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HHV-6B reduces autophagy and induces ER stress in primary monocytes impairing their survival and differentiation into dendritic cells

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ABSTRACT

HHV-6A and HHV-6B are ubiquitous human betaherpesviruses sharing more than 80% homology. HHV-6B is the most common cause of encephalitis in transplant patients and its primary infection may cause the exanthema subitum and febrile seizures in infants. HHV-6A and HHV-6B are able to infect several immune cell types such as T cells, monocytes and dendritic cells (DCs). In this study we found that HHV-6 B derived from patients affected by exanthema subitum impaired monocyte differentiation into DCs, as the infected cells acquired less CD1a DC marker and retained more CD14 monocyte marker. In agreement with the previous finding that HHV-6B dysregulated autophagy and induced endoplasmic reticulum (ER) stress in cells in which it replicated, here we found that these effects occurred also in differentiating monocytes and that ER stress relief, by using the chemical chaperone sodium 4-phenylbutirate (PBA), partially restored DC formation. This suggests that the induction of ER stress, likely exacerbated by autophagy inhibition, could contribute to the immune suppression induced by HHV-6B derived from exanthema subitem patients.

1. Introduction

HHV-6A and HHV-6B belong to betaherpesvirus family and share more than 80% homology (Ablashi et al., 2014). HHV-6B, the causative agent of exanthema subitum, is a major cause of febrile seizures and febrile status epilepticus (Epstein et al., 2012; Mohammadpour Touserkani et al., 2017) and is also the most common cause of encephalitis in transplant patients (Ongradi et al., 2017). On the other hand, HHV-6A has been associated with neurodegenerative diseases such multiple sclerosis (MS) (Alvarez-Lafuente et al., 2002) and more recently with Alzheimer's disease (Eimer et al., 2018; Readhead et al., 2018). The receptor for HHV-6A is CD46, and although this molecule is used as receptor also by HHV-6B, its main receptor is CD134 (OX40), molecule playing a key role in HHV-6B replication (Nagamata et al., 2018). HHV-6A and HHV-6B have been reported to have a strong tropism for CD4+ T-lymphocyte, both *in vitro* and *in vivo* (Lusso et al., 1988; Takahashi et al., 1989). HHV-6A can productively infect CD8 + T cells, natural killer cells and gamma/delta T cells and induce *de novo* expression of CD4 that is otherwise not expressed in these cell subsets (Lusso et al., 1995, 1991) while HHV-6B infects these cells less efficiently (Grivel et al., 2003). Besides T lymphocytes, HHV-6B can infect B cells (Lusso et al., 1988), monocytes (Kondo et al., 1991) and dendritic cells (Asada et al., 1999). Moreover, human progenitor derived astrocytes can be infected by HHV-6A that successfully replicates in these cells while HHV-6B leads to abortive infection (Dietrich et al., 2004; Donati et al., 2005) and a similar infection occurs oligodendrocyte progenitor cells (Ahlqvist et al., 2005; Dietrich et al., 2004; Donati et al., 2005). Several other differences have been found between the two HHV-6 variants (Cuomo et al., 1995) even if both HHV-6A and HHV-6B may induce immune suppression (Dagna et al., 2013), as for example it has been reported that HHV-6A can accelerate progression of AIDS in macaques (Lusso et al., 2007) and that these viruses may inhibit dendritic cell (DC) maturation, reducing their allostimulatory capacity and the production of IL-12p70 (Smith et al., 2005). The interference with DC function is a common strategy adopted by viruses that establish lifelong infection (Cirone et al., 2007; Li et al., 2002). Indeed DCs play a

Abbreviations: ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; eIF2 alpha, eukaryotic initiation factor 2 alpha; BIP, binding immunoglobulin protein; HHV-6, human herpesvirus 6; IRE1 alpha, inositol requiring 1 alpha; TH, thapsigargin; UPR, unfolded protein response

