**Temporal In Vitro Raman Spectroscopy for Monitoring Replication Kinetics of Epstein–Barr Virus Infection in Glial Cells**

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**ABSTRACT:** Raman spectroscopy can be used as a tool to study virus entry and pathogen-driven manipulation of the host efficiently. To date, Epstein–Barr virus (EBV) entry and altered biochemistry of the glial cell upon infection are elusive. In this study, we detected biomolecular changes in human glial cells, namely, HMC-3 (microglia) and U-87 MG (astrocytes), at two variable cellular locations (nucleus and periphery) by Raman spectroscopy post-EBV infection at different time points. Two possible phenomena, one attributed to the response of the cell to viral attachment and invasion and the other involved in duplication of the virus followed by egress from the host cell, are investigated. These changes corresponded to unique Raman spectra associated with specific biomolecules in the infected and the uninfected cells. The Raman signals from the nucleus and periphery of the cell also varied, indicating differential biochemistry and signaling processes involved in infection progression at these locations. Molecules such as cholesterol, glucose, hyaluronan, phenylalanine, phosphoinositide, etc. are associated with the alterations in the cellular biochemical homeostasis. These molecules are mainly responsible for cellular processes such as lipid transport, cell proliferation, differentiation, and apoptosis in the cells. Raman signatures of these molecules at distinct time points of infection indicated their periodic involvement, depending on the stage of virus infection. Therefore, it is possible to discern the details of variability in EBV infection progression in glial cells at the biomolecular level using time-dependent in vitro Raman scattering.

**INTRODUCTION**

Raman spectroscopy (RS) is a sensitive enough tool to capture the characteristic bond vibrations of molecules and elucidate minute biochemical changes occurring in complex biological systems such as cells, tissue, and other body fluids. Notably, any alteration in the biochemical homeostasis of the body may inevitably be associated with a disease state, mark disease initiation, or represent the aftermath of an illness. For instance, biochemical changes due to manipulation in the carbohydrate metabolism may cause various pathological conditions such as galactosemia, diabetes, diabetic ketoacidosis, hyperglycemia, hereditary fructose intolerance, and glycogen storage disorders. Besides, conditions like phenylketonuria, tyrosinemia, maple syrup urine syndrome, etc. appear due to alterations in the protein expression. Additionally, alterations in lipid metabolism may lead to conditions such as familial hypercholesterolemia, hypertriglyceridemia, and low high-density lipoprotein (HDL). RS is used to analyze human serum samples for various biochemical components such as glucose, cholesterol, lipids, phenylalanine, etc. for the detection of respective metabolic disorders mentioned above. The use of RS has been so far extended as a diabetes management tool to determine the blood glucose level subcutaneously. Thus, the technique presents an immense potential in the field of disease diagnosis. Also, it is widely utilized to understand cancer, infection, and inflammation in the human body. RS can differentiate between invasive and noninvasive breast cancer based on Raman spectral markers. It is also employed to detect inflammation in the colon of patients suffering from inflammatory bowel disease (IBD) in in vitro Tumor necrosis factor or TNF-α (pro-inflammatory cytokine)-treated endothelial cells, and in in vivo experiments on tympanic membrane of a murine model. Signature Raman spectra arising from lipids, collagen, or DNA content of the respective samples identified the disease-associated alterations. Infections with similar clinical symptoms like dengue and malaria can easily be distinguished using RS based on differentially regulated metabolites.
Moreover, the technique is used to study various virus-associated modalities of Kaposi’s sarcoma-associated herpesvirus (KSHV) infection in BCBL-1, BC-1, and BJAB (B-cell lineage)\(^1\) and rotavirus\(^1\) and human papillomavirus (HPV) infection in primary human keratinocytes (PHKs) and CaSki cells.\(^2\) In the last few decades, the prevalence of ubiquitous herpesviruses has accounted for a major disease burden in the human population worldwide.\(^2\,4\) Human herpesvirus-4 (HHV-4) also known as Epstein–Barr virus (EBV) is an important member of the \textit{Herpesviridae} family. It infects about 90% of the world population.\(^3\) Primary infection during childhood largely remains asymptomatic; however, infection in adults could cause infectious mononucleosis (IM).\(^3\) Moreover, reactivation of the virus later in life after the initial exposure has been linked to serious conditions such as IM-like disease, hemophagocytic syndrome, chronic active EBV infection, and lymphomas.\(^4\) It is also a recognized oncogenic virus known to infect lymphocytes and cause B-cell lymphomas (Burkitt’s and Hodgkin’s lymphoma) or lymphoproliferative disorders. EBV can infect epithelial cells resulting in nasopharyngeal carcinoma or EBV-associated gastric cancer.\(^5\) Interestingly, the presence of EBV transcripts and DNA in the cerebrospinal fluid (CSF) of patients with neurodegenerative disorders such as Alzheimer’s disease (AD),\(^6\) Parkinson’s disease (PD),\(^6\) multiple sclerosis (MS),\(^7\) etc. provides clues toward the possibility of EBV infecting the brain cells (neurons and glial cells).

In general, to successfully infect any cell a virus must first attach to its surface and then penetrate through the outer cell membrane to insert its genomic content inside the cell. The entry of the virus inside a cell essentially depends upon the interaction between the virus surface proteins and the host cell receptors. It, therefore, limits the virus infectivity to host cells possessing the receptor and thereby defines its cell tropism.\(^8\) Nonetheless, several recent studies have shown the plausibility of viruses such as SARS-CoV2\(^9\) and Zika\(^10\) virus to infect cells of multiple origins, i.e., exhibiting the property of multitropism. Studies demonstrated that EBV shows the property of multitropism by infecting B-cells, epithelial cells, and possibly neural cells as well.\(^11\) EBV infects B-cells with the help of interaction between gp350, a glycoprotein present on its surface, and CD-21 surface marker expressed on the host cells.\(^11\) However, EBV attaches and enters inside the epithelial cells in a CD-21-independent manner. The predicted cell receptors that may facilitate EBV entry inside the epithelial cells via gHgL (a viral glycoprotein) include integrin and ephrin molecules.\(^12\) Fascinatingly, Jha et al. for the first time characterized \textit{in vitro} EBV infection in a neuroblastoma cell line (ShSy-5y), teratocarcinoma neurons (Ntera2), and primary human fetal neurons.\(^13\) Further, we successfully demonstrated EBV infection in glial cells, namely, HMC-3 (microglia) and U-87 MG (astroglia).

Moreover, the virus may take advantage of diverse pathways in cells of different origins to gain entry and carry out its replication.\(^14\) The type of genetic material (RNA or DNA) of the virus dictates further processing, which is accompanied by major alterations in the biochemistry of the host cell. Briefly, these processes involve viral genome replication, transcription, and translation. This disparity in biomolecular pathways adopted by the virus in different cells may alter the kinetics of infection,\(^15\) the duration of time elapsed from attachment to gaining entry, and further carrying out of the replication.\(^16\) For instance, in B-cells, postinfection transcriptional changes are detected within the first 24 h preceding metabolic and phenotypic changes. EBV-initiated transcriptional reprogramming in B-cells is broadly categorized under three stages: RNA synthesis, manipulation of metabolic pathways, followed by cell division that occurs subsequently on the second, third, and fourth days post infection (dpi), respectively.\(^17\) In contrast, \textit{de novo} (cell-in-cell) EBV infection in epithelial cells is initiated as early as 4 h post infection (hpi), and activation of EBV can be traced by 6 hpi. In SHSy-5y and Ntera2 cells, the infection
could be detected by 1 dpi and proceed to the lytic cycle by 9 dpi. Therefore, the same virus may take different time durations to establish a successful infection in cells of different origins.

