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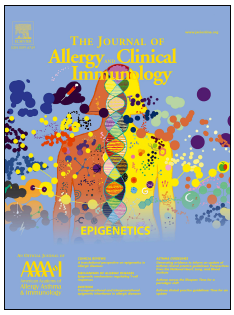

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Human TBK1 is required for early autophagy induction upon HSV1 infection

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To the Editor:

Mutations disrupting the Toll-like receptor 3 (TLR3)-dependent-interferon (IFN) pathway can underlie herpes simplex encephalitis (HSE) of childhood caused by herpes simplex virus-1 (HSV1) infection. These otherwise healthy HSE patients carry germline mutations in the TLR3-IFN circuit including *TRIF* and *TBK1*[1, 2]. Their dermal fibroblasts show impaired IFN production following HSV1 infection and poly(I:C) stimulation. A number of these genes (*TLR3, TRIF, TBK1*) have also been implicated in the process of autophagy. On the other hand, HSV1 is known to antagonize the antiviral IFN pathway and the autophagy machinery in part via TBK1. Specifically, TBK1 is targeted by the viral encoded proteins ICP34.5, ICP27, VP24 and UL46, compromising anti-viral IFN signalling[3, 4]. In the context of autophagy, TBK1 has been reported to phosphorylate autophagy receptors such as p62 to promote clearance of intracellular pathogens including HSV1 *in vitro*[5]. Herein, we study the role of autophagy in HSV1 infection using dermal fibroblasts from control and HSE patients with autosomal dominant (AD) TBK1 (p.G159A/WT) and autosomal recessive TRIF (p.R141X/R141X) deficiencies.

Despite showing normal autophagy activation after rapamycin and poly(I:C) stimulation, $TBK1^{+/-}$ fibroblasts showed no induction of autophagy following multiple stimuli: cyclic di-guanylate monophosphate (c-di-GMP), HSV1 60mer-dsDNA (60mer-dsDNA), and HSV1 infection. Following rapamycin, LC3B punctate signal increased by 3-fold in both control (media: 20.0%, rapamycin: 72.3%) and $TRIF^{-/-}$ (media: 21.8%, rapamycin: 70.0%) fibroblasts, and by 6-fold (media: 11.0%, rapamycin: 61.1%) in $TBK1^{+/-}$ fibroblasts, suggesting that TRIF and TBK1 were not required for rapamycin-induced autophagy (Fig 1, *A* and *B*). To assess autophagy induced via TLR3, poly(I:C) was used to stimulate fibroblasts leading to a 12-fold (media: 7.1%, poly(I:C): 86.5%) increase of LC3B puncta in control fibroblasts. $TRIF^{-/-}$ fibroblasts were unable to induce LC3B puncta, implicating TRIF in poly(I:C)-induced autophagy. $TBK1^{+/-}$ fibroblasts however showed a moderate 8-fold (media: 4.0%, poly(I:C): 31.3%) induction of autophagy suggesting its partial role in poly(I:C)-induced autophagy consistent with its partial impairment of poly(I:C)-induced IFN production (Fig 1, *A* and *C*)[2]. Although the role of dsRNA-TLR3 pathway in regulating autophagy has been documented in other cell lines, its involvement in infection remains elusive. In addition to TLR3-IFN signaling, TBK1 is also involved in the HSV1 DNA recognition pathway via STING-TBK1-IRF3 which serves to activate type I IFNs, and STING-dependent autophagy[6, 7]. To evaluate induction of autophagy via this pathway, fibroblasts were transfected with c-di-GMP and 60mer-dsDNA, known to stimulate STING-induced autophagy and IFN production[6, 7]. Whilst

mock treated fibroblasts did not show significant LC3B induction, we observed a 2-fold (c-di-GMP control: 20.7%, c-di-GMP: 45.2%) and 1.8-fold (c-di-GMP control: 18.5%, c-di-GMP: 33.6%) increase in punctate LC3B in control and TRIF$^{-/-}$ fibroblasts respectively when transfected with c-di-GMP, compared to the c-di-GMP control. Significant induction of LC3B puncta following 60mer-dsDNA transfection was also observed in control and TRIF$^{-/-}$ fibroblasts by 4-fold (media: 19.6%, 60mer-dsDNA: 84.8%) and 2-fold (media: 28.0%, 60mer-dsDNA: 67.6%) respectively. However, c-di-GMP and 60mer-dsDNA stimulation failed to induce LC3B puncta in TBK1$^{+/-}$ fibroblasts (Fig 1, *A*, *D* and *E*) suggesting TBK1 is essential for dsDNA-induced autophagy.