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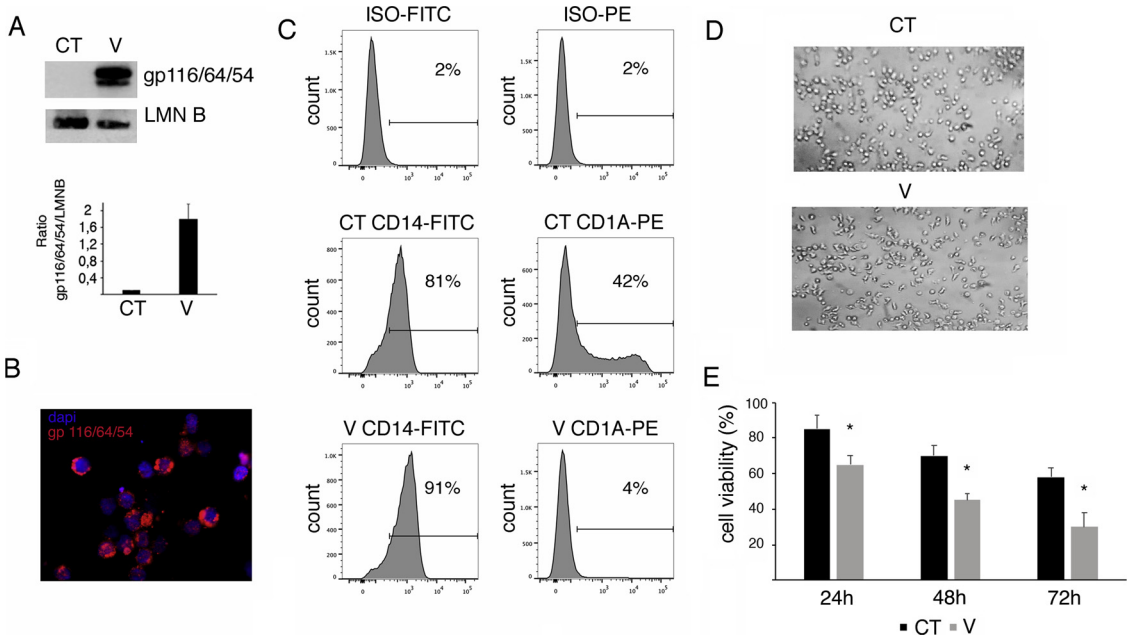

Fig. 1. Infection by HHV-6B derived from exanthema subitum patients reduces monocyte differentiation into DC and cell survival. Primary monocytes isolated from healthy donors were infected with serum derived from exanthema-subitum affected patients and differentiated in DCs for 72 h to evaluate: The expression of the lytic viral antigen complex gp116/64/54 by A) western blot analysis. Lamin B (LMNB) was used as loading control; Histograms represent the ratio of the mean plus SD of densitometric analysis of three different experiments and B) immunofluorescence of infected monocytes: nuclei are stained in blue with DAPI and the lytic viral antigen complex in red; C) The expression of CD14 and CD1A was assessed by FACS analysis. The percentage of positive cells is reported. One representative experiment out of three is shown; D) Cellular morphology was evaluated by optical microscopy; E) Cell survival of mock and infected DCs was evaluated by MTT assay after 24, 48,72 h of culture. Histograms represent the mean plus SD of three independent experiments. * p value ≤ 0.05. Mock-infected monocytes (CT), HHV-6B-infected monocytes (V).

pivotal role in initiating and regulating the immune response and the impairment of their function promotes viral escape from immune recognition. Autophagy, a catabolic process required to maintain cellular homeostasis, is essential for antigen presentation and has been reported to be required for proper monocyte differentiation (Zhang et al., 2012). Accordingly, we have demonstrated that the reduction of autophagy, achieved through different strategies, allowed EBV and KSHV to prevent DC (Gilardini Montani et al., 2018b; Santarelli et al., 2016) or macrophage (Gilardini Montani et al., 2019) formation. We have recently shown that HHV-6A and B induced endoplasmic reticulum (ER) stress and activated the unfolded protein response (UPR) during their replication in T cell lines as well as in PBMCs (Romeo et al., 2019). Autophagy and UPR are two strongly inter-connected responses to stress, as autophagy represents a route for protein degradation (Cai et al., 2016) and therefore may help cells to cope with ER stress. UPR is initiated by the activation of three major ER sensors, namely IRE1 alpha, ATF6 and PERK that promote the adaption to stress by reducing protein translation, activating the antioxidant response and up-regulating chaperones such as BIP. However, depending on the duration and on the intensity of ER stress, the activation of UPR may induce the expression of pro-death molecules such as CHOP, that mainly occurs through the PERK-eIF2alpha-ATF4 axis (Oakes and Papa, 2015). ER stress and UPR activation may dysregulate immune response, since it has been reported that ER stressor tunicamycin may impair the differentiation process of THP1 monocytoid cells into macrophages (Komura et al., 2013) or that the increases activation of PERK and IRE1alpha or CHOP up-regulation may occur in M2 alternatively activated macrophages (Oh et al., 2012). Based on these evidences, in this study we investigated whether HHV-6B derived from exanthema subitum patients could infect primary monocytes and impair monocyte survival and differentiation by dysregulating autophagy and activating ER stress/ UPR.