Therefore, we investigated the biomolecular changes in the glial cells upon EBV infection at different time intervals using RS. We intended to identify the unique Raman signals originating from the EBV invasion and infection in the glial cells, in particular astrocytes and microglia. Also, in our study, we observed differential regulation of various biochemical molecules in the cell body (periphery) and the nucleus. These modulations in the biomolecules also led to alterations in the signaling pathways associated with them. The temporal (infection progression with time) and spatial (infection condition at different locations on the cell) Raman analysis provides a consolidated study on the dynamics of EBV infection.

**RESULTS**

**Raman Spectra.** We observed different peaks for HMC-3 and U-87 MG, pre- and postinfection, on plotting the raw data points (wavenumber vs intensity) of the Raman spectra using Origin-2018b software at various time durations (2, 4, 6, 12, 24, 36, and 48 h). For HMC-3, five major Raman peaks were observed in the wavenumber ranges of $547-560, 1097-1109, 2047-2054, 2669-2676,$ and $3825-3840 \text{ cm}^{-1}$ in the nucleus (Figure 1A-I), whereas at the periphery, five peaks were documented in the ranges of $548-554, 1097-1125, 2043-2051, 2673-2679,$ and $3828-3843 \text{ cm}^{-1}$ (Figure 1B-I). For U-87 MG, five major Raman peaks in the nucleus were observed in the ranges of $472-494, 999-1005, 1442-1450, 1655-1664,$ and $2879-2890 \text{ cm}^{-1}$ (Figure 1C-I). Three major peaks corresponding to the ranges of $2892-483, 998-1004,$ and $2872-2892 \text{ cm}^{-1}$ were obtained at the peripheral region of U-87 MG (Figure 1D-I).

For ease of further processing and validating the number of major peaks, the dimensionality of the raw data was reduced using principal component analysis (PCA). We obtained probable solutions, i.e., the principal components in our data in the range of 3–8, thus validating the number of peaks observed in the graph.

**Biomolecules’ Peak Identification in Glial Cells.** The distinct peaks that we obtained from the graph correspond to the signature spectra of unique biomolecules (Table 1). On comparing the Raman signals of these molecules in the infected samples with those of the uninfected (UI) ones, we observed variations in vibrational intensity at different time points. The positive variation, i.e., upregulation of intensity above the basal level of uninfected cells, represented an enhancement of anabolic activity. Also, a decline of signal intensity of increase in the number of molecules probably due to their consumption/catabolism. The positive variation, i.e., upregulation of intensity above the basal level of uninfected cells, represented an enhanced anabolic activity. Also, a decline of signal intensity of increase in the number of molecules probably due to their consumption/catabolism. The positive variation, i.e., upregulation of intensity above the basal level of uninfected cells, represented an enhanced anabolic activity. Also, a decline of signal intensity of increase in the number of molecules probably due to their consumption/catabolism.
| Wavenumber range (cm\(^{-1}\)) | Associated Biomolecule | Probable function in cell metabolism | Reports on association with virus infection |
|---------------------------------|------------------------|--------------------------------------|---------------------------------------------|
| 460–480                         | DNA                    | genetic material                      | 1. HCMV enhances glycolytic flux to fuel fatty acid synthesis\(^a\)\(^b\) |
| 484–490                         | glycogen               | energy-storage molecule               | 2. HCV enhances glycolytic metabolism toward the production of pyrimidine nucleotide components\(^a\)\(^b\) |
| 540–560                         | cholesterol/cholesterol esters | cell membrane constituent (maintains membrane fluidity), involved in cell signaling, transport processes, and nerve conduction | 3. EBV-infected NPC cell lines show increased glycolysis levels; LMP-1 of EBV induces hexokinase-2 to induce glycolysis and upregulation of GLUT-1\(^a\)\(^b\) |
| 540                             | glucose-saccharide band | energy currency of the cell           | 1. cellular cholesterol facilitates the postentry replication cycle of herpes simplex virus-1\(^a\)\(^b\) |
| 573                             | tryptophan             | essential amino acid, involved in synthesis of brain serotonin and kynurenine | 2. a nuclear receptor involved in cholesterol metabolism regulates herpesvirus latency and reactivation\(^a\)\(^b\) |
| 776–800                         | phosphatidylinositol   | interacts with proteins, involved in cell signaling cascades, and intracellular membrane trafficking; primary source of arachidonic acid (in brain) | 3. EBV: LMP-2A is secreted in exosomes in a cholesterol-dependent manner\(^a\)\(^b\) |
| 782                             | thymine/ cytosine, guanine | basic unit of genetic material, molecules like ATP, NADH, etc. | 1. cellular cholesterol facilitates the postentry replication cycle of herpes simplex virus-1\(^a\)\(^b\) |
| 784                             | phosphodiester, cytosine | components of the genetic material (forming backbone of DNA/RNA) | 2. a nuclear receptor involved in cholesterol metabolism regulates herpesvirus latency and reactivation\(^a\)\(^b\) |
| 786                             | pyrimidine ring        | components of the genetic material (forming backbone of DNA/RNA) | 3. EBV: LMP-2A is secreted in exosomes in a cholesterol-dependent manner\(^a\)\(^b\) |
| 787                             | phosphatidylethanolamine | components of the genetic material (forming backbone of DNA/RNA) | 4. KSHV: decreased cholesterol synthesis\(^a\)\(^b\) |
| 788                             | O–P–O stretching DNA   | components of the genetic material (forming backbone of DNA/RNA) | 1. HCMV: increased lipid biosynthesis\(^a\)\(^b\) |
| 850–855                         | proline/hydroxyproline, tyrosine | involved in cell signaling pathways regulating cell proliferation and mTOR pathway; can scavenge ROS | 2. HCV: increased anaplerotic use of glutamine\(^a\)\(^b\) |
| 1112–1124                       | glucose and saccharide band | energy currency of the cell           | 1. HCMV: increased lipid biosynthesis\(^a\)\(^b\) |
| 1122                            | polysaccharides        | energy storage or structural support  | 2. HCV: increased lipid synthesis\(^a\)\(^b\) |
| 1124                            | C–C stretching mode of lipids | conformational structure of lipids; involved in long-term energy storage, cell membrane constitution, and intercellular transmembrane transport | 3. KSHV: increased anaplerotic use of glutamine\(^a\)\(^b\) |
| 1257–1263                       | amide III              | structural constituent of proteins in the body | 1. HCMV: increased lipid biosynthesis\(^a\)\(^b\) |
| 1260                            | protein band           | plays multiple roles in the cells; involved in cell signaling, proliferation, etc.; structural role | 2. HCV: increased lipid synthesis\(^a\)\(^b\) |
| 1270–1280                       | triglycerides          | main component of dietary fats, act as long-term energy-storage molecules | 3. KSHV: increased anaplerotic use of glutamine\(^a\)\(^b\) |
| 1270                            | unsaturated fatty acids | structural constituent of the cell; involved in signaling and transport | 1. HCMV, EBV, and CMV: infection facilitates cytokine-induced alterations in lipid and lipoprotein metabolism, leading to decreased serum levels of total cholesterol (TC), HDL-C, LDL-C, apoA1, apoB, and Lp(a), as well as increased triglyceride (TG) and apoE concentrations\(^a\)\(^b\) |
| 1270                            | phospholipids          | structural constituent of the cell; involved in signaling and transport | 2. HCV: disturbed lipid synthesis\(^a\)\(^b\) |
| 2860–2880                       | lipids and proteins    | structural constituent of the cell; involved in signaling and transport | 3. HCV: decreased lipid secretion\(^a\)\(^b\) |