In control fibroblasts, HSV1 infection triggered a 10-fold (non-infected: 0.2 a.u., infected MOI 5: 1.8 a.u.) increase in LC3BII:I and a 2-fold (non-infected: 1.0 a.u., infected MOI 5: 0.5 a.u.) reduction in p62 protein indicating activation of autophagy, as assessed by western blot (Fig 1, *F*). TRIF$^{-/-}$ fibroblasts also showed a 6-fold (non-infected: 0.2 a.u., infected MOI 5: 1.2 a.u.) LC3BII:I increase following infection at MOI 5. TBK1$^{+/-}$ fibroblasts however showed no change in LC3BII:I or p62 following HSV1 infection suggesting impaired HSV1-induced autophagy. Depletion of endogenous TBK1 using siRNA in control fibroblasts recapitulated this impairment (Fig 1, *G*). Using immunofluorescence imaging, we found that HSV1 infection triggers two LC3B phenotypes in control fibroblasts: perinuclear LC3B puncta in infected cells and cytoplasmic LC3B puncta in antigen-negative-plaque-neighbouring ('antigen-negative') cells (Fig 2, *A*). Whilst the former occurs later in infection and is likely the phenomenon termed nuclear develop-derived autophagy (NEDA) as it also stained with LC3A[8] (Fig 2, *A*), cytoplasmic LC3B formed early in infection (up to 3 hours post-infection) (Fig 2, *B*). Strikingly, TBK1$^{+/-}$ fibroblasts failed to form cytoplasmic LC3B puncta in antigen-negative cells, despite being able to form perinuclear LC3B later in infection (Fig 2, *A* and *B*). Furthermore, inhibiting TBK1 in control fibroblasts using BX795 resulted in significant reduction in cytoplasmic LC3B formation (see Fig E1 in this article's Online Repository at www.jacionline.org). Whilst the lack of early autophagic induction was specific to TBK1$^{+/-}$ fibroblasts, TRIF$^{-/-}$ fibroblasts only showed delayed induction of autophagy (see Fig E2 in this article's Online Repository at www.jacionline.org), suggesting its partial involvement in HSV1-induced autophagy. The antigen-negative LC3B puncta has been previously reported in HSV1-infected mice trigeminal neurons but was shown to be cGAMP-independent and IFN-dependent[9]. In contrast to this, we find this phenomenon to be TBK1-dependent and IFNβ-independent since TRIF$^{-/-}$ fibroblasts, shown to have undetectable IRF3 phosphorylation and IFNs after HSV1 infection[1] (see Fig E3, *A* and E4 in this article's Online Repository at www.jacionline.org), were able to induce this phenotype. Furthermore, IFN treatment was able to induce

autophagy in TBK1$^{+/-}$ fibroblasts, ruling out the role of IFN in inducing cytoplasmic LC3B puncta in HSV1 infection (see Fig E5 in this article's Online Repository at www.jacionline.org). TBK1$^{+/-}$ fibroblasts also failed to reduce STING following HSV1 infection in contrast to control and TRIF$^{-/-}$ fibroblasts suggesting HSV1 induction is STING dependent (see Fig E3, *B*). We confirmed similar HSV1-induced autophagy phenotypes in primary fibroblasts from which these SV40-immortalized cell lines were derived from (see Fig E6 in this article's Online Repository at www.jacionline.org). These results show that the two types of autophagy differ in localization (cytoplasmic vs perinuclear) and temporal response to HSV1 infection, implying that they have different functions. We decided to focus on the TBK1-dependent early cytoplasmic phenotype as the later perinuclear LC3B, likely NEDA, was induced in all cells and has been reported to be a generalized stress response to viral late protein production[8].

