Virus Research 313 (2022) 198726

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The dysregulation of autophagy and ER stress induced by HHV-6A infection activates pro-inflammatory pathways and promotes the release of inflammatory cytokines and cathepsin S by CNS cells

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ARTICLE INFO

Keywords:
HHV-6A
Neuroinflammation
ER stress
4-PBA
DMF
Astrocytes

ABSTRACT

HHV-6A is a neurotropic herpesvirus able to infect several CNS cells including astrocytes and primary neurons. Here we found that HHV-6A infection of astrocytoma cells, by reducing autophagy, increased ROS and induced ER stress, promoting the release of inflammatory cytokines such as IL-6 and IL-1β and activating pathways such as STAT3, NF-kB and mTOR. Moreover, HHV-6A infection increased the production of CXCL13, a B lymphocyte attracting chemokine, whose recruitment in the CNS could further enhance neuroinflammation. Interestingly, HHV-6A also increased the release of cathepsin S by infected astrocytoma cells as well as by primary neurons. As this enzyme is involved in the degradation of MBP, this effect could contribute to the onset/progression of MS, a neurodegenerative disease that, besides inflammation, is characterized by a progressive demyelination process. In conclusion, this study unveils new molecular mechanisms through which HHV-6A may promote important aspects involved in several neurodegenerative diseases.

1. Introduction

We have previously shown that Human Herpesvirus (HHV) 6A and B, which share about 95% nucleotide identity (Ablashi et al., 2014; Krug and Pellett, 2014) but differ for several biological characteristics (Cuomo et al., 1995), dysregulated autophagy and induced Endoplasmic Reticulum (ER) stress in HSB2 and Molt-3 cells (Romeo et al., 2019b). We then extended this study and evaluated the impact of viral infection on autophagy in other cell types, showing that HHV-6B dysregulated autophagy and led to a pro-death activation of unfolded protein response (UPR) in infected monocytes. This resulted in an impairment of their survival and differentiation into dendritic cells (DCs) (Romeo et al., 2019a), effects induced also by other Herpesviruses, as an immune-escape mechanism (Cirone et al., 2008; Gilardini Montani et al., 2019; Gredmark and Soderberg-Naucler, 2003; Santarelli et al., 2016). Moreover, through induction of ER stress, HHV-6B up-regulated programmed death ligand 1 (PD-L1) on the surface of infected monocytes (Romeo et al., 2019a), an immune checkpoint inhibitor that induces T cell exhaustion and immune dysfunction (Blackburn et al., 2010). Other Herpesviruses such as Epstein Barr virus (EBV) (Gilardini Montani et al., 2018) and Kaposi Sarcoma Herpesvirus (KSHV) (Gilardini Montani et al., 2020; Host et al., 2017) are able to up-regulate PD-L1 to induce immunosuppression.

Several human Herpesviruses can be considered neurotropic, being able to infect CNS cells such as glial cells, microglia or primary neurons. Previous scientific evidences support the hypothesis that Herpesvirus infection, particularly that mediated by Herpes simplex virus 1 (HSV-1) (Marcocci et al., 2020), Varicella-zoster virus (VZV) (Tsai et al., 2017) or HHV-6A (Readhead et al., 2019, 2018) might play a role in Alzheimer's disease (Itzhaki et al.), a neurological disorder afflicting a high percentage of persons among the elderly population, especially in the developed countries, in which the life expectance is longer (Long and

Abbreviations: HHV, human herpesvirus; ER, endoplasmic reticulum; UPR, unfolded protein response; AD, Alzheimer's disease; MBP, myelin basic protein; MS, multiple sclerosis; STAT3, signal transducer and activator of transcription 3; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; mTOR, mammalian target of rapamycin.

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https://doi.org/10.1016/j.virusres.2022.198726
Received 23 November 2021; Received in revised form 1 March 2022; Accepted 2 March 2022
Available online 4 March 2022

Holtzman, 2019). However, the association between viruses and AD remains controversial issue (Rizzo, 2020), and indeed, in the case of HHV-6, other studies have found no correlation between the presence of the virus and AD, contradicting the above reported findings (Agostini et al., 2016; Allnutt et al., 2020; Chorlton, 2020; Jeong and Liu, 2019). In a previous study, we have shown that HHV-6A infection of astrocytoma cells and primary neurons reduced autophagy and increased ER stress in these cells, effects that promoted the accumulation of intracellular and extracellular β-amyloid (Aβ) and the increase of protein tau phosphorylation (Romeo et al., 2020a). Other authors have also evidenced the capacity of HHV-6A to increase inflammatory markers as well as Aβ expression and tau phosphorylation in microglia cells (Bortolotti et al., 2019). Neuroinflammation (Heneka et al., 2015) together with β-amyloid accumulation and tau hyper-phosphorylation are the hallmarks of AD (Serrano-Pozo et al., 2011), therefore the above reported findings suggest that HHV-6A infection could be associated with AD pathogenesis.