\(^a\)The table enlists the specific Raman signature of the biomolecules that are known to be altered on infection with different viruses and how the virus utilizes them. \(^b\)HCMV, human cytomegalovirus; HSV-1, herpes simplex virus-1; EBV, Epstein–Barr virus; KSHV, Kaposi’s sarcoma-associated herpesvirus; HBV, hepatitis B virus; and DENV, dengue virus.
proteins \((2928−2942 \text{ cm}^{-1})\) showed their maximum assimilation at both the cellular locations at 2 and 36 hpi (Figure 2B-6(I)). Apart from this, at 6 hpi, the nucleus showed catabolic activity, whereas at the periphery, anabolic activity restored the amount of these molecules (Figure 2B-6(II)).

Varying Biomolecular Expression at the Nucleus and the Periphery of Glial Cells with Continuing EBV Infection. To understand the alterations occurring in biomolecular expression in the continuous cycle of infection progression, we compared the signal intensities at subsequent time points. The gradual increase in intensity was analyzed at successive time frames throughout the infection in terms of predefined intervals, i.e., \(0−2, 2−4, 4−6, 6−12, 12−24,\) and \(24−36 \text{ hpi}\) for HMC-3 cells and an additional interval of \(36−48 \text{ hpi}\) for U-87 MG cells.

For HMC-3 cells, in the interval of \(0−2 \text{ hpi}\), we observed an increase in the signal intensities of glucose and lipids (Figure 3A) in the nucleus, whereas at the periphery, an increase in the intensity of PIP, DNA, and its phosphodiester backbone was obtained (Figure 3B). In the time span of \(4−6 \text{ hpi}\), the amount of polysaccharide was increased at the cell periphery (Figure 3B). Later during \(6−12 \text{ hpi}\), the nuclei of microglial cells showed maximum activity with increased levels of DNA and its phosphodiester backbone, glycogen, nucleotides (guanine and cytosine), and amino acids such as proline and tyrosine (Figure 3A). However, the peripheral region maintained its status quo without any changes (Figure 3B).

During the last leg of our experimental infection from \(24 \text{ to } 36 \text{ hpi}\), the activity of amide III, nucleotides of DNA (guanine, cytosine, adenine, and thymine), and fatty acids was highest in the nucleus (Figure 3A). On the contrary, the amount of cholesterol and polysaccharides was highest in the peripheral region (Figure 3B).

During the early phase of infection, i.e., from \(0 \text{ to } 2 \text{ hpi}\), purines of DNA and tryptophan showed a considerable increase at both the cellular locations at 2 and 36 hpi (Figure 2B-6(I)). Apart from this, at 6 hpi, the nucleus showed catabolic activity, whereas at the periphery, anabolic activity restored the amount of these molecules (Figure 2B-6(II)).
Figure 3. Representative view of changes in biomolecular activity upon continuous infection progression. Briefly, the biomolecules represented by their corresponding wavenumber values are plotted against the time intervals of their occurrence. The recorded data was plotted for consequent time intervals of 0–2, 2–4, 4–6, 6–12, 12–24, and 24–36 for HMC-3 and an additional 36–48 hpi for U-87 MG cells at both cellular locations (nucleus and periphery) separately. The maximum molecular activity was recorded (A) at the nucleus of HMC-3 cells during 0–2, 6–12, and 24–36 hpi, (B) at the periphery of HMC-3 cells during 0–2, 4–6, and 24–36 hpi, (C) at the nucleus of U-87 MG cells during 4–6, 6–12, 12–24, and 36–48 hpi, and (D) at the periphery of U-87 MG cells during 0–2 and 4–6 hpi.

Pathway Analysis of Selected Biomolecules. Further, the information regarding the biomolecules was uploaded to Qijen’s IPA system. The global molecular network available in the Ingenuity pathway knowledge base (IPKB) identified canonical pathways and gene networks associated with particular biomolecule-related neuropathologies. We obtained a connectome with the set members of virus infection and associated diseases including neurodegeneration, suggesting high involvement of cholesterol in various pathways. Prominent molecules obtained on infection of microglia (HMC-3) were cholesterol, cholesterol esters, PIP, 3-nitrotyrosine, lactic acid, lipids, and glucose (Figure 4A). Notably, cholesterol trafficking is prominently affected in AD and MS pathologies via ABCA-1 (ATP binding cassette subfamily A member 1) transporter and modulation of apolipoprotein (APOA and APOE) metabolism. \(^5\) Yet another group of molecules involved in cholesterol metabolism and implied in MS, \(^5\) AD, and PD pathologies is that involved in membrane trafficking. These molecules, sterol-O-acyltransferase-1 (SOAT-1), sphingosine-1-phosphate lyase-1 (SGPL-1), and synuclein \(\alpha\) (SNCA), are involved in cholesterol transport in and out of the cell and in presynaptic signaling. The connectome indicated various other membrane proteins such as matrix-associated protein-2 (MAP-2), CD44 (a cell surface marker), proteolipid protein-1 (PLP-1), and amyloid precursor protein (APP) directly or indirectly related to cholesterol metabolism in development of neurodegenerative pathologies. These molecules mainly act as cell surface receptors and are responsible for maintaining cell integrity, cell–cell interactions, adhesion, and migration. APP, PLP-1, and MAP-2 are widely recognized in AD, whereas MBP (myelin basic protein) linked with lipid metabolism is a primary marker for neurodegeneration observed in MS and ALS. Cell damage caused by oxidative stress generated due to reactive oxygen species (ROS) is one of the prevailing hypotheses of neurodegenerative pathology. Being rich in peroxidation susceptible substrates and having high oxygen consumption, the brain is more prone to damage caused by ROS. Molecules involved in the oxidative stress hypothesis of neurodegeneration were also interlinked with the ones we found in our study. For example, PSEN-1 (presenilin-1), which is thoroughly studied in MS, \(^6\) AD, and PD pathologies, is the connecting link among PIP, 3-nitrotyrosine, lactic acid, cholesterol ester, and cholesterol. Additionally, molecules involved in the oxidative stress hypothesis such as BACE-1 (\(\beta\) site APP cleaving enzyme-1) and \(\alpha\)PP also interlink with the cholesterol metabolism.