2. Materials and methods

2.1. Monocytes isolation, infection and treatments

Monocytes isolated from human peripheral blood mononuclear cells (PBMCs) of healthy donors as previously described (Gilardini Montani et al., 2018a) infected with HHV-6B derived from exanthema subitum-patient sera for 2 h at 37 °C and cultured at a density of 2×10^6 cells/mL in 12-well plates in RPMI 1640 containing 10% Fetal Bovine Serum (FBS) (Corning), glutamine (300 mg/ml), 100 U/ml penicillin G and 100 mg/ml streptomycin. As mock-infected control, monocytes were infected with filtered 0,22 µm serum derived from exanthema subitum-affected patient. To quantify virus DNA extraction was carried out from plasma samples by Nuclisens EasyMag instrument (BioMerieux S.p.A.) according to manufacturer instructions. Extracted samples were then analyzed for the presence of HHV6-DNA by quantitative TaqMan real-time 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). Differentiation into dendritic cells was performed by adding every two days, 50 ng/ml GM-CSF (Miltenyi Biotec,) and 25 ng/ml IL-4 (Miltenyi Biotec,) (differentiation cocktail). In some experiments, monocytes were pre-treated with sodium 4-phenylbutirate (4-PBA, PBA) (4,8 mM) for 2 h (Sigma-Aldrich), infected with serum derived from exanthema subitum-affected patient and then cultured with the differentiation cocktail for additional 48 h. The experiments were undertaken with the understanding and written consent of each subject.

2.2. Cell viability

Cell viability was evaluated by a trypan blue (Sigma Aldrich) exclusion assay after 72 h of culture. Cells were counted by light microscopy using a Neubauer emocytometer. The experiments were performed in triplicate and repeated at least three times.