In addition to AD, neuroinflammation plays a role in other neurodegenerative diseases such Multiple Sclerosis (MS), an autoimmune disease in which, one of the main targets of immune recognition is represented by Myelin Basic Protein (MBP) (Bjelobaba et al., 2017). MS is indeed characterized by a progressive demyelination, process in whose triggering viral infection may also be involved (Mechelli et al., 2021). There are indeed several reports investigating the possible link between Herpesvirus infection and MS and, among those, a particular attention has been given to Epstein Barr virus (EBV) (Bjornevik et al., 2022; Robinson and Steinman, 2022), which remains the top candidate as causal agent for such disease, HHV-6A/B (Leibovitch and Jacobson, 2014) and Human Endogenous Retrovirus (HERVs) (Tao et al., 2017), viruses that could also work together in promoting MS (Komaroff et al., 2020). Interestingly, as HHV-6 encodes for U24 membrane protein shares some amino acids with MBP protein, in a previous study we have investigated a possible T cell cross-reactivity between HHV-6A and MBP (Cirone et al., 2002). As neuroinflammation plays a role in the pathogenesis of several different neurodegenerative diseases and given that autophagy and ER stress/UPR activation strongly influence the inflammatory response (Hooper et al., 2019), in the present study, we investigated whether HHV-6A infection of astrocytoma cells could affect the release of inflammatory cytokines, chemokines and the production of reactive oxygen species (ROS), molecules also involved with the inflammatory process (Naik and Dixit, 2011). We also explored the involvement of ER stress/UPR activation by HHV-6A-infection in promoting the inflammatory response by using sodium 4-phenylbutyrate (4-PBA), a molecule displaying numerous beneficial functions, including that to reduce ER stress (Kusaczuk et al., 2015; Rellmann et al., 2019). We also used dimethyl fumarate (DMF), a drug that activates nuclear factor erythroid 2–related factor 2 (NRF2) and displays anti-inflammatory properties (McGuire et al., 2016), as another possible strategy to counteract virus-induced inflammation. The release of pro-inflammatory cytokines by HHV-6A infection was correlated with the activation of signal transducer and activator of transcription 3 (STAT3), Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) and mammalian target of rapamycin (mTOR), as these pathways regulate and are regulated by inflammatory cytokines. Finally, we investigated whether HHV-6A infection could affect the release of cathepsin S, an enzyme that has been previously shown to mediate MBP degradation (Beck et al., 2001), process strongly involved in the pathogenesis of MS.

2. Materials and methods

2.1. Cell culture, infection and treatments

U373 (a glioblastoma-astrocytoma cell line) was grown in RPMI 1640 (Thermo Fisher Scientific), 10% Fetal Bovine Serum (FBS) (Corning), L-glutamine, streptomycin (100 μg/ml) (Corning) and penicillin (100 U/ml) (Corning) in 5% CO_2 at 37 °C.

Neural Stem Cells (NSCs) (Thermofisher scientific) were grown as spheroid culture in Gibco KnockOut™ DMEM/F-12 Basal Medium (Thermofisher scientific), StemPro® NSC SFM Supplement 2% (Thermofisher scientific), FGF Basic Recombinant Human 20 ng/mL (Thermofisher scientific), EGF Recombinant Human 20 ng/mL (Thermofisher scientific), GlutaMAX -I supplement 2 mM (Thermofisher scientific), heparin 6 u /mL (Sigma Aldrich) and ascorbic acid 200 μM (Sigma Aldrich) in 5% CO_2 at 37 °C. Neural Stem cells were differentiated in neurons on Geltrex® hESC-qualified Reduced Growth Factor Basement Membrane Matrix Substrate (Thermofisher scientific) for 10 day in Neurobasal Medium (Thermofisher scientific), B-27 Serum-Free Supplement 2% (Thermofisher scientific) and GlutaMAX -I supplement 2 mM (Thermofisher scientific) in 5% CO_2 at 37 °C. Differentiation into neurons was verified by using the neuronal marker Dcx (Invitrogen) by Western Blot according to the recommended protocol by Thermofisher Scientific. HHV-6A (GS) was propagated in HSB-2 cells. The virus stock (titer, 105 50% tissue culture- infective doses) was obtained from 7-day supernatant of infected cells, when more than 80% of the cells showed a cytopathic effect. Cell-free culture fluid was harvested, filtered through a 0.45-mm-pore-size filter, and pelleted by centrifugation at 25,000 x g for 90 min at 4 °C. U373 and neurons were plated and the day after infected with an appropriate dilution of the virus stock. After 4 h at 37 °C, the cells were washed once and suspended in complete medium. U373 and neurons treated with supernatant of uninfected HSB-2 cells were used as mock.

In some experiments U373 and neurons were pre-treated or not with sodium phenylbutyrate (4-PBA) (10 μM) (Sigma Aldrich) or Dimethyl Fumarate (DMF) (5 μM) (Sigma Aldrich) for 1 h and then infected with HHV-6A for 72 h. Mock-pre-treated and mock-infected cells were used as appropriate control.

Human peripheral blood mononuclear cells (PBMCs) from buffy coats of healthy donors were isolated by Lympholyte cell separation medium (Cedarlane, CL5020). B lymphocytes were isolated from PBMCs by immunomagnetic cell separation using anti-CD19-conjugated microbeads according to the manufacturer's instructions (Miltenyi Biotec, 130–050–301) and cultured in RPMI 1640 (Sigma Aldrich, R0883), 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, F7524), L-glutamine (Aurogene, AU-X0550) and streptomycin (100 μg/ml) and penicillin (100 U/ml) (Aurogene, AU-L0022), complete medium, in 5% CO_2 saturated humidity at 37 °C.

This research involving human subjects has been performed in accordance with the Declaration of Helsinki and has been approved by the ethic committee of Policlinico Umberto I, Rome, Italy (847/19).

2.2. Quantitative real time-polymerase chain reaction (qRT-PCR)

To evaluate the infection, DNA extraction was carried out from 72 h-infected cells (150.000 cells) by ELITe Galaxy system (ELITechGroup S. p.A.) according to manufacturer's instructions. Extracted samples were then analyzed for the presence of HHV6-DNA by quantitative TaqMan RT-PCR using a commercially available kit that amplifies a sequence relative to ORF 13R region, U67 gene (ELITechGroup S.p.A.) using ABI 7300 real-time PCR System (Applied Biosystem).