In the case of astrocytes, we found alterations in pathways implied in sterol biosynthesis, lipid transport, microtubule...
cytoskeleton constitution, and various endoplasmic reticulum (ER) proteins, along with the molecules involved in transcription regulation, metal ion binding, and various kinases (Figure 4B). Cholesterol was found to be related to most of the molecules compared to any other biomolecule under investigation. It is known that cholesterol metabolism is affected via modulation of the enzymes involved in sterol biosynthesis, such as sterol-C5-desaturase (SC5D), 24-dehydrocholesterol reductase (DHCR24), squalene monooxygenase (SQLE), and lecithin-cholesterol acyltransferase (LCAT). Dysregulation of sterol biosynthesis is strongly implied in neurodegenerative pathology like AD. Other molecules concerned with AD and MS pathologies are also affected in astrocytes on EBV infection such as those involved in lipid transport pathway (involving ABCA-1, APOE, and APOA1) and microtubule cytoskeleton proteins (PLP-1, MAP-2, and APP) in addition to PMP-22 (peripheral myelin protein-22) and other membrane proteins (matrix metallo-endopeptidases or MME, BACE-1, and sonic hedgehog or SHH). Some other molecules including glutamate ionotropic receptor NMDA type subunit 1 (GRIN1) and sodium voltage-gated channel α subunit 11 (SCN11A) were also reported in this regard. In addition, certain kinases such as protein kinase Cζ (PRKCZ), erb-b2 receptor tyrosine kinase 2 (ERBB2), and neurotrophic receptor tyrosine kinase 2 (NTRK2), also associated to cholesterol metabolism, were observed in astrocytes upon EBV infection. Interestingly, these molecules are reported to be associated with AD, PD, and other neurodegenerative pathologies. Cholesterol metabolism pathways associated with transcription regulator factors such as cAMP-responsive element-binding protein 1 (CREB1), cAMP response element mediator (CREM), MAF (transcription factor), and methyl CpG binding protein (MECP) contemplated in our study were also implied in Huntington’s disease (HD) and AD, respectively. Apart from cholesterol, hyaluronic acid (HA) is another molecule modulated on EBV infection. Nonetheless, glycosaminoglycan-binding proteins such as BCAN (brevican core protein), CD44 (CD44 antigen), and HAPLN4 (hyaluronan and proteoglycan link protein 4) related to the HA metabolism are involved in PD pathology. Various other molecules related to HA metabolism such as insulin-like growth factor (IGF1), kinases protein tyrosine kinase 2 (PTK2), matrix metallo-proteinases (MMP14, MMP9), and hyaluronan synthase 3 (HAS3) could be responsible for altered HA metabolism on EBV infection ultimately leading to neurodegeneration.

Figure 4. (A) Connectome representing the interlinks of biomolecules observed to be modulated on EBV infection in HMC-3 (microglia). The network depicts the molecules implied in various neurodegenerative pathologies (according to the IPA knowledge database). Molecular metabolisms related to (A) cholesterol, lipids, lactic acid metabolism, and PIP signaling cascade were recorded to be modulated in HMC-3 cells. (B) Connectome representing the interlinks of biomolecules observed to be modulated on EBV infection in U-87 MG (astroglia). The network depicts the molecules implied in various neurodegenerative pathologies (according to the IPA knowledge database). Molecular metabolisms related to (B) sterol biosynthesis, lipid transport, and microtubule cytoskeleton were recorded to be altered in U-87 MG cells.
DISCUSSION

The presence of EBV has been prevalently reported in CSF samples and postmortem brain tissue of patients suffering from neurodegenerative disorders, such as MS, AD, PD, etc., although how the virus gains entry and propagates inside the cells of the host brain is yet to be elucidated at the biomolecular level. Traditional biomolecular techniques used to detect the virus in infection any cell utilize the expression of viral transcript or proteins in the host. Detection of infection, thereby, becomes organism- and individual-specific, costly, time-consuming, and handling-dependent, along with a low reproducibility rate. RS on the other hand can trace the infection process with minute biomolecular changes occurring in the host cell on virus infection non-invasively and more robustly in lesser time and is also independent of handling constraints. This advantage gives an upper edge to the technique for the early detection of the virus during the infection process. As discussed previously, a virus infection cycle in a mammalian cell follows the steps of attachment, penetration, uncoating, genomic replication, assembly, and finally viral egress. All of these processes are biochemically driven and can be related to changes in the signature Raman spectrum. We observed sequential biomolecular changes occurring in the astrocytes and microglial cell lines post-EBV infection using RS. The information thus obtained provided an insight into the probable virus control and the host cell modulations. Our data suggested differences in the infection pattern of the virus in the glial cell repertoire.

Raman signals obtained during initial phases of infection, i.e., in the time duration between causing EBV infection to a healthy cell and 2 hpi, showed increased levels of glucose and lipid molecules in the nucleus and enhanced DNA and PIP activity at the periphery of the microglial cells, whereas Raman bands relating to PIP, lipids (cholesterol and cholesterol esters), and proteins were observed at the periphery of astroglial cells. It is suggested that lipids might be involved in the process of cell membrane modulation to facilitate EBV entry in the cells. In addition, until the elapse of 2 hpi, EBV (a DNA virus) was present at the cell periphery of the glial cells, and alterations in the signaling processes involving PIP were ongoing, implying that signaling molecules such as PIP might help in attachment and entry of EBV inside the glial cells. Many viruses have developed mechanisms to take advantage of phosphatidylinositol (PI)-mediated signaling cascade to gain entry and trigger modulations in the cell microenvironment beforehand to favor the virus’s survival. Although PI is the least abundant phospholipid in the cell membrane, it plays a crucial role in modulating the traffic in and out of the cell. It does so by getting differentially phosphorylated and giving rise to various PIP species. Class-I PI-3 kinase (PI-3K) is the most extensively exploited signaling pathway by various viruses during the entry process. For example, HIV-1 is known to employ the PI-3K-mediated micropinocytosis mechanism to modulate the cell cytoskeleton for gaining entry into the cell. Various other studies have also reported the alteration of pathways involving PIP in the successful establishment of virus infections of KSHV, human herpesvirus-8 (HHV-8), HSV-1, HCV, Zaire Ebola virus, and VZV. Thus, our experiments suggested its role in EBV’s entry inside the glial cells. Interestingly, PIP is implied in the processing of APP as well and thereby in the development of AD pathologies such as plaque formation or neurofibrillary tangle (NFT) deposition. It also suggested a probable PIP-mediated role of EBV in neurodegeneration. Our experiments also indicated that EBV infection manipulates sterol metabolism in astroglial cells on entry. Interestingly, the brain is the most cholesterol-rich organ, consisting of about 20–25% of the total cholesterol content of the whole body. Sterol metabolism naturally being one of the cornerstones of the energy cycle in the brain plays an important role in numerous neural metabolic pathways. Disruption of sterol homeostasis in the brain may be linked with multiple neurodegenerative pathologies such as MS, AD, PD, HD, and ALS. Any disturbance in the enzymatic mechanism of cholesterol synthesis, trafficking, or dissolution would lead to abnormal deposition of various sterol moieties in the intracellular or extracellular matrix. This might cause hindrance in impulse transmission through neurons, leading to atypical pathology of neurodegenerative diseases as AD, PD, or Lewy body dementia. The capacity of EBV to modulate sterol homeostasis in astroglia is observed in our study, and therefore it is suggested that EBV could also mediate neurodegeneration by dysregulation of the cholesterol metabolism.