We next sought to understand how the different triggers of autophagy affect HSV1 infection. Following pre-treatment with poly(I:C), HSV1 replication was significantly reduced in control fibroblasts which can be attributed to the production of IFNβ (Fig 2, *C* and *D*). Consistently, with a low dose of HSV1, no viral plaque was observed in control fibroblasts which exhibited cytoplasmic puncta in response to the poly(I:C) treatment (Fig 2, *C, D, E* and *G*). Interestingly, poly(I:C)-induced LC3B puncta in TBK1$^{+/-}$ fibroblasts was detectable following HSV1 infection. This pre-enhanced autophagy and IFNβ production in TBK1$^{+/-}$ fibroblasts however did not improve cell viability or viral replication in contrast to control fibroblasts (Fig 2, *C*-*G*). TRIF$^{-/-}$ fibroblasts failed to induce autophagy or IFNs following poly(I:C) treatment[1] and hence were not protected against HSV1 infection (Fig 2, *D*). Notably however, cytoplasmic LC3B puncta was present upon HSV1 infection of TRIF$^{-/-}$ fibroblasts, confirming that the formation of cytoplasmic LC3B puncta is IFN-independent (Fig 2, *C, D* and *G*, Fig E3, *A* and E4). Rapamycin pre-treatment led to the induction of autophagy in control, TRIF$^{-/-}$ and TBK1$^{+/-}$ fibroblasts as expected (Fig 2, *C* and *G*). In control and TRIF$^{-/-}$ fibroblasts, upregulating autophagy using rapamycin prior to HSV1 infection resulted in the same proportion of cytoplasmic LC3B puncta post-infection (non-treated infected vs rapamycin infected) (Fig 2, *C* and *G*). In contrast, rapamycin pre-treated TBK1$^{+/-}$ fibroblasts showed a 4-fold (non-treated infected: 8.7% vs rapamycin-infected: 35.5%) increase of cytoplasmic LC3B puncta in antigen-negative fibroblasts following HSV1 infection (Fig 2, *C* and *G*). Rapamycin pretreatment did not affect viral replication in all cells however it significantly improved cell viability of TBK1$^{+/-}$ cells (non-treated: 55.0% vs rapamycin treated: 76.7%) (Fig 2, *E*, *F* and *G*). Taken together, this shows that rapamycin-induced autophagy selectively increased the number of cytoplasmic LC3B, which

confers a cytoprotective effect by increasing cell viability in TBK1$^{+/-}$ fibroblasts. This protective effect of rapamycin could not be attributed to IFNβ as rapamycin did not induce IFNβ (Fig 2, *D*).

In conclusion, we show that in addition to its antiviral role in IFN production via TLR3 and STING[1, 2, 6], TBK1 induces autophagy upon HSV1 infection. We demonstrate that TBK1-induced autophagy occurs early during HSV1 infection in antigen-negative fibroblasts, can be mediated by c-di-GMP or HSV1 dsDNA and is TLR3- and IFN-independent. TBK1$^{+/-}$ fibroblasts derived from a HSE patient harboring a dominant negative mutation had a selective impairment of autophagy induction early in infection represented by the lack of cytoplasmic LC3B puncta formation. We believe that host or viral induced factors, possibly acting as danger signals, can trigger autophagy in antigen-negative fibroblasts promoting cell survival without influencing viral replication. This study highlights a possibly cytoprotective role for TBK1 in HSV1-induced autophagy which may serve to control inflammation and has potential implications for patients with HSE.