2.3. Indirect immunofluorescence assay (IFA)

Infected U373 and primary neurons were washed with PBS, applied on multispot microscope slides and air-dried. Cells were then incubated with 2% paraformaldehyde (Electron Microscopy Science) for 20 min and permeabilized with 0.1% Triton X-100 (SIGMA) for 5 min. After 3 washes with PBS, cells were incubated with 1% glycine, 3% BSA for a further 30 min. Then cells were incubated with the primary monoclonal antibody against p41/38 (kindly provided by HHV-6 Foundation) for 1 h at room temperature. Slides were then washed 3 times with PBS and cells were further incubated with a polyclonal conjugated-Cy3 sheep

anti-mouse antibody (Jackson ImmunoResearch) for 30 min at room temperature. After 3 washes in PBS, cells were stained with DAPI (SIGMA) for 1 min at room temperature. Slides were further washed in PBS, mounted with glycerol:PBS (1:1) and observed by a fluorescence microscope (Olympus BX53, USA).

2.4. Western blot analysis

1×10^6 cells were washed with PBS and lysed in a RIPA buffer containing 150 mM NaCl, 1% NP-40 (Calbiochem), 50 mM Tris–HCl, pH 8, 0.5% deoxycholic acid (SIGMA), 0.1% SDS, protease and phosphatase inhibitors. 12 μg of protein lysates were subjected to protein electrophoresis on 4–12% NuPage Bis-Tris gels (Sigma Aldrich), according to the manufacturer's instructions. The gels were blotted on nitrocellulose membrane (Biorad) for 1 h in Tris-Glycine buffer. The membranes were blocked in PBS 0.1% Tween20 solution containing 3% of BSA, probed with specific antibodies and developed using ECL Blotting Substrate (Advansta).

The quantification of protein bands was performed by densitometric analysis using Image J software.

2.5. Antibodies

To evaluate protein expression on western blot membranes the following antibody were used: rabbit polyclonal anti-p62 (1:1000) (Cell Signaling, 5114T), mouse monoclonal anti-NRF2 (1:100) (Santa Cruz Biotechnology Inc., sc-365,949), mouse monoclonal anti-NQO1 (1:100) (Santa Cruz Biotechnology Inc., sc-32,793), rabbit polyclonal anti p-eIF2α (Cell Signaling, 9721S), rabbit polyclonal anti-eIF2α (Cell Signaling, 9722S), rabbit polyclonal anti-p-4E-BP1 (1:500) (Cell Signaling, 2855T), rabbit polyclonal anti-4E-BP1 (1:500) (Cell Signaling, 9452S), mouse monoclonal anti-p-STAT3 (1:100) (BD Bioscience, 612,356), mouse monoclonal anti-STAT3 (1:500) (BD Bioscience, 610,189), mouse monoclonal anti-p-NF-kB (1:100) (Santa Cruz Biotechnology Inc. sc-101,752), mouse monoclonal anti-NF-kB (1:100) (Santa Cruz Biotechnology Inc., sc-136,548) and mouse monoclonal anti-MBP (1:100) (Santa Cruz Biotechnology Inc., sc-271,524). Mouse monoclonal anti-β-actin (1:5000) (Sigma Aldrich) and mouse monoclonal anti-GAPDH (1:500) (Santa Cruz Biotechnology Inc. sc-47,724) were used as loading control. The goat anti-mouse IgG-HRP (1:10,000) (Bethyl Laboratories, A90–116P) and goat anti-rabbit IgG-HRP (1:10,000) (Bethyl Laboratories, A120–101P) were used as secondary antibodies. All the primary and secondary antibodies were diluted in PBS-0.1% Tween20 solution containing 3% of BSA (SERVA).

2.6. Measurement of intracellular reactive oxygen species (ROS)

To measure reactive oxygen species (ROS) production, 10 μM 2,7-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich D6883) was added to cell cultures for 15 min. After that, cells were detached by trypsin, washed with PBS and then analyzed by FACScalibur flow cytometer (BD Transduction Laboratories, Franklin Lakes, NJ, USA) using CELLQuest Pro-software (version 6.0, BD Biosciences, Franklin Lakes, NJ, USA). For each analysis, 10,000 events were recorded.

2.7. Chemiluminescent immunometric assay

After 72 h of infection, supernatants derived from infected cells were analyzed using a magnetic Luminex assay using a human pre-mixed multi-analyte kit (R&D Systems Bio-Techne, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8. Transmigration assay

To study B cells transmigration, supernatants derived from 72 h infection of U373 cells pre-treated or not were inserted on the bottom of modified Boyden chambers (Thermo Scientific, Denmark) with a 0.8 μm porous filter and 2×10^6 B lymphocytes were plated on the filter in complete medium. The transmigration was performed overnight and then the cells migrated were counted by trypan blue exclusion assay (Sigma Aldrich) with a light microscopy using a Neubauer hemocytometer. The experiments were performed in triplicate and repeated at least three times.

2.9. Myelin basic protein (MBP) degradation assay

Supernatants derived from U373 infection and treatment, in which Cathepsin S was measured by Luminex assay, were collected. Supernatants were concentrated with Centricon Plus 70 (Merck Millipore) following the manufacturer's instructions.

10 μg of purified MBP protein (Sigma Aldrich) was incubated overnight in each supernatant in a Thermomixer (Eppendorf) at 37 °C. The day after, MBP protein was subjected to protein electrophoresis on 4–12% NuPage Bis-Tris gels (Sigma Aldrich), according to the manufacturer's instructions. The gels were blotted on nitrocellulose membrane (Biorad) for 1 h in Tris-Glycine buffer. The membranes were blocked in PBS 0.1% Tween20 solution containing 3% of BSA, probed with specific antibodies and developed using ECL Blotting Substrate (Advansta).

2.10. Densitometric analysis

The quantification of proteins bands was performed by densitometric analysis using the Image J software (1.47 version, NIH, Bethesda, MD, USA), which was downloaded from NIH website http://imagej.nih.gov).

2.11. Statistical analysis

Results are represented by the mean ± standard deviation (SD) of at least three independent experiments and a two-tailed Student's *t*-test was used to demonstrate statistical significance. Difference was considered as statistically significant when p-value was at least < 0.05.