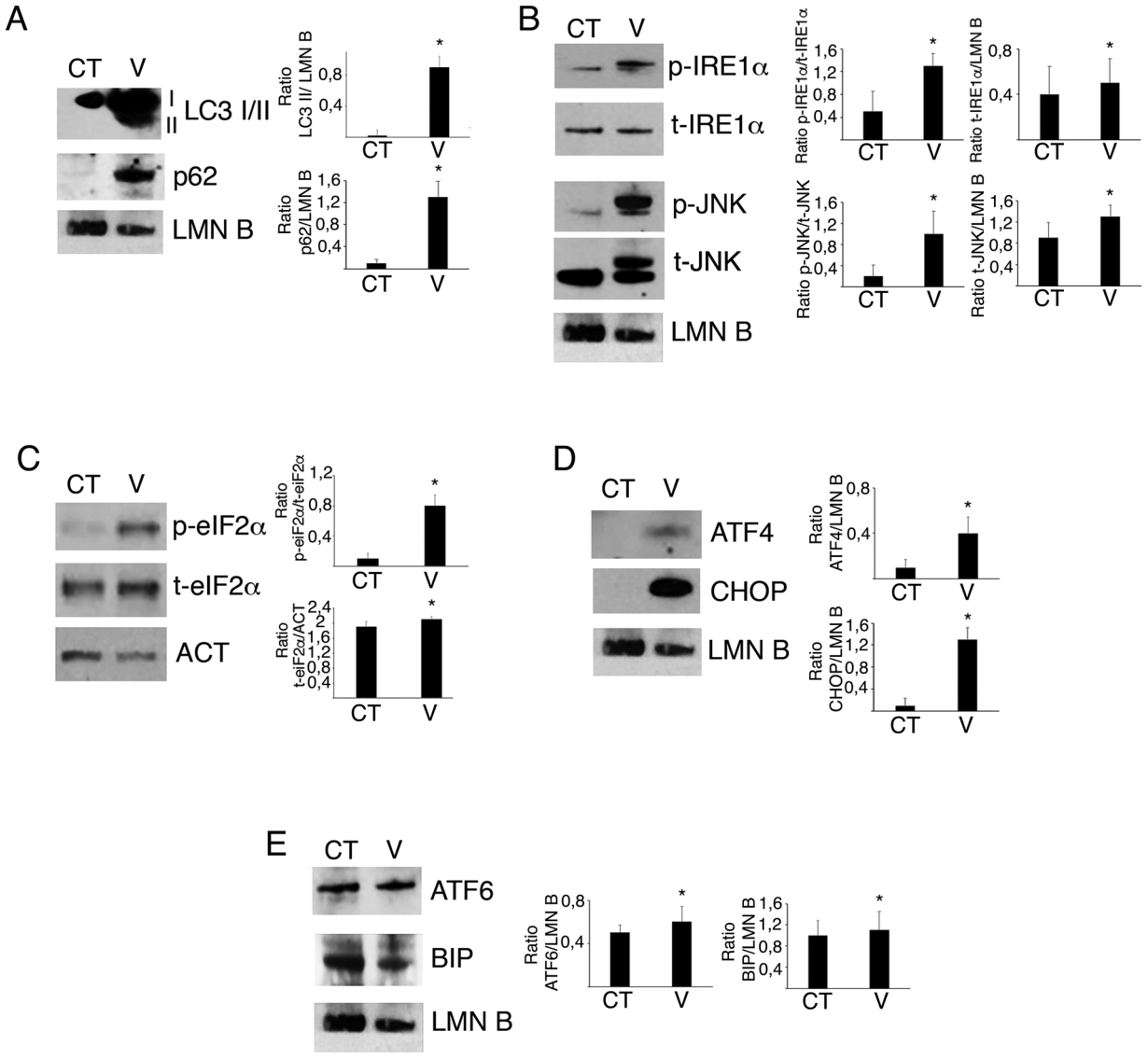

Fig. 2. HHV-6B derived from exanthema subitum patients reduces autophagy and activates ER stress/UPR in primary monocytes. By western blot analysis the expression of A) LC3 I/II and p62 B) phospho- and total IRE1 alpha, phospho- and total JNK1/2 C) phospho- and total eIF2 alpha D) ATF4, CHOP and E) ATF6 and BIP was evaluated in mock- and serum-(derived from exanthema-subitum affected patients) infected differentiating monocytes. Lamin B (LMNB) and Actin (ACT) were used as loading control; Histograms represent the mean plus SD of densitometric analysis of specific proteins to the loading control of three different experiments. Mock-infected monocytes (CT), HHV-6B-infected monocytes (V).

2.3. MTT assay

Cell viability was also evaluated by monotetrazolium (MTT) assay (Roche Diagnostics GmbH). 2×10^5 monocytes mock-infected and infected with HHV-6B derived from exanthema subitum-patient sera were seeded in 96-well plates in 100 µl growth medium with the differentiation cocktail. After 24, 48 and 72 h of culture, 10 µl of 5 mg/ml MTT in 1X PBS was added to each well for 4 h at 37 °C. After that period, 100 µl of solubilization solution was added to dissolve formazan crystals. After O.N. incubation in the incubator in a humidified atmosphere, the optical densities were determined at 590 nm using as microplate reader (BIO-RAD Microplate Reader). The experiments were performed in triplicate. The absorbance values were normalized to those of the vehicle-treated cells. In some experiments, monocytes were pre-treated with sodium 4-phenylbutirate (4-PBA, PBA) (4,8 mM) for 2 h (Sigma-Aldrich) before infection with serum derived from exanthema subitum-affected patient and then cultured with the differentiation cocktail for 72 h before the MTT procedure described.

2.4. FACS analysis

After 72 h, mock- infected, infected or treated monocytes were stained with anti CD14 (Miltenyi Biotec), anti CD1A (Miltenyi Biotec) and isotype control antibody (Miltenyi Biotec) and analyzed by EPICS XL Coulter (Hialeah, FL), FACSC Calibur, using CELLQuest (BD Biosciences) and FlowJo v9.3.2 (Treestar) softwares. For each analysis 5000 events were recorded.

