB cells, the lower expression of ISG, which are known to possess crucial antiviral activity against CHIKV, is observed [26e29] and could permit high virus production compared to SLC and MT. This observation is supported by the correlation between viral RNA quantity and the expression of viperin protein ($P = 0.006$) and IFIT1 protein ($P = 0.04$) in SLC, MB, and MT cells. Additionally, in MB cells, there is a correlation between viral RNA, virus titer, and the expression of viperin protein ($P = 0.04$ and $P = 0.01$, respectively) and IFIT1 protein ($P = 0.02$ and $P = 0.007$, respectively). In MB, our results are in favor of the limitation of IFN production during CHIKV infection. Interestingly, in MT cells, despite exhibiting relatively high levels of ISG, there is a significant production of viral particles compared to SLC cells, suggesting an inhibition of the antiviral activity of ISG in MT by CHIKV. These findings confirm that the cellular environment plays a crucial role in the antiviral response to CHIKV. Notably, CHIKV is known to induce pro-viral proteins that hinder the antiviral activity of ISG [58,59]. It is important to note that the antiviral activity of some ISG, such as viperin, varies depending on the cell type and specific region of the organ [60].

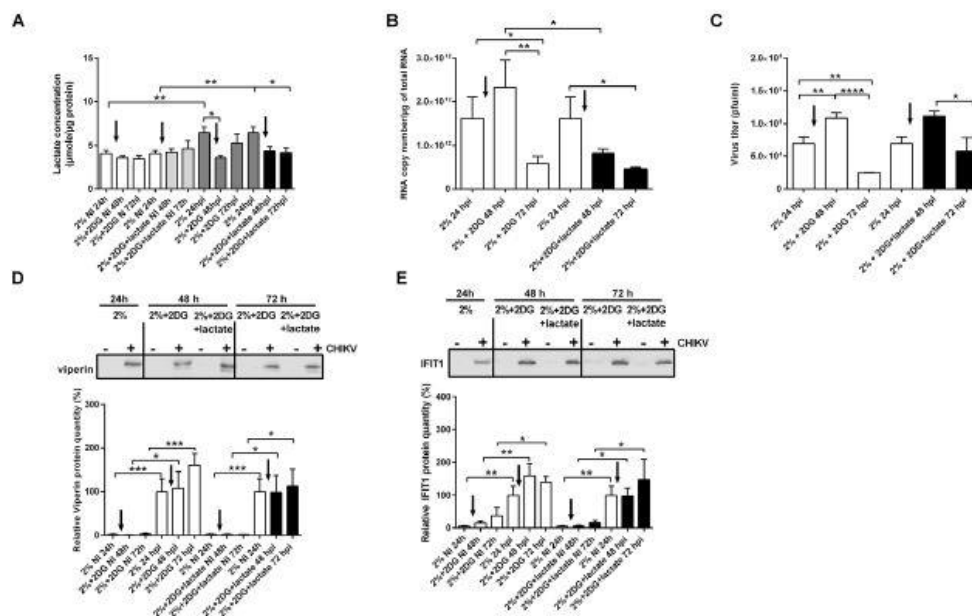


Fig. 7. Lactate concentration, viral production and ISG induction in SLC cells treated with 2DG and lactate. SLC were infected or not with CHIKV at MOI of 2. At 24 hpi, cells were switched to differentiation medium supplemented with 2DG (2%+2DG) or to differentiation medium supplemented with 2DG and lactate (2%+2DG + lactate). A) Lactate level, expressed in $\mu\text{mol}/\mu\text{g}$ protein measured in non-infected (NI) and infected cells growing in differentiation medium supplemented with 2DG (2%+2DG) or in differentiation medium supplemented with 2DG and lactate (2%+2DG + lactate) as indicated by an arrow. B) Viral RNA level, expressed in copy number/ μg of total RNA, in infected cells growing in differentiation medium supplemented with 2DG (2%+2DG) or to differentiation medium supplemented with 2DG and lactate (2%+2DG + lactate) as indicated by an arrow. C) Infectious viral particles (pfu/ml) in infected cells growing in differentiation medium supplemented with 2DG (2%+2DG) or in differentiation medium supplemented with 2DG and lactate (2%+2DG + lactate) as indicated by an arrow. D) Viperin and E) IFIT1 protein levels. Upper panels: representative blots of viperin and IFIT1 proteins expression in infected and non-infected cells growing in differentiation medium supplemented with 2DG (2%+2DG) or to differentiation medium supplemented with 2DG and lactate (2%+2DG + lactate) as indicated. Lower panels: Quantification of the Viperin and IFIT1 proteins levels in infected and non-infected (NI) cells growing in differentiation medium supplemented with 2DG (2%+2DG) or in differentiation medium supplemented with 2DG and lactate (2%+2DG + lactate) as indicated by an arrow. Viperin and IFIT1 proteins levels are expressed relatively to their level in SLC infected cells at 24 hpi that was set at 100%. Data are expressed as mean \pm SEM. Multiple groups comparisons were performed using 2-way ANOVA followed by Tukey's post-hoc test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Another important feature of our present work is that SC could serve as a CHIKV reservoir as suggested in several previous studies in human and animal showing the presence of CHIKV in SM biopsies and particularly in SC several months post-infection. These results, lead to the hypothesis that SM and SC could serve as viral reservoir during CHIKV chronic infection [10]. In fact, in our study, SLC produce very low amount of viral RNA and of infectious particles. This low amount of virus production is correlated to high lactate, viperin, and IFIT1 levels. Consequently, the elevated levels of lactate and the increased expression of ISG may