3. Results

3.1. HHV-6A induces p62 accumulation, up-regulates NRF2 and increases ROS in infected astrocytoma cells

In previous studies, we have shown that HHV-6A infection reduced autophagy and induced ER stress in astrocytoma cells (Romeo et al., 2020a). Given the intricate interplay between autophagy, UPR and oxidative stress (Zhang et al., 2019) here we evaluated whether viral infection could affect the level of intracellular ROS. We first assessed HHV-6A infection of astrocytoma cells by performing qRT-PCR (Fig. 1A) and IFA (Fig. 1B) and confirmed that viral infection reduced autophagy in these cells, by evidencing the accumulation of p62 (Fig. 1C). We then evaluated intracellular ROS by performing DCFDA staining and FACS analysis and, as shown in Fig. 1D, ROS level increased following viral infection. ROS may stabilize NRF2 (Itoh et al., 1999) and such effect could also be induced by p62 that is able to sequester Keap-1, a NRF2 inhibitor (Jiang et al., 2015). Therefore, we next evaluated NRF2 expression level in HHV-6A-infected astrocytes and found that, although NRF2 was up-regulated (Fig. 1C), the expression level of NQO1, one of the most important anti-oxidant enzymes whose transcription is under NRF2 control, increased only slightly following viral infection (Fig. 1C). This could be one of the reasons why ROS level increased in viral-infected cells.

3.2. ER stress activation promotes IL-6 and IL-1β release by infected astrocytoma cells

Pro-inflammatory cytokines play a key role in neuroinflammation.

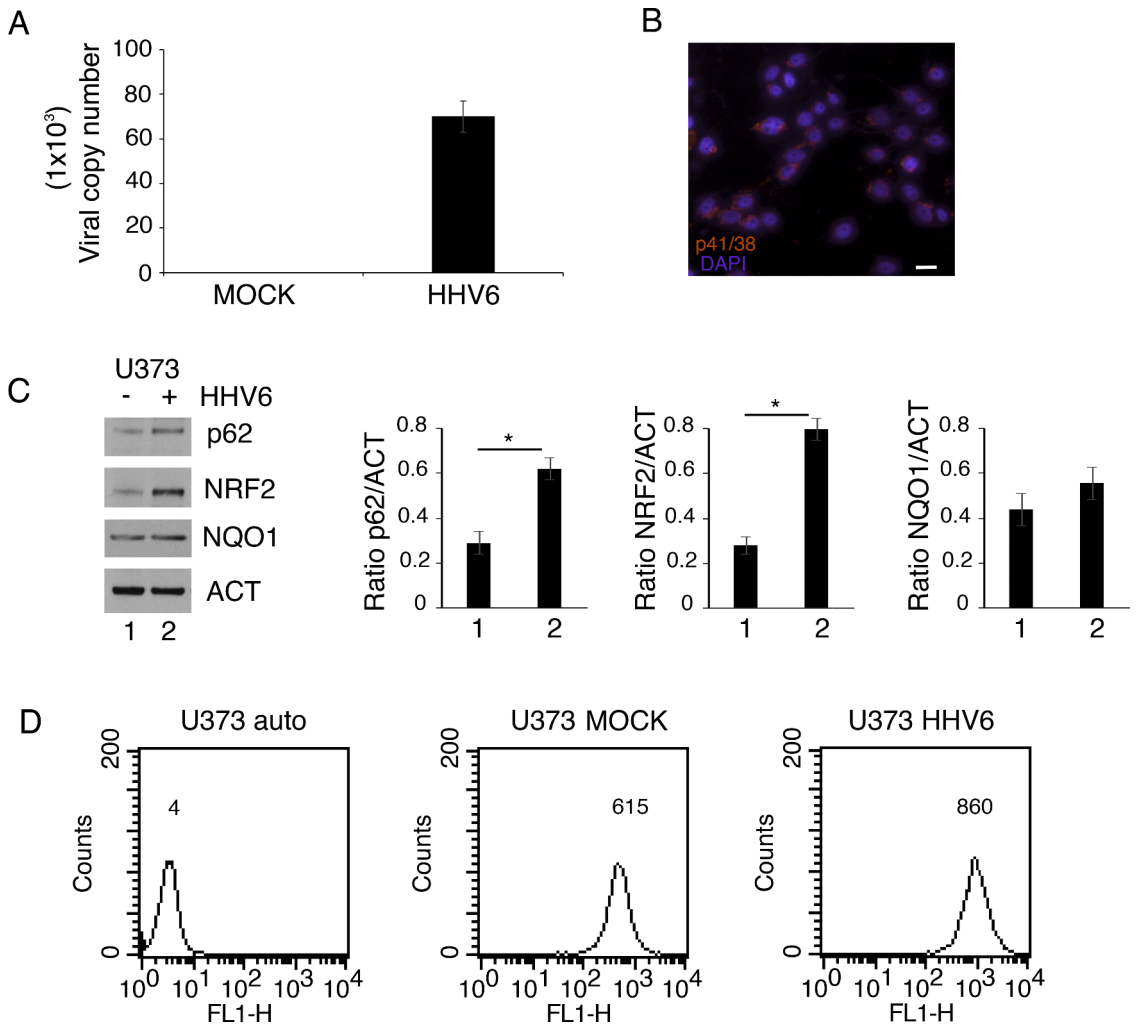

Fig. 1. HHV-6A infection promotes p62 accumulation, NRF2 and ROS up-regulation. U373 cells were infected with HHV-6A for 72 h. The infection was evaluated by A) qRT-PCR analysis and by B) IFA studying the expression of p41/38 protein. Bars = 10 μ C) after 72 h of infection, expression level of p62, NRF2 and NQO1 was evaluated by western blot analysis. β Actin was used as loading control. Mock-infected cells were used as control. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio between the protein and the appropriate control *p-value < 0.05. D). Intracellular ROS level was measured by FACS analysis using DCFDA as staining. Mean of fluorescence intensity (MFI) is indicated. One representative experiment out of three is reported. Auto means autofluorescence.