By the end of 2 hpi, the Raman signals obtained from the periphery of microglial cells coincide with those obtained at the nucleus. In addition to the continued glucose and lipid metabolism signals from the nucleus, we observed an increase in the expression of PIP and DNA at both locations. The nuclear signals decline slightly thereafter till 4 hpi. These observations point toward an early start of an increase in the DNA activity inside the nucleus of microglial cells by 2 hpi, accompanied by an enhancement in signaling processes throughout the cell, as indicated by the increased signals for PIP or lipid molecules such as cholesterol. Moreover, by the end of 4 hpi, signals from the periphery showed the presence of polysaccharide, protein-related moieties, and purines of nucleic acids. Most biomolecular signals from the nucleus showed a decline in the period of 4–6 hpi, except for a slight increase in the expression of fatty acids at 6 hpi. However, we noticed a probable increase in cellular traffic at the periphery during 4–6 hpi based on the increased activity of cholesterol, PIP, and amino acids (Phe, Tyr, and Trp) at the periphery of microglia. Contrastingly, we recorded a high activity of astroglial nucleus during 4–6 hpi. We surmise that by the end of 4 hpi the virus must have gained entry into the astroglial cell nucleus. Later, upon reaching the nucleus, it probably utilized cholesterol, polysaccharides, and lipid metabolism to carry out its replication. Besides, few reports suggest the exploitation of cholesterol and saccharide moieties to attach and gain entry into the host cell by the herpesvirus family.

We speculated that after entering the nucleus of a microglial cell, during 6–12 hpi, the virus most likely manipulates nuclear metabolism of glycerogen and amino acids to facilitate its replication cycle. It is noteworthy to state that abnormal glucose metabolism is often associated with MS disease pathology and various neuro-inflammatory disorders. The microglial nucleus showed maximum activity post 6 hpi till 12 hpi. Enhanced signals for amino acids (Pro and Tyr), glycerogen, and DNA metabolism (in the form of signals for DNA, phosphodiester bond, and purines-guanine and cytosine) are observed past 6 h in the nucleus of microglial cells and are maintained till the end of 12 hpi. This coincided with the presence of polysaccharides and amino acids in the nucleus. Nonetheless, in microglia, the activity of most biomolecules at the periphery was downregulated from 6 to

https://dx.doi.org/10.1021/acsomega.0c04525
ACS Omega 2020, 5, 29547−29560
12 hpi, except a slight increase in polysaccharides and amino acids (Pro and Val) signals, whereas in the astroglial cells at 6–12 hpi, further processing of the virus inside the nucleus is speculated, as implied by the expression of various molecules such as triglycerides, fatty acids, lipids, and proteins. Various viruses like dengue (DENV), human cytomegalovirus (HCMV), herpes simplex virus-1 (HSV-1), and EBV are known to manipulate the glycolytic pathway for their benefit. These viruses could either utilize glycogen for glycogenolysis to sustain increased energy requirements during replication or fuel fatty acid synthesis (HCMV) or enhance pyrimidine production (HSV-1) as required. The manipulation of glycogen metabolism by various viruses to facilitate their replication cycle enabled us to conclude that EBV may modulate glycogen metabolism for aiding its replication cycle in the microglia occurring at 6–12 hpi. Importantly, Pro and Tyr are involved in cell signaling pathways such as those regulating proliferation and ROS scavenging. Modulation of amino acid metabolism similar to that of Tyr and Phe is known to be associated with HIV-1 and HBV infections.

Then, in the duration of 12–24 hpi, we hypothesized that successive steps of the virus packaging and transport occurred in the astrocytes indicated by the presence of nucleotide molecules at the periphery. However, the virus replication cycle repeated after completion of 24 hpi, as suggested by the repetition of signals obtained for PIP and cholesterol from the nucleus, whereas signals collected from the periphery indicate the presence of nucleotides and proteins, which might denote the viral egress from the cell at later time points (36–48 hpi). Thereafter, in the microglial cell line, plausibly, the process of viral packaging is initiated (periphery) at 24 hpi and is carried out until 36 hpi. Enhancements in signals produced from amide bond and amino acids (Phe, Tyr, and Trp), nucleotides, and fatty acids corroborate our speculations, as the molecules falling in the broad category of protein and lipids are believed to be utilized by certain viruses such as HCMV to facilitate their survival inside the host. In fact, a wide variety of viruses, such as HCMV, HSV, EBV, DENV, hepatitis B virus (HBV), hepatitis-C virus (HCV), vaccinia virus, and KSHV to name a few, reportedly alter fatty acid metabolism. Lipids and fatty acids being the secondary preferred energy source after glucose for the brain play a major role in neurophysiology. Thus, any disturbance in the homeostasis of the lipid metabolism in the brain could result in grave consequences. For instance, impaired enzymatic cascades (such as sphingolipid pathway) or oxidative stress resulting in dysregulation of lipid metabolism and lipid rafts are well described to be associated with amyloid plaque and NFT deposition causing AD, PD, or HD. Notably, various lipid molecules are enhanced in the brain of MS patients, thus giving rise to a specific lipidomic profile. Also, a perturbed cholesterol pathway is associated with MS. After 36 hpi, we suspect that many cellular processes simultaneously occur as the Raman signals thus obtained are mixed (pertaining to the obtained signals corresponding to molecules such as phosphodiester, Pro, Tyr, and lipids). These processes may occur to facilitate the initiation of the next virus replication cycle and/or carry on the ongoing cycle.

## CONCLUSIONS

Time-dependent in vitro spatial Raman spectroscopy carried out on different regions of glial cells shows the temporal evolution of EBV infection, enabling one to understand the virus influencing mechanism. In brief, the investigation directed us to believe that EBV enters the glial cells probably in the first 2 hpi by utilizing PIP-dependent signaling pathways, whereas further processing of the virus differs slightly among microglial and astroglial cells. To reach inside and manipulate the nuclear microenvironment of microglial cells, it takes up to 6 hpi and up to 4 hpi in the astroglial cells. Moreover, during its nuclear hijack process from 6 to 12 hpi, the virus manipulates glycogen and amino acid metabolism in the microglial cells. While in the case of the astroglial cells, fatty acids and triglyceride metabolism is affected at 6–12 hpi. Later, during 12–24 hpi, the processes of replication and cellular transport are still carried on in the microglia, whereas EBV showed signs of viral packaging and transport being initiated in astroglial cells. It is only after 24 hpi that viral packaging and egress is initiated in microglial cells. However, in astroglia, from 24 hpi onward we noticed overlapping signals for replication and viral egress. Thus, the study aided us to further our understanding of the involvement of different biomolecules at various stages of EBV infection progression in two different glial cells. With further advances in technology in the future, the application of RS could extend to differentiating the viral infection stages in clinical settings and help in noninvasive and early disease diagnosis. The recent outbreak of SARS-CoV2 has brought forth the importance of such rapid diagnostic tools in the detection of virus infection. The temporal and spatial Raman spectroscopic technique appears to be a step forward toward understanding the viral biology after infection in host cells and also assisting in a comparative analysis of replication kinetics in different cells on infection with multitropic viruses such as EBV.