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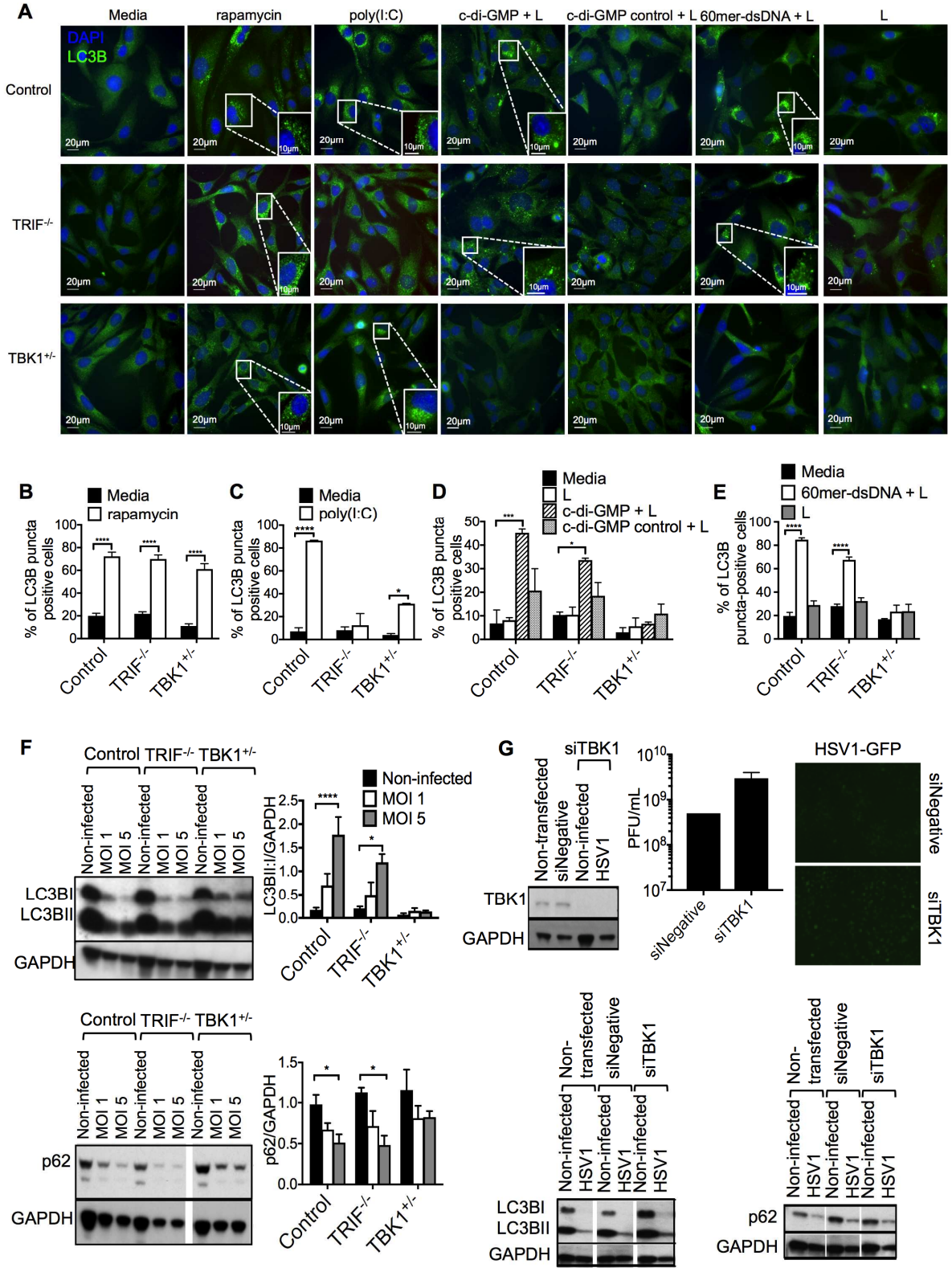
FIGURE LEGENDS

FIG 1. TBK1$^{+/-}$ fibroblasts show impaired cytosolic-dsDNA- and HSV1-induced autophagy. (A) Immunofluorescence images of LC3B puncta (green) and DAPI (blue) in fibroblasts. Quantification of LC3B puncta positive fibroblasts stimulated with **(B)** rapamycin, **(C)** poly(I:C), **(D)** cdi-GMP or cdi-GMP control, and **(E)** 60mer-dsDNA. **(F)** Immunoblots and densitometric graphs of HSV1-infected fibroblasts. **(G)** Immunoblot confirming TBK1 siRNA knockdown in control fibroblasts; immunoblots for LC3B, p62, GAPDH in HSV1-infected TBK1 knockdown fibroblasts. Viral titre and immunofluorescence images of infected TBK1 knockdown fibroblasts. L; Lipofectamine, (all experiments were performed at least three times; means ± SEM; ****P <0.0001, ***P <0.001 and *P <0.05).

FIG 2. TBK1$^{+/-}$ fibroblasts lack cytoplasmic LC3B puncta induced early in HSV1 infection. (A, B, C) Immunofluorescence images of fibroblasts stained for HSV1 ICP4 (red), LC3B or LC3A (green), and DAPI (blue). White arrows indicate cytoplasmic LC3B, yellow arrows indicate perinuclear LC3A/B. Dashed lines mark the plaque boundary. **(D)** IFNβ production, **(E)** viral titre, **(F)** cell viability and **(G)** cytoplasmic LC3B puncta positive fibroblasts were quantified. n.d., not-detectable, (n=3; means ± SEM; ****P <0.0001, ***P <0.001, **P <0.01 and *P <0.05).