Their release has been shown to be also strongly interconnected with ER stress/UPR activation (Gilardini Montani et al., 2020; Romeo et al., 2020b; Smith, 2018) as previously evidenced also in other HHV-6A infected cells (Romeo et al., 2019a). Therefore, we then investigated the production of cytokines known to play a key role in the inflammatory process, namely IL-1β and IL-6. As shown in Fig. 2A, the production of both cytokines increased following HHV-6A infection of astrocytoma cells. Interestingly such effect was reduced by 4-PBA (Fig. 2A), a molecule able to reduce ER stress and counteract inflammation (Kusaczuk et al., 2015). We then used DMF, a drug reported to reduce secretion of cytokines (McGuire et al., 2016) and ER stress induced by these molecules (Zhu et al., 2020), to evaluate if it could counteract inflammatory cytokine release induced by HHV-6A infection. We found that DMF reduced IL-6 and IL-1β production even more efficiently than 4-PBA (Fig. 2A). In correlation with the effect on cytokine release, DMF also reduced the level of pEIF2 alpha phosphorylation, a marker of ER stress induced by HHV-6A infection in astrocytoma cells (Fig. 2B).

3.3. STAT3, p65 NF-kB and mTOR activation by HHV-6a infection can be prevented by 4-PBA and DMF

IL-6 is known to activate several pathways, particularly STAT3 that, in turn, promotes the release of this as well as other inflammatory cytokines, in a positive regulatory circuit (Garbers et al., 2015). Therefore, we next evaluated the status of STAT3 activation in viral-infected astrocytoma cells and found that its phosphorylation increased in comparison to the mock-infected control (Fig. 3). Interestingly 4-PBA and even more efficiently DMF were able to reduce its activation in HHV-6A-infected cells (Fig. 3). Another pathway with a central role in the inflammatory network is NF-kB. Therefore, we next assessed the activation of p65, member of NF-kB pathway, and found that its phosphorylation increased following HHV-6A infection (Fig. 3). As NF-kB has been reported to inhibit NRF2 activity (Liu et al., 2017), this may be one of the reasons why NQO1 expression slightly increased although NRF2 was up-regulated following viral infection (Fig. 2C).

Finally, the activation of mTOR was investigated, given that this pathway can be also activated by cytokines such IL-6 (Bhatt et al., 2010; Granato et al., 2017). As read-out of its activation, the phosphorylation status of mTOR target Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4-EBP1) was assessed and the results shown in Fig. 3 indicate that following HHV-6A infection 4-EBP1 was hyper-phosphorylated. Both mTOR and NF-kB phosphorylation were reduced by 4-PBA and DMF (Fig. 3) in correlation with the inhibition on pro-inflammatory cytokine release mediated by these drugs. Of note, mTOR activation could contribute to autophagy reduction induced by HHV-6A, mTOR being the master negative regulator of autophagy

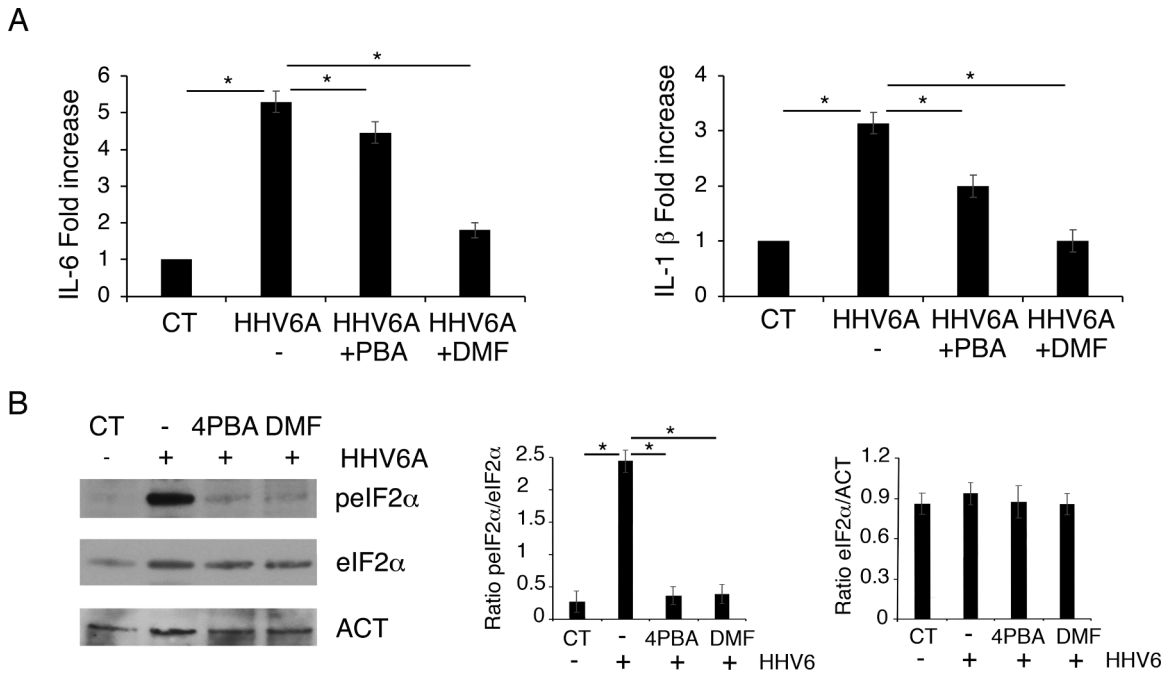

Fig. 2. ER stress activation promotes IL-6 and IL-1β release by infected astrocytoma cells. U373 cells were pre-treated or not with 4-PBA and DMF and then infected with HHV-6A or Mock (CT). After 72 h post-infection, supernatants were collected and analyzed for IL-6 and IL-1β amount by Chemiluminescent Immunometric Assay. Histograms representing the mean plus S.D. are indicated and one out of three independent experiments is shown *p-value < 0.05. B). eIF2α phosphorylation was evaluated by western blot analysis. βActin was used as loading control. Mock-infected cells were used as control. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio between the protein and the appropriate control *p-value < 0.05.