2.5. Indirect immunofluorescence assay (IFA)

Monocytes infected with HHV-6B derived from exanthema subitum-patient were washed with PBS, applied onto multispot microscope slides and air-dried. Cells were then incubated with 2% paraformaldehyde (Electron Microscopy Science, 157-8) for 20 min and permeabilized with 0.1% Triton X-100 (SIGMA, T-8787) for 5 min. After 3 washes, cells were incubated with 1% glycine, 3% BSA for a further 30 min. Then cells were incubated with the primary monoclonal antibody against gp116/64/54 (1:200 in PBS) (kindly provided by HHV-6

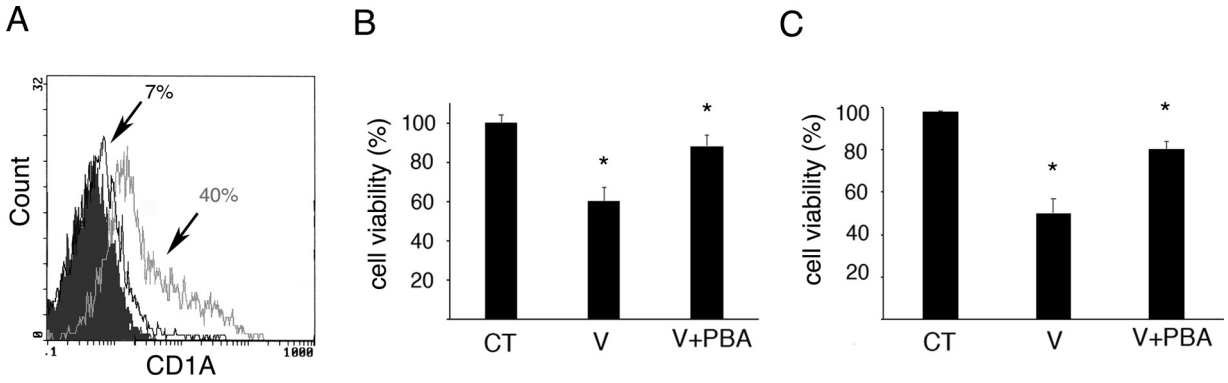

Fig. 3. ER stress plays a role in the reduction of monocyte differentiation and survival. Primary monocytes isolated from healthy donors were pretreated or not with PBA, infected with serum derived from exanthema-subitum affected patients and differentiated in DCs for 72 h. A) The expression of CD1A was assessed by FACS analysis in presence or absence of PBA. The filled black histogram represents isotype control Ab, the empty black histogram represents the serum-infected monocytes and the grey histogram represents the PBA-pretreated serum-infected monocytes. The percentage of positivity is reported. One representative experiment out of three is shown. B) Cell survival of mock- and infected differentiating monocytes pretreated or not with PBA was evaluated by trypan blue exclusion assay or C) by MTT assay. Histograms represent the mean plus SD of three independent experiments. * p value ≤ 0.05. Mock-infected monocytes (CT), HHV-6B-infected monocytes (V). sodium 4-phenylbutirate (PBA; 4-PBA).

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; 515-165-062) for 30 min at room temperature. After 3 washes in PBS, cells were stained with DAPI (SIGMA, D9542) for 1 min at room temperature. Slides were further washed in PBS, mounted with glycerol:PBS (1:1) and observed under a fluorescence microscope (Olympus BX53, USA).

2.6. Western blot analyses

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.

2.7. Antibodies

To evaluate the expression of viral proteins we used the following antibodies: mouse monoclonal anti-gp116/64/54 (1:300) (kindly provided by HHV-6 Foundantion), rabbit polyclonal anti-LC3 (1:1000) (Novus Biologicals), mouse monoclonal anti-p62 (1:500) (BD Transduction Laboratories), rabbit polyclonal anti-BiP (1:100) (Cell Signaling), mouse monoclonal anti-CHOP (1:100) (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-p-IRE1α (1:100) (Abcam), mouse monoclonal anti-IRE1α (1:100) (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-pJNK (p46 and p54) (1:300) (Cell Signaling), rabbit polyclonal anti-JNK (p46 and p54) (1:500) (Cell Signaling), mouse monoclonal anti-ATF6 (1:100) (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti p-eif2α (1:300) (Cell Signaling), rabbit polyclonal anti-eif2α (1:300) (Cell Signaling), mouse monoclonal anti-ATF4 (1:100) (Santa Cruz Biotechnology Inc.). Mouse monoclonal anti-β-actin (1:10,000) (Sigma Aldrich) and goat polyclonal Lamin B (Santa Cruz Biotechnology Inc.) were used as loading control. The goat anti-mouse IgG-Horseradish Peroxidase (Santa Cruz Biotechnology Inc.), goat anti-rabbit IgG- HRP (Santa Cruz Biotechnology Inc.), rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology Inc.) were used as secondary antibodies. All the primary and secondary antibodies were diluted in PBS-0.1% Tween20 solution containing 3% of BSA (SERVA).