be responsible for the long-lasting nature of CHIKV infection in these cells. Activation of glycolysis in these specific cell types results in a reduction in lactate levels, a decrease in ISG expression, and a significant increase in viral production, leading to acute infection. In vivo, such SC activation occurs whenever there is a need to restore muscle mass and function, such as in the case of muscle damage caused by injuries, burns, or intense exercise [61, 62]. In SC, CHIKV could be in dormancy waiting a favourable environment. When glycolysis increases following muscle damage and SC activation, CHIKV could be produced with all the negative consequences on muscle physiology observed during acute/chronic CHIKV infection. As an example, 84% of CHIKV-infected patients presented myalgia during outbreaks in La Réunion island and in the Indian Ocean region [63]. Symptoms can last for several months or even years, which makes CHIKV infection particularly disabling, stressing the importance of understanding the mechanisms underlying CHIKV infection.

Some viruses like dengue virus (DV), SARS-CoV2 or IAV are known to manipulate glucose metabolism and particularly glycolysis to increase disposable energy and stimulate their own reproduction [53, 55, 64]. However, to the best of our knowledge, this is the first time that a study show that CHIKV modulates glycolysis and lactate production during infection. It has been observed since a long time that glucose metabolism dysregulation as observed during T2D, negatively affects the immune response and promotes infections [65]. Epidemiologic studies have clearly identified T2D as major comorbidities associated with CHIKV infection [51, 52]. Moreover, increase in glucose metabolism could leads to insulin resistance (IR) or to a progression of T2D in pre-diabetic subjects as Sestan et al. recently shown in a model of obese mice infected with the murine cytomegalovirus (MCMV) [51,57]. Therefore, in agreement with these observations and our results it would be interesting to determine whether T2D is a risk factor for CHIKV infection and/or whether CHIKV infection is a risk factor for development of T2D particularly during chronic infection. Based on our findings, it would be valuable to investigate not only the impact of glucose metabolism on CHIKV production but also the concentration of lactate since we found that lactate seems to limit CHIKV production and induce CHIKV chronicity in SLC. Exercise is known to affect lactate concentration, especially high-intensity exercise [66]. An elevation in lactate concentration could contribute to the persistence of CHIKV chronicity. However, as mentioned earlier, if muscular lesions are induced during high-intensity exercise, the activation of SC would facilitate viral replication and result in acute infection. Exercise is recognized to have diverse and advantageous effects on the prevention and treatment of diseases. Nevertheless, when it comes to infectious diseases, further experimental data are required to determine the specific recommendations or limitations of exercise for individuals infected with CHIKV.

5. Conclusion

Our work allowed a better understanding of the mechanisms involved in CHIKV infection of human SM. We identify the possible role of SC in chronic CHIKV infection. Importantly, our work shows that CHIKV infection and the antiviral response of SM cells relies on their energetic metabolism. Another important point highlights here is that alteration of the glucose metabolism of SM cells by CHIKV depends precisely of their glucose metabolism before infection.

Authors' contributions

M. D and B. C: funding acquisition, project administration, supervision of the project. J. M, B. M, L.K, C. G, B. C: experimental work and data analysis. B. C wrote the first draft of the manuscript and all the authors review the manuscript.

Declaration of competing interest

None.

Acknowledgments

This work was supported by the French National Research Agency under the "Investissements d'avenir programme" with the reference ANR-16-IDEX-0006. This work was also supported by grants from the French National Research Agency ANR-17-CE15- 00029 and ANR-10-LABX-25-01.

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