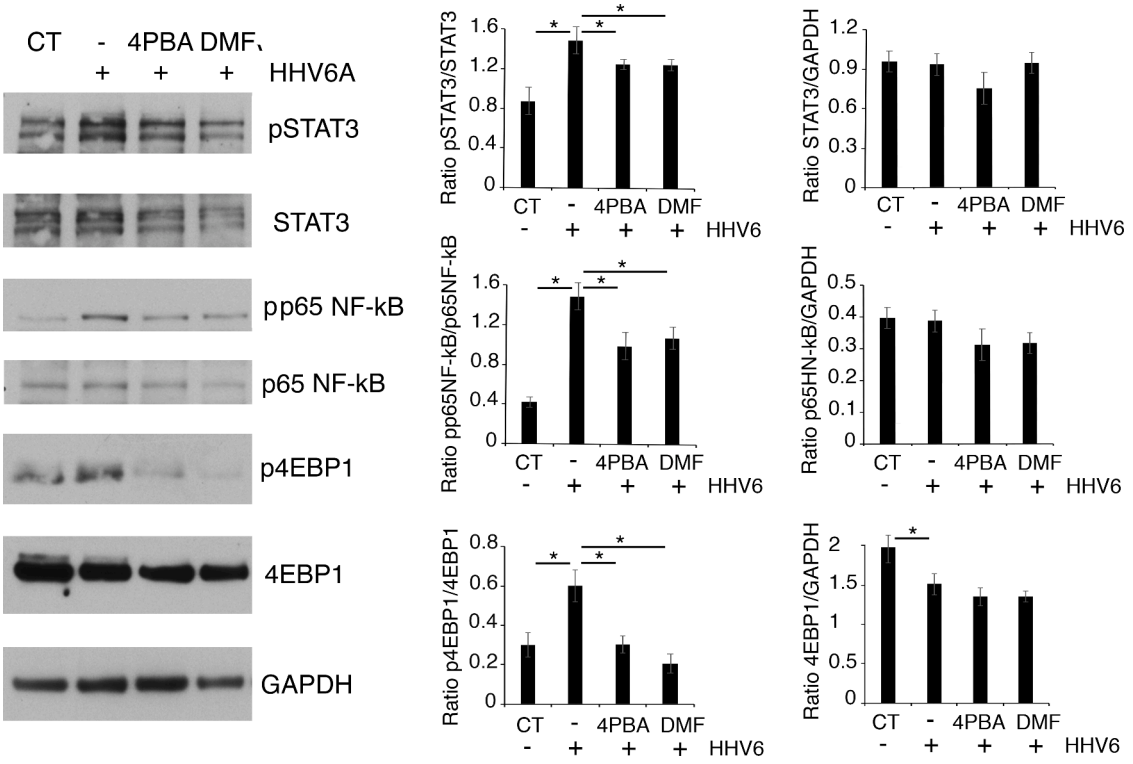

Fig. 3. STAT3, p65 NF-kB and mTOR are activated by HHV-6A infection and reduced by 4-PBA and **DMF.** U373 cells were pre-treated or not with 4-PBA and DMF and then HHV-6A-infected. Protein phosphorylation of STAT3, NF-kB and 4EBP1 (mTOR target) was evaluated by western blot analysis. GAPDH was used as loading control. Mock-infected cells were used as control. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio between the protein and the appropriate control *p-value < 0.05.

(Cirone, 2021).

3.4. HHV-6A infected-astrocytoma cells release a higher amount of CXCL13 able to attract B lymphocytes, effect reduced by 4-PBA and DMF

Besides resident cells, immune cells recruited into the CNS by chemotactic factors, may promote neuroinflammation. Here we found that HHV-6A infection increased the production of C-X-C Motif Chemokine Ligand 13 (CXCL13) (Fig. 4A). This chemokine can attract B lymphocytes, whose important role in promoting neuroinflammation has been previously highlighted (Kowarik et al., 2012). In correlation with CXCL13 release, a higher number of primary B lymphocytes migrated in response to HHV-6A-infected astrocyte cell supernatant as compared to the uninfected astrocyte supernatant (Fig. 4B). Interestingly, B cell infiltration in the brain, migrated through a porous membrane barrier, has been reported to play a role in the pathogenesis of MS (Sospedra, 2018) and to strongly contribute to the progression of AD (Kim et al., 2021).

3.5. Astrocytoma cells as well as primary neurons release higher amount of cathepsin S following HHV-6A-infection

As cathepsin S release has been shown to be promoted by the activation of pathways such as STAT3 (Yan et al., 2016), we next assessed the production of this enzyme by HHV-6A-infected astrocytoma cells. We found that a higher amount of cathepsin S was released by these cells following viral infection (Fig. 5A) and interestingly such effect was also observed in primary neurons (Fig. 5A) in which infection was demonstrated by qRT-PCR (Fig. 5C) and IFA (Fig. 5D). Interestingly, PBA e DMF were able to counteract cathepsin S release by both cell types (Fig. 5B). Cathepsin S has been reported to efficiently mediate MBP proteolysis (Beck et al., 2001), therefore we next investigated whether the supernatant of HHV-6A-infected cells could promote the degradation of MBP. The results shown in Fig. 5E and F evidenced a reduction of MBP following exposure to such supernatant and this effect was reverted by 4-PBA and DMF. This suggest that these drugs may have a protective role in counteracting several pathologic effects induced by HHV-6A infection in CNS cells.

4. Discussion

Neuroinflammation is known to contribute to the onset and progression of several different neurodegenerative disorders (Kwon and Koh, 2020). As in other organs, while a transient inflammation is mainly a protective response, chronic inflammatory processes can be dangerous. Astrocytes and microglia are the cells most involved in neuroinflammation, particularly when infected by neurotropic viruses.