2.8. 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-6B derived from exanthema subitum patients impairs monocyte differentiation into DC and reduces cell survival

Primary monocytes, isolated from healthy donors, were exposed to the sera derived from exanthema subitum-affected children containing at least 1200 copies of HHV-6 /ml or, as control, to the same sera 0.22 µM filtered to remove viral particles. The virus present in the sera was not able to infect HSB2 cells while it efficiently infected Molt3 cells, suggesting that HHV-6B and not HHV-6A was present in the sera (Ablashi et al., 2014; Romeo et al., 2019). After 72 h, monocyte infection was assessed by evaluating the expression of the lytic viral antigen complex gp116/64/54 by western blot analysis (Fig. 1A) and by IFA (Fig. 1B). We then investigated whether HHV-6B could alter monocyte differentiation into DCs, driven by GM-CSF and IL-4. According to previous findings (Niiya et al., 2006), we found that the virus derived from exanthema subitum patients induced a reduction monocyte differentiation into DCs, as the infected cells displayed lower expression of the dendritic cell marker CD1A while maintained the expression of CD14 monocyte marker (Fig. 1C). The reduction of monocyte differentiation was then confirmed by optical microscopy observations that evidenced more elongated cells (typical DC shape) in the control in comparison with virus-infected culture (Fig. 1D). We next evaluated whether, besides impairing monocyte differentiation, HHV-6B could reduce cell survival. The results shown in Fig. 1E indicate that the virus induced a time-dependent reduction of the number of live monocytes in comparison to the mock-controls, as evaluated by MTT assay.

3.2. HHV-6B derived from exanthema subitum patients reduces autophagy and induces ER stress/UPR activation in primary monocytes