FIGURE 1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poly(I:C)
c-di-GMP + L
c-di-GMP control + L
60mer-dsDNA + L
L
DAPI
LC3B
Control
TRIF-/-
TBK1+/-
20μm
10μm
B
Media
rapamycin
% of LC3B puncta positive cells
C
Media
poly(I:C)
D
Media
L
c-di-GMP + L
c-di-GMP control + L
E
Media
60mer-dsDNA + L
L
% of LC3B puncta-positive cells
F
Control TRIF-/- TBK1+/-
Non-infected
MOI 1
MOI 5
LC3BI
LC3BII
GAPDH
LC3BII/GAPDH
Non-infected
MOI 1
MOI 5
p62
p62/GAPDH
G
siTBK1
Non-transfected
siNegative
Non-infected
HSV1
TBK1
GAPDH
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HSV1-GFP
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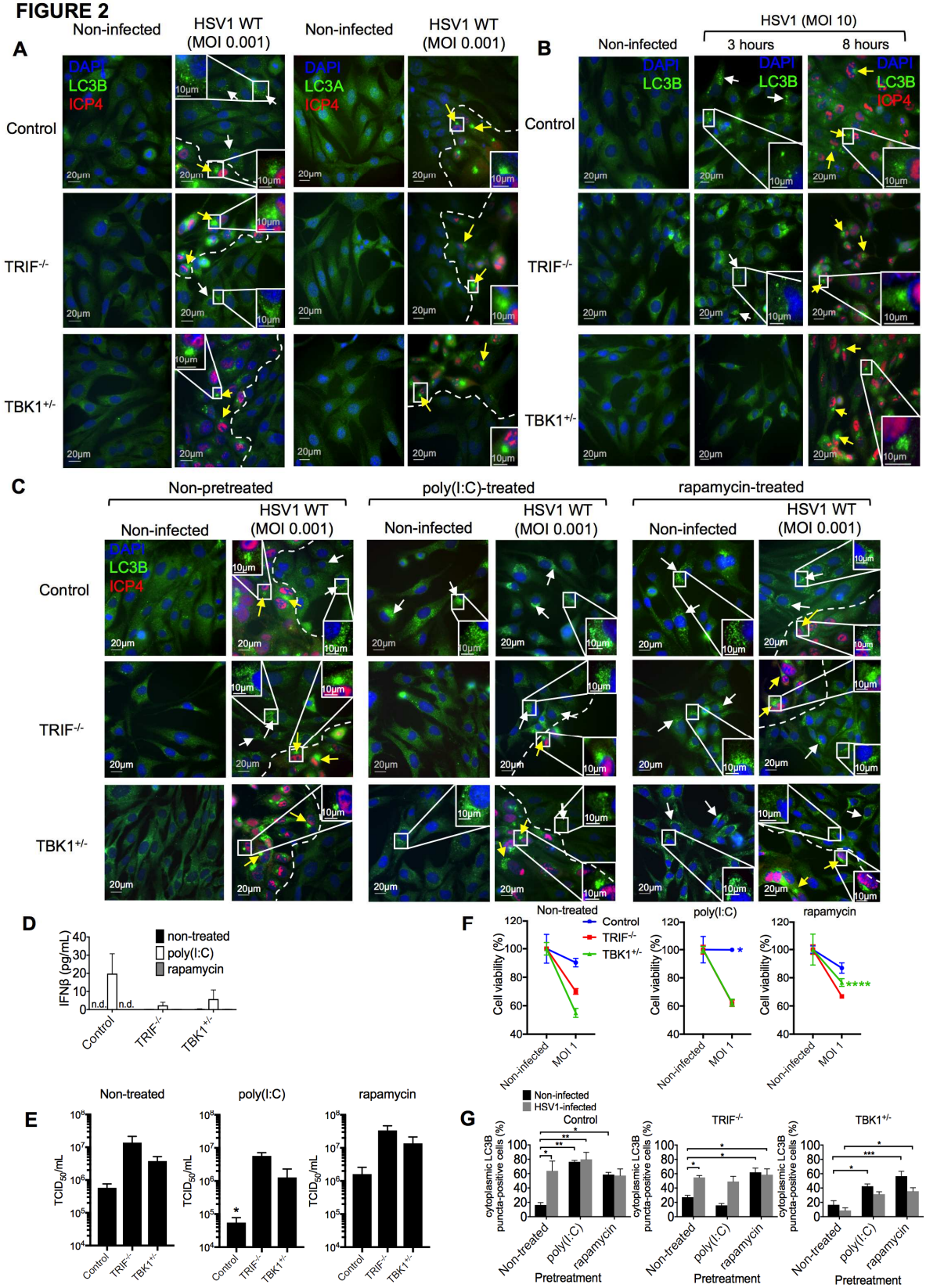
HSV1
LC3BI
LC3BII
GAPDH
p62