Several viruses may localize in the CNS and, among those, the most studied neurotropic herpesviruses are HSV-1 (Duarte et al., 2019), EBV (Soldan and Lieberman, 2020) and HHV-6A (Santpere et al., 2020) that can infect and persist within several cells present in the brain (Komaroff et al., 2020). It has long been debated whether HHV-6A is etiologically involved in the pathologically distinct diseases, AD and MS, both of which connect to inflammatory processes. Despite the number of studies carried out in the last years, the link between microbial agents and neurodegenerative diseases remains an open issue (Itzhaki et al., 2020; Komaroff et al., 2020; Rizzo, 2020).

Interestingly, in the present study, we found that HHV-6A infection promotes the release of pro-inflammatory cytokines such as IL-6 and IL-1β and CXCL13 chemokine by astrocytoma cells. Intracellular ROS also increased following HHV-6A infection and these molecules may also contribute to the inflammatory process (Chelombitko, 2018), i.e. by sustaining the activation of molecular pathways promoting the production of pro-inflammatory cytokines (Naik and Dixit, 2011). In particular, STAT3, NF-kB and mTOR pathways may play an important role in bridging ROS to inflammatory cytokine production and here, we showed that all these pathways were activated by HHV-6A infection. STAT3 may be phosphorylated by UPR activation, mainly through the protein kinase RNA-like endoplasmic reticulum kinase (PERK) sensor (Meares et al., 2014). Interestingly, STAT3 has been reported to contribute to astrocyte activation in the course of neurodegenerative diseases and has been identified as a potential target for the treatment of these diseases (Ben Haim et al., 2015). Moreover, the role of STAT3 activation in neurodegeneration has been demonstrated also in animal models (Reichenbach et al., 2019). Although with a more controversial role, NF-kB could contribute to oligodendrocyte pathology (Bonetti et al., 1999) and regarding mTOR, it has been shown that its activation promotes neurodegeneration, likely through its inhibitory effect on the autophagic process (Perluigi et al., 2015). The pathogenic role of mTOR has been also confirmed by studies in which its inhibition was able to counteract neurodegeneration (Jahrling and Laberge, 2015). The activation of all these pro-inflammatory pathways by HHV-6A suggests that viral infection of CNS cells could play a role in promoting neuroinflammation and neurodegeneration.

Importantly, neuroinflammation may also promote neuronal dysfunction and could be one of the processes linking viral infection to neurodegeneration (Guzman-Martinez et al., 2019). Therefore, treatments able to counteract inflammation or inhibit the activation of molecular pathways promoting it, could greatly improve the course of neurodegenerative disorders.

DMF (Rosito et al., 2020) and 4-PBA (Kusaczuk et al., 2015) are drugs known to counteract ER stress, inflammation and oxidative stress

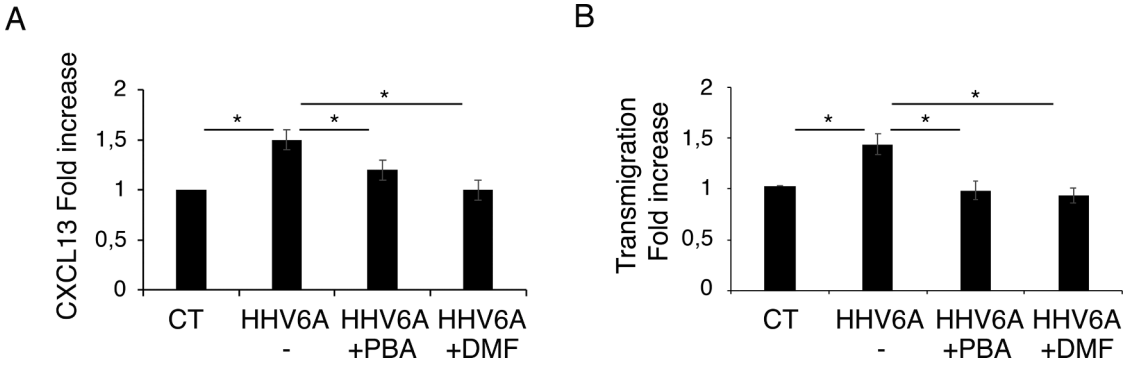

Fig. 4. The supernatant obtained from HHV-6A infected-astrocytes contains a higher amount of CXCL13 that promotes the trans-well migration of B lymphocytes. U373 cells, pre-treated or not with 4-PBA and DMF, were HHV6A-infected. The supernatants were collected and A) the amount of CXCL13 was evaluated by Chemiluminescent Immunometric Assay. Histograms representing the mean plus S.D. are indicated and one out of three independent experiments is shown *p-value < 0.05. B). Transmigration of lymphocytes B was studied using modified Boyden chambers. Histograms representing the mean plus S.D. are indicated and one out of three independent experiments is shown *p-value < 0.05.