## MATERIALS AND METHODS

### Cell Culture.

HMC-3 (SV-40 transformed, immortalized microglial cell lines were obtained from Dr. Anirban Basu’s laboratory, National Brain Research Centre, Delhi) and U-87 MG cell lines (glioblastoma cells of epithelial origin from the human brain were purchased from the National Centre for Cell Sciences, Pune) were cultured in high-glucose-containing Dulbecco’s modified Eagle’s medium (HiMedia) supplemented with 10% fetal bovine serum (Invitrogen), 50 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) over coverslips. The cells were incubated at 37 °C with 5% CO2.

### Virus Isolation and Purification.

HEK-293T cells (a kind gift from Prof. Erle S. Robertson’s laboratory, University of Pennsylvania) transformed with GFP-tagged virus-containing bacmid were used to obtain EBV particles. Briefly, to obtain the virus particles, lytic induction was given to cells with 20 ng/mL tetracyclnophorol phorbol acetate and 3 mM butyric acid at 60% cell confluence for 4–5 days. The cell suspension was centrifuged at 775g for 20 min, and the supernatant was then filtered through a 0.45 μm membrane (Millipore). The filtrate was ultracentrifuged at 65 291g for 90 min at 4 °C. The concentrated virus pellet was resuspended in a suitable amount of culture media and stored at −80 °C until further use.

### EBV Infection in Glial Cells.

An appropriate concentration of EBV for each cell line was investigated. The U-87 MG cells (astrocytes) cultured onto the coverslips were infected with EBV at an MOI (multiplicty of infection) of 2.5 and incubated for the duration of 2, 4, 6, 12, 24, 36, and 48 h. Also, 5.0 MOI of EBV was used for infecting HMC-3 cells (microglia). The microglial cells were incubated with the virus for the duration of 2, 4, 6, 12, 24, and 36 h. After collecting the coverslips at the
respective time points, the cells grown on these coverslips were fixed using 4% paraformaldehyde (PFA) for 20 min at room temperature. The coverslips were mounted onto glass slides before visualization on the Raman spectrometer.

**Raman Spectroscopy.** Raman spectroscopy was performed on a LabRAM HR Evolution (Horiba-Jobin Yvon) spectrometer using a He-Ne laser ($\lambda_{\text{exc}} = 633$ nm, $\sim$10 mW) excitation source. The sample was focused with the help of a microscope objective to probe the nucleus and the periphery of the cell. The Raman measurement was performed using minimum possible laser power controlled using an ND filter to avoid any laser-related damage to the cells. The Raman study was performed on uninfected (UI) and 2, 4, 6, 12, 24, 36, and 48 hpi of respective cells. The laser was focused onto the nuclei and cell body (periphery) separately as visualized on the microscope to acquire signals from respective places.

**Data Analysis. Graphical Analysis.** All of the raw data of Raman vibrational intensity obtained from the Raman spectroscope within wavenumber ranges of 400–3500 cm$^{-1}$ (HMC-3) and 400–4000 cm$^{-1}$ (U-87 MG) was smoothened by 20 points for better visibility. Signals from common cell culture artifacts such as culture medium, phosphate-buffered saline (PBS), PFA, coverslips, and glass slides were subtracted from all of the samples. Thereafter, we selected the most relevant wavenumber peaks from the graph based on intensity.

**Statistical Analysis.** The dimensionality of the raw data was reduced using principal component analysis (PCA) on SPSS software. The total numbers of selected peaks were verified using PCA. Mann–Whitney U test ($\alpha = 0.05$) was applied to the screened data consisting of wavenumber ranges corresponding to the peaks for comparison of Raman intensities at subsequent time points. Additionally, we also included the odds ratio (OR) analysis to enhance data confidence.

**Interactionome Study.** The selected wavenumber peaks were cross-referenced with the available literature to verify the corresponding biomolecules. The biomolecules were then uploaded on Qiagen’s Ingenuity Pathway Analysis (IPA) software to obtain an interconnection between them. Thereafter, a global molecular network available on the Ingenuity pathway knowledge base (IPKB) was used as a reference to perform analysis of canonical pathways, diseases, functions, and gene networks. The analysis gave us hits that are most significantly related to the biomolecular changes occurring in viral infection and neurodegenerative diseases.

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**Funding**

We thank the Council of Scientific and Industrial Research grant no. 37(1693)/17/EMR-II, Department of Science and Technology, and Ramanujan fellowship grant no. SB/S2/RJN-132/20/S, the Ministry of Human Resource, for fellowship to S.J. and D.T. in the form of research stipend. The funding organization has not played any role in the study design or the preparation of the manuscript.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Facilities received from the Department of Science and Technology (DST), Government of India, under FIST Scheme (grant number SR/FST/PSI-225/2016) are acknowledged. We are thankful to Prof. Earl S. Robertson (U. Penn.) and Dr. Anirban Basu (NBRC, Delhi) for their kind gift of the cell lines. One of the authors (R.K.) acknowledges financial support from the Science and Engineering Research Board, Government of India (Grant CRG/2019/000371). One of the authors (D.K.P.) acknowledges the Council of Scientific and Industrial Research (CSIR) for financial assistance (File 09/1022(0039)/2017-EMR-I). We appreciate our laboratory colleagues (from Infection-bioengineering group), in particular Omkar Indari, for insightful discussions and advice and Buddhadev Baral for his help in performing the experiments. We gratefully acknowledge the Indian Institute of Technology Indore for providing facilities and support.

**REFERENCES**

(1) Kuhar, N.; Sil, S.; Verma, T.; Umapathy, S. Challenges in application of Raman spectroscopy to biology and materials. RSC Adv. 2018, 8, 25888–25908.

(2) Barr, A. J. The biochemical basis of disease. Essays Biochem. 2018, 62, 619–642.

(3) Taylor, H. L.; Wu, C. L.; Chen, Y. C.; Wang, P. G.; Gonzalez, J. T.; Betts, J. A. Post-Exercise Carbohydrate-Energy Replacement Attenuates Insulin Sensitivity and Glucose Tolerance the Following Morning in Healthy Adults. Nutrients 2018, 10, 123.

(4) Boyer, S. W.; Barclay, L. J.; Burrage, L. C. Inherited Metabolic Disorders: Aspects of Chronic Nutrition Management. Nutr. Clin. Pract. 2015, 30, 502–510.