Having previously shown that HHV-6B reduced autophagy in

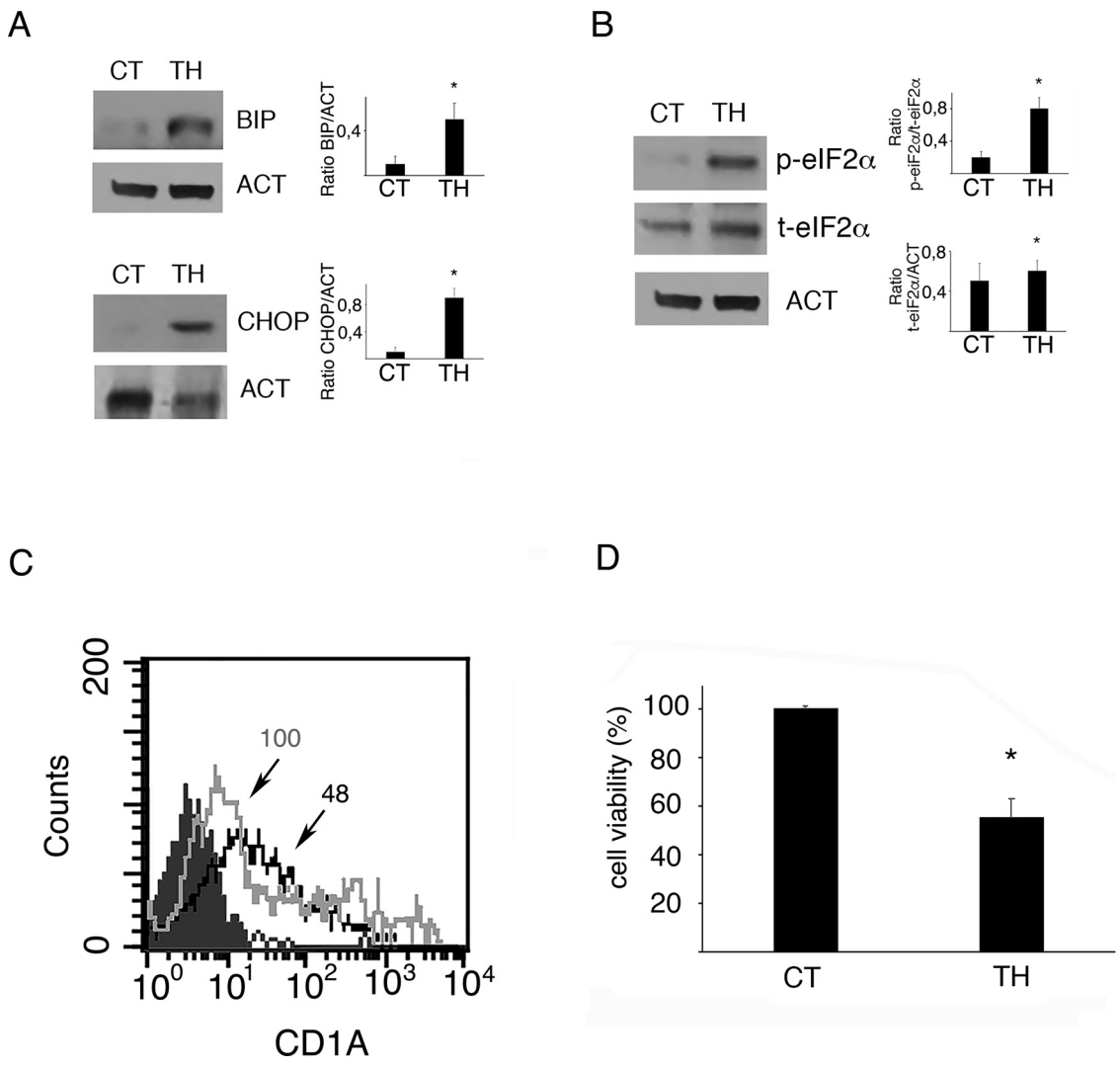

Fig. 4. Thapsigargin treatment reduces monocyte differentiation and survival. Primary monocyte isolated from healthy donors were treated with 25 nM of thapsigargin (TH), ER stressor and cultured in presence of GM-CSF and IL-4 for 72 h to induce DCs differentiation. The expression of A) BIP, CHOP, B) phospho- and total eIF2 alpha was evaluated by western blot. Actin (ACT) was used as loading control. Histograms represent the mean plus SD of densitometric analysis of three different experiments; C) the expression of CD1A was evaluated by FACS analysis in treated or mock differentiating monocytes. The filled black histogram represents isotype control Ab, the empty black histogram represents the TH-treated monocytes and the grey histogram represents the mock monocytes. The mean of fluorescence intensity is reported. One representative experiment out of three is shown. D) cells survival of TH-treated and mock differentiating monocytes was evaluated by trypan blue exclusion assay. Histograms represent the mean plus SD of three independent experiments. * p value ≤ 0.05. Mock monocytes (CT), Thapsigargin (TH).

MOLT-3 cells, we investigated if this effect could be induced also by the virus derived from exanthema subitum patients in infected primary monocytes. The results shown in Fig. 2A indicate that LC3II, the lipidated form of LC3 that is formed and degraded through autophagy increased, and that p62, a protein mainly degraded through autophagy, accumulated in infected cells, suggesting that the autophagic flux was reduced by viral infection. A strong inter-connection between autophagy and ER stress exists (Cai et al., 2016), as it is known that autophagy activation may represent a degradation route that alleviates ER stress. However, ER stress could be induced in virus-infected cells by the large number of proteins expressed during viral replication. Therefore, we next assessed the impact of HHV-6B infection on ER stress and UPR activation in primary differentiating monocytes. At this aim, the activation of the three sensors of the UPR, IRE1 alpha, PERK and ATF6 was investigated. As shown in Fig. 2B, an increased phosphorylation of IRE1 alpha UPR sensor was observed in HHV-6-infected cells and one of the molecules activated down-stream of it, JNK, was phosphorylated. JNK is a key molecule in regulating fundamental processes as autophagy and apoptosis (Dhanasekaran and Reddy, 2017). Next, we found that eIF2 alpha was also phosphorylated by HHV-6B (Fig. 2C), suggesting that the PERK UPR sensor was activated too. ATF4 and the pro-apoptotic molecule CHOP, mainly up-regulated downstream of PERK-eIF2 alpha axis, also increased in HHV-6B-infected differentiating monocytes (Fig. 2D) while ATF6 and the pro-survival molecule BIP were minimally affected by HHV-6B infection (Fig.2E). The preferential activation of CHOP could correlate with cell death induction in infected monocytes, as this molecule mainly mediates the pro-apoptotic functions of UPR (Szegezdi et al., 2006).