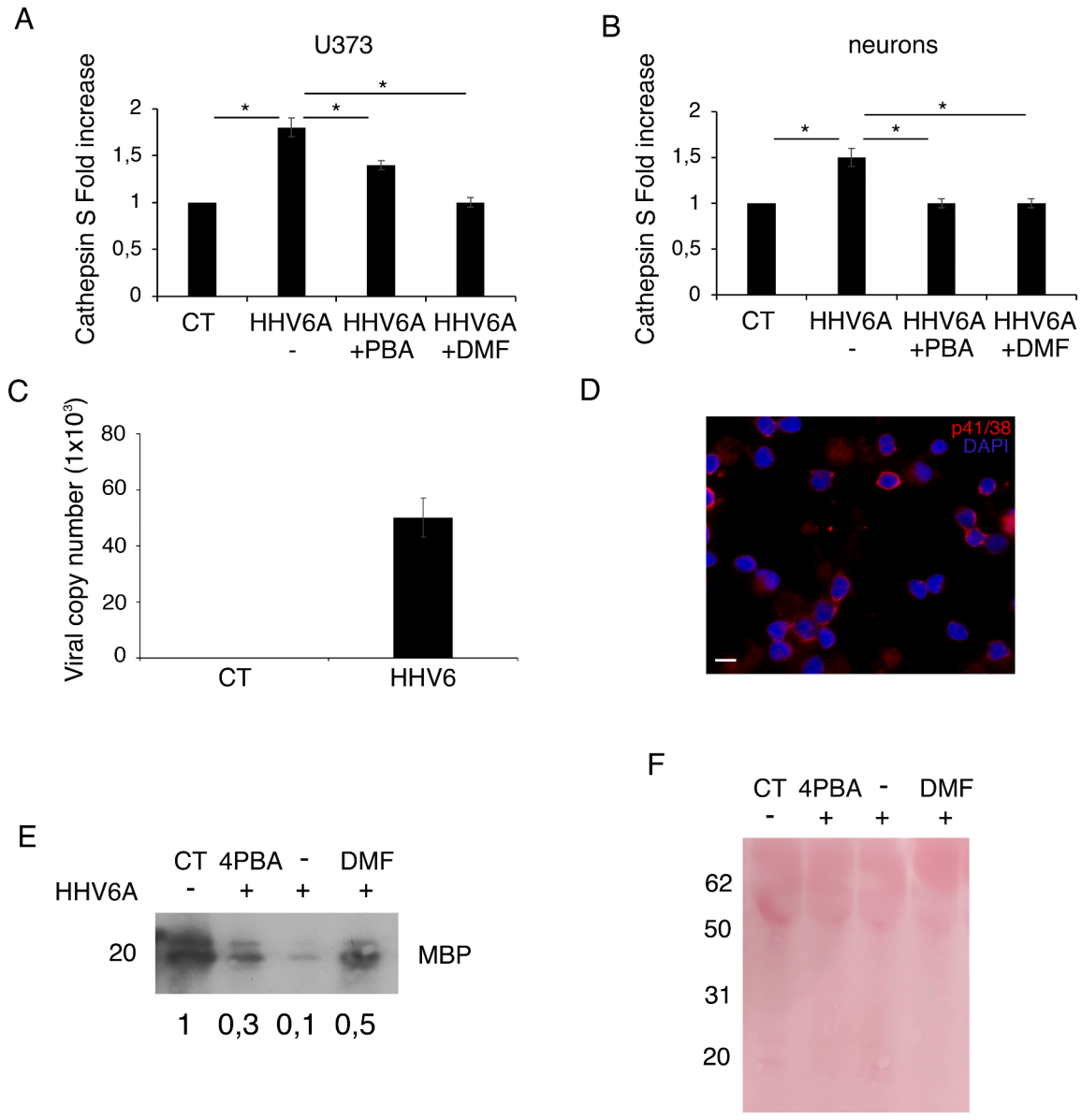

Fig. 5. HHV-6A-infection induces higher amount of Cathepsin S release in both primary neurons and astrocytes, causing MBP degradation. U373 cells and neurons were pre-treated or not with 4-PBA and DMF and then infected. (A, B) 72 h post-infection of pre-treated or not U373 cells, supernatants were collected and production of Cathepsin S was evaluated by Chemiluminescent Immunometric Assay. Histograms representing the mean plus SD are indicated and one out of three independent experiments is shown *p-value < 0.05. Primary neuron-infection was evaluated by (C) qRT-PCR analysis and (D) IFA studying the expression of p41/38 protein. Bars = 10 μ (E) supernatants from infected or uninfected U373 cells were collected, concentrated and used to evaluate MBP degradation with western blot analysis. Numbers represent the densitometric analysis of the amount of MBP assuming the control mock cell (CT) = 1; (F) Ponceau staining of western blot shown in E was used as loading control.

and may therefore exert several benefic effects against inflammatory-based diseases. The findings of this study suggest that 4-PBA and even more DMF counteracted the release of pro-inflammatory cytokines, the activation of pro-inflammatory pathways and the secretion of CXCL13, by lowering ER stress induced by HHV-6A infection. Counteracting the release of CXCL13 is particularly important as this chemokine may recruit B lymphocytes into the CNS. Indeed, these cells are known to contribute to inflammation in the course of both AD and MS (Kim et al., 2021; Sospedra, 2018). The other interesting finding of this study is that the secretion of cathepsin S, enzyme known to degrade MBP, increased following HHV-6A-infection of astrocytoma cells and primary neurons. This effect was also counteracted by 4-PBA and DMF, further indicating the potential of these drugs to limit several pathologic processes involved in MS pathogenesis.

5. Conclusions

In conclusion, this study provides evidence that, by triggering ER stress, HHV-6A (1) promotes inflammation, characteristic of several distinct neurodegenerative diseases, and (2) that 4-PBA and DMF may inhibit this process and limit the release of cathepsin S by viral-infected CNS cells. Considering that we have previously shown that 4-PBA could reduce pro-inflammatory cytokine production by HHV-6A-infected monocytes (Romeo et al., 2019a) and given that monocytes recruited from peripheral blood could contribute to microglia formation, the use of this drug could be even more promising for the treatment of neurodegenerative diseases.

CRediT authorship contribution statement

Maria Anele Romeo: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **Maria Saveria Gilardini Montani:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Rossella Benedetti:** Formal analysis, Writing – review & editing. **Andrea Arena:** Software, Formal analysis, Writing – review & editing. **Aurelia Gaeta:** Methodology, Formal analysis, Writing – review & editing. **Mara Cirone:** Conceptualization, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This research was funded by HHV6 Foundation, AIRC (IG 2019 Id.23040), PRIN 2017 (2017 K55HLC), Istituto Pasteur Italia-Fondazione Cenci Bolognetti Grant, Ateneo 2019.

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and has been approved by the ethic committee of Policlinico Umberto I, Rome, Italy (847/19).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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