(5) Parhofer, K. G. The Treatment of Disorders of Lipid Metabolism. Dtsch. Arztebl. Int. 2016, 113, 261–268.

(6) Borges, R. D.; Navarro, R. S.; Giana, H. E.; Tavares, F. G.; Fernandes, A. B.; Silveira Junior, L. Detecting alterations of glucose and lipid components in human serum by near-infrared Raman spectroscopy. Res. Biomed. Eng. 2015, 31, 160–168.

(7) Javanmard, M.; Davis, R. W. Surface-Enhanced Raman Scattering (SERS) for Detection of Phenylketonuria for Newborn Screening. In Nanoscale Imaging, Sensing, and Actuation for Biomedical Applications XI; International Society for Optics and Photonics, 2014; Vol. 8954.

(8) Kang, J. W.; Park, Y. S.; Chang, H. Z.; Lee, W.; Singh, S. P.; Choi, W.; et al. Direct observation of glucose fingerprint using in vivo Raman spectroscopy. Sci. Adv. 2020, 6, 5206.
(49) Stone, N.; Kendall, C.; Smith, J.; Crow, P.; Barr, H. Raman spectroscopy for identification of epithelial cancers. Faraday Discuss. 2004, 126, 141–157.
(50) Binoy, J.; Abraham, J. P.; Hubert Joe, I.; Jayakumar, V. S.; Petti, G. R.; Nielsen, O. F. NIR-FT Raman and FT-IR spectral studies and ab initio calculations of the anti-cancer drug combretastatin-A4. J. Raman Spectrosc. 2004, 35, 939–946.
(51) Cheng, W.-T.; Liu, M.-T.; Liu, H.-N.; Lin, S.-Y. Micro-Raman spectroscopy used to identify and grade human skin pilomatrixoma. Microsc. Res. Tech. 2005, 68, 75–79.
(52) Talari, A. C.; Movasaghi, Z.; Rehman, S.; Rehman, I. U. Raman spectroscopy of biological tissues. Appl. Spectrosc. Rev. 2015, 50, 46–111.
(53) Mahadevan-Jansen, A.; Richards-Kortum, R. In Raman Spectroscopy for Cancer Detection: A Review, Proceedings of the 19th Annual International Conference of the IEEE Engineering in Medicine and Biology Society. "Magnificent Milestones and Emerging Opportunities in Medical Engineering", 1997; pp 2722–2728.
(54) Hanlon, E. B.; Manoharan, R.; Koo, T. W.; Shafer, K. E.; Motz, J. T.; Fitzmaurice, M.; et al. Prospects for in vivo Raman spectroscopy. Phys. Med. Biol. 2000, 45, R1–R59.
(55) Bonnier, F.; Byrne, H. J. Understanding the molecular information contained in principal component analysis of vibrational spectra of biological systems. Analyst 2012, 137, 322–332.
(56) Sigurdsson, S.; Philipsen, P. A.; Hansen, L. K.; Larsen, J.; Gnaidecka, M.; Wulf, H. C. Detection of Skin Cancer by Classification of Raman Spectra. IEEE Trans. Biomed. Eng. 2004, 51, 1784–1793.
(57) Chan, J. W.; Taylor, D. S.; Zwingder, T.; Lane, S. M.; Ihara, K.; Huser, T. Micro-Raman spectroscopy detects individual neoplastic and normal hematopoietic cells. Biophys. J. 2006, 90, 648–656.
(58) Koljenović, S.; Schut, T. B.; Vincent, A.; Kros, J. M.; Puppels, G. J. Detection of meningioma in dura mater by Raman spectroscopy. Anal. Chem. 2005, 77, 7958–7965.
(59) Hirsch-Reinschagen, V.; Zhou, S.; Burgess, B. L.; Bernier, L.; McIsaac, S. A.; Chan, J. Y.; Tansley, G. H.; Cohn, J. S.; Hayden, M. R.; Wellington, C. L. Deficiency of ABCA1 impairs apolipoprotein E metabolism in brain. J. Biol. Chem. 2004, 279, 41197–4207.
(60) Consonati, S.; Novellino, E. The first sphingosine 1-phosphate lyase inhibitors against multiple sclerosis: a successful drug discovery tale. J. Med. Chem. 2014, 57, 5072–5073.
(61) Cummings, M.; Arumanayagam, A. C.; Zhao, P.; Kannanganat, S.; Stuve, O.; Karandikar, N. J.; Eagar, T. N. Presenilin1 regulates Th1 and Th17 effector responses but is not required for experimental autoimmune encephalomyelitis. PLoS One 2018, 13, No. e0200752.
(62) Martiskainen, H.; Paldanius, K. M. A.; Natunen, J.; Takalo, M.; Marttinen, M.; Leskelä, S.; et al. DHR24 exerts neuroprotection upon inflammation-induced neuronal death. J. Neuroinflammation 2017, 14, 215.
(63) Gardner, L. A.; Levin, M. C. Importance of apolipoprotein AI in multiple sclerosis. Front. Pharmacol. 2015, 6, 278.
(64) Fitz, N. F.; Tapias, V.; Cronican, A. A.; Castronovo, E. L.; Saleem, M.; Carter, A. Y.; et al. Osmosing effects of ApoE/ApoA1 double deletion on amyloid-β pathology and cognitive performance in APP mice. Brain 2015, 138, 3699–3715.
(65) Chew, H.; Solomon, V. A.; Fonteh, A. N. Involvement of Lipids in Alzheimer’s Disease Pathology and Potential Therapies. Front. Physiol. 2020, 11, 598.
(66) Bamburg, J. R.; Bloom, G. S. Cytoskeletal pathologies of Alzheimer disease. Cell Motil. Cytoskeleton 2009, 66, 635–649.
(67) Cloake, N. C.; Yan, J.; Aminian, A.; Pender, M. P.; Greer, J. M. PLP1 mutations in patients with multiple sclerosis: Identification of a new mutation and potential pathogenicity of the mutations. J. Clin. Med. 2018, 7, 347.
(68) Jana, M.; Pahan, K. Astrocytes, Oligodendrocytes and Schwann Cells. Neuroimmun. Pharmacol. 2017, 117–140.
(69) Lyons, P. D.; Benveniste, E. N. Cleavage of membrane-associated ICAM-1 from astrocytes: involvement of a metalloprotease. Glia 1998, 22, 103–112.
(89) Zheng, K.; Xiang, Y.; Wang, X.; Wang, Q.; Zhong, M.; Wang, S.; et al. Epidermal Growth Factor Receptor-PI3K Signaling Controls Coflin Activity To Facilitate Herpes Simplex Virus 1 Entry into Neuronal Cells. *mBio* 2014, 5, No. e00560.

(90) Prestwich, G. D. Phosphoinositide signaling: from affinity probes to pharmacological targets. *Chem. Biol.* 2004, 11, 619–637.

(91) Rocha-Peregrini, V.; Gordon-Alonso, M.; Sánchez-Madrid, F. PI3P: choreographer of actin-adaptor proteins in the HIV-1 dance. *Trends Microbiol.* 2014, 22, 379–388.