3.3. ER stress is involved in the impairment of monocyte differentiation and survival

To evaluate whether the induction of ER stress/UPR activation in differentiating monocytes by HHV-6B derived from exanthema subitum patients could contribute to the impairment of differentiation and survival, we used sodium 4-phenylbutirate (PBA), a chemical

chaperone that stabilizes the folded proteins and preventing protein aggregation leads to a reduction of ER stress (Kaur et al., 2018). As shown in Fig. 3A, this molecule partially restored DC formation, as it increased CD1A expression, suggesting that ER stress was involved the impairment of monocyte differentiation induced by HHV-6B. Furthermore, we observed that cell survival was partially restored by 4-PBA as evaluated with trypan blue assay (Fig. 3B) and MTT assay (Fig. 3C). These results indicate that ER stress played a role in the immune suppressive effects induced by this virus. To support the hypothesis that ER stress could alter monocyte differentiation and survival, we treated monocytes with the ER stressor Thapsigargin (TH) (Oslowski and Urano, 2011). The results shown in Fig. 4A and B indicated that Thapsigargin activated UPR in differentiating monocytes, as the expression of BIP and CHOP as well as eIF2 alpha phosphorylation increased. We then found that monocyte differentiation into DC was reduced by Thapsigargin, as CD1A expression was reduced (Fig. 4C) and that monocyte survival was also impaired (Fig. 4D). All together these results suggest that the induction of ER stress by HHV-6B derived from exanthema subitum patients played a role in the reduction of monocyte survival and differentiation into DCs.

4. Discussion

Previous studies have indicated that among other immune suppressive effects, HHV-6A and HHV-6B infection of monocytes reduced DC function (Niiya et al., 2006). We expanded this study and used HHV-6B derived from exanthema subitum patients to investigate the effect of viral infection on monocyte differentiation, also investigating the molecular mechanisms possibly involved in such effect. As suggested by the results obtained in this study, ER stress induction that correlated with the reduction of autophagy was involved in HHV-6B-mediated reduction of DC formation driven by GM-SCF and IL-4. This virus induced a selective activation of UPR, as it increased the phosphorylation of IRE1 alpha and eIF2 alpha and up-regulated the expression of ATF4 and CHOP, while did not affect ATF6 and BIP expression. Interestingly the PERK/ATF4/CHOP axis mainly activates the pro-death function of the UPR (Szegezdi et al., 2006) which may correlate with the reduction of monocyte survival also induced by virus infected. Moreover, the use of the chemical chaperone 4-PBA that reduces ER stress, partially restored cell survival and differentiation. However, besides the reduction of autophagy, ER stress in infected monocytes could be due to the expression of the large amount of viral proteins that are normally produced during viral infection or replication. Similar effects have been previously observed in Molt 3 T cell lines and PBMCs infected by HHV-6B (Romeo et al., 2019). Regarding the impact of ER stress/UPR on the differentiation and function of immune cells different and sometimes opposite findings have been reported. It has been reported that the activation of Xbp1, occurring downstream of IRE1 alpha is required for DC function (Iwakoshi et al., 2007; Osorio et al., 2014) but another study has also shown the activation of Xbp1 in DCs exposed to the oxidative stress of the tumor environment induced DC dysfunction (Cubillos-Ruiz et al., 2015). Since ER stress is often induced by viral infection (Zhang et al., 2012), it is possible that this represents a common mechanism through which viruses interfere with immune response, thus promoting their own survival and persistence in the infected host. Elucidating the mechanisms through which viruses induce immune dysfunction is of pivotal importance to find new strategies able to counteract such effect and to improve the outcome of antiviral treatments. The finding that HHV-6B dysregulates autophagy and induces ER stress/UPR in monocytes encourages to extend such investigations to evaluate whether these effects could be induced by HHV-6A/B also in microglia, CNS cells that in part derive from monocytes crossing the brain-blood barrier. This is important since HHV-6 has been linked to Alzheimer's Disease (AD) and the microglial cell infection could dysregulate autophagy and induce ER stress also in these cells, effects known to promote inflammation, beta amyloid accumulation and also protein tau phosphorylation, all hallmarks of AD.

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Author contributions

M.C. and A.F. planned experiments; M.A.R., M.S.G.M. and L.F. performed experiments; M.C., M.A.R. and M.S.G.M. analysed data; A.G. and C.N. contributed essential material and M.C. wrote the paper.

Declaration of Competing Interest

The authors declare no conflict of interest.

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