(92) Bhattacharya, S.; Damania, B. AKTivation of PI3K/AKT/mTOR signaling pathway by KSHV. *Front. Immunol.* 2012, 3, 401.

(93) Berger, K. L.; Cooper, J. D.; Heaton, N. S.; Yoon, R.; Oakland, T. E.; Jordan, T. X.; et al. Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication. *Proc. Natl. Acad. Sci. U.S.A.* 2009, 106, 7577–7582.

(94) Saeed, M. F.; Kolokoltsov, A. A.; Freiberg, A. N.; Holbrook, M. R.; Davey, R. A. Phosphoinositide-3-Kinase-Akt Pathway Controls Cellular Entry of Ebola Virus. *PLoS Pathog.* 2008, 4, No. e1000141.

(95) Izmailian, R.; Hsiao, J.-C.; Chung, C.-S.; Chen, C.-H.; Hsu PW, C.; Liao, C.-L.; et al. Integrin 1 Mediates Vaccinia Virus Entry through Activation of PI3K/Akt Signaling. *J. Virol.* 2012, 86, 6687–6697.

(96) Morel, E.; Chamoun, Z.; Lasiecka, Z. M.; Chan, R. B.; Williamson, R. L.; Vanevotzov, C.; et al. Phosphatidylinositol-3-phosphate regulates sorting and processing of amyloid precursor protein through the endosomal system. *Nat. Commun.* 2013, 4, No. 2250.

(97) Browne, R. W.; Jakimovski, D.; Ziliotto, N.; Kuhle, J.; Bernardi, F.; Weinstock-Guttman, B.; Zavidov, R.; Ramanathan, M. High-density lipoprotein cholesterol is associated with multiple sclerosis fatigue: A fatigue-metabolism nexus? *J. Clin. Lipidol.* 2019, 13, 654–663.

(98) Lim, L.; Shui, G.; Wenk, M. R. Lipid Metabolism in Neurodegenerative Diseases. *Lipidomics* 2012, 269–296.

(99) Jin, U.; Park, S. J.; Park, S. M. Cholesterol Metabolism in the Brain and Its Association with Parkinson’s Disease. *Exp. Neurobiol.* 2019, 28, 554–567.

(100) Chang, T.-Y.; Yamauchi, Y.; Hasan, M. T.; Chang, C. Cellular cholesterol homeostasis and Alzheimer’s disease. *J. Lipid Res.* 2017, 58, 2239–2254.

(101) Alecu, I.; Bennett, S. A. L. Dysregulated Lipid Metabolism and Its Role in α-Synucleinopathy in Parkinson’s Disease. *Front. Neurosci.* 2019, 13, 328.

(102) Wudiri, G. A.; Pritchard, S. M.; Li, H.; Liu, J.; Aguilar, H. C.; Li, H.; Liu, J.; et al. Rapid Detection of COVID-19 Causative Virus (SARS-CoV-2) in Human Nasopharyngeal Swab Specimens Using Field-Effect Transistor-Based Biosensor. *ACS Nano* 2020, 14, 5135–5142.

(103) Xiao, L.; Hu, Z. Y.; Dong, X.; Tan, Z.; Li, W.; Tang, M.; Chen, L.; Yang, L.; Tao, Y.; Jiang, Y.; Li, J. Targeting Epstein-Barr virus oncoprotein LMP1-mediated glycosylation sensitizes nasopharyngeal carcinoma to radiation therapy. *Oncogene* 2014, 33, 4568–4578.

(104) Seo, G.; Lee, G.; Kim, J.; Baek, S.-H.; Choi, M.; Ku, K. B.; et al. Rapid Detection of COVID-19 Causative Virus (SARS-CoV-2) in Human Nasopharyngeal Swab Specimens Using Field-Effect Transistor-Based Biosensor. *ACS Nano* 2020, 14, 5135–5142.

(105) Seo, G.; Kim, J.; Park, Y.-H.; Kim, S.-U.; Moon, H.-B.; Park, D. S.; et al. Hepatitis B virus X protein regulates hepatic glucose homeostasis via activation of inducible nitric oxide synthase. *J. Biol. Chem.* 2011, 286, 29872–29881.

(106) Ivanova, L.; Buch, A.; Döhner, K.; Pohlmann, A.; Binz, A.; Pranke, U.; et al. Conservation of Cysteinyl Motifs in the Large Tegument Protein pUL36 Are Required for Efficient Secondary Replication of Herpes Simplex Virus Capsids. *J. Virol.* 2016, 90, 5368–5383.

(107) Adams, O.; Besken, K.; Oberdörfer, C.; MacKenzie, C. R.; Skalka, O.; Däuber, W. Role of indoleamine-2,3-dioxygenase in alpha/beta and gamma interferon-mediated antiviral effects against herpes simplex virus infections. *J. Virol.* 2004, 78, 2632–2636.

(108) Feng, L.; Wu, T.-T.; Tchieu, J. H.; Feng, J.; Brown, H. J.; Feng, J.; et al. Inhibition of the phosphatidylinositol 3-kinase-Akt pathway enhances gamma-2 herpesvirus lytic replication and facilitates reactivation from latency. *J. Gen. Virol.* 2010, 91, 463–469.
Yu, Y.; Maguire, T. G.; Alwine, J. C. Human cytomegalovirus infection induces adipocyte-like lipogenesis through activation of sterol regulatory element binding protein 1. J. Virol. 2012, 86, 2942–2949.

Delgado, T.; Sanchez, E. L.; Camarda, R.; Lagunoff, M. Global Metabolic Profiling of Infection by an Oncogenic Virus: KSHV Induces and Requires Lipogenesis for Survival of Latent Infection. PLoS Pathog. 2012, 8, e1002866.

Chambers, J. W.; Maguire, T. G.; Alwine, J. C. Glutamine metabolism is essential for human cytomegalovirus infection. J. Virol. 2010, 84, 1867–1873.

Gazi, I. F.; Elisaf, M. S. Effect of infection on lipid profile: focus on Epstein–Barr virus. Clin. Lipidol. 2010, 5, 607–610.

Heaton, N. S.; Perera, R.; Berger, K. L.; Khadka, S.; LaCount, D. J.; Kuhn, R. J.; et al. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. Proc. Natl. Acad. Sci. U.S.A. 2010, 107, 17345–17350.

Greseth, M. D.; Traktman, P. De novo Fatty Acid Biosynthesis Contributes Significantly to Establishment of a Bioenergetically Favorable Environment for Vaccinia Virus Infection. PLoS Pathog. 2014, 10, e1004021.

Teng, C.-F.; Wu, H.-C.; Hsieh, W.-C.; Tsai, H.-W.; Su, I.-J. Activation of ATP citrate lyase by mTOR signal induces disturbed lipid metabolism in hepatitis B virus pre-S2 mutant tumorigenesis. J. Virol. 2015, 89, 605–614.

Perlemuter, G.; Sabile, A.; Letteron, P.; Vona, G.; Topilco, A.; Christien, Y.; et al. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. FASEB J. 2002, 16, 185–194.