FIGURE 2


A
Non-infected
HSV1 WT (MOI 0.001)
Non-infected
HSV1 WT (MOI 0.001)
DAPI
LC3B
ICP4
LC3A
Control
TRIF-/-
TBK1+/-
20µm
10µm
B
HSV1 (MOI 10)
3 hours
8 hours
C
Non-pretreated
poly(I:C)-treated
rapamycin-treated
D
IFNβ (pg/mL)
non-treated
poly(I:C)
rapamycin
n.d.
E
Non-treated
poly(I:C)
rapamycin
TCID50/mL
F
Control
TRIF-/-
TBK1+/-
Cell viability (%)
Non-infected
MOI 1
G
Non-infected
HSV1-infected
cytoplasmic LC3B puncta-positive cells (%)
Pretreatment

Legends for Online Repository Figures

Fig E1 - TBK1 inhibition reduced cytoplasmic LC3B puncta formation but did not affect perinuclear LC3B formation following HSV1 infection (A) Control and TBK1$^{+/-}$ fibroblasts were pre-treated with 1 µM of BX795 for 16 hours before infecting with HSV1 (MOI 10) for indicated length of time, and being fixed and stained for LC3B (green) and/or ICP4 (red). DAPI (blue) was used the nuclear stain. The scale bar of each representative image is 20 µm. Inset represents the magnified view of the indicated area and has a scale bar of 10 µm. White arrows indicate cytoplasmic LC3B, while yellow arrows indicate perinuclear LC3B. **(B)** The percentage of cells positive for cytoplasmic LC3B puncta in (A) was counted on a minimum number of >100 cells. Images are representative of three independent experiments (n=3). Data are represented as mean ± SEM and were analysed by two-way ANOVA; $^{**}P<0.01$.

Fig E2 - TRIF$^{-/-}$ fibroblasts showed delayed cytoplasmic LC3B puncta formation – (A) Fibroblasts grown on coverslips were infected with HSV1 (MOI 10) for indicated lengths of time before being fixed and stained for endogenous LC3B (green) and/or HSV1 ICP4 (red) proteins. DAPI (blue) was used as the nuclear stain. The scale bar of each representative image is 20 µm. Inset represents the magnified view of the indicated area and has a scale bar of 10 µm. White arrows indicate cytoplasmic LC3B, while yellow arrows indicate perinuclear LC3B. **(B)** The percentage of cells positive for cytoplasmic LC3B puncta in (A) was counted on a minimum number of >100 cells. Images are representative of three independent experiments (n=3). Data are represented as mean ± SEM and were analysed by two-way ANOVA; $^{***}P<0.001$ and $^{****}P<0.0001$.

Fig E3 – Endogenous protein levels of TBK1, IRF3, phosphorylated IRF3 and STING during HSV1 infection. Control, TRIF$^{-/-}$ and TBK1$^{+/-}$ fibroblasts were infected with HSV1 (MOI 1) for indicated lengths of time. Whole-cell lysates were electrophoresed and probed

for endogenous (A) TBK1, IRF3 phosphorylated IRF3 and (B) STING proteins. GAPDH was used a loading control. Relative level of STING to GAPDH was measured by densitometry.

Fig E4 – TRIF$^{-/-}$ and TBK1$^{+/-}$ fibroblasts showed impaired IFNβ production following HSV1 infection. Control, TRIF$^{-/-}$ and TBK1$^{+/-}$ fibroblasts were infected with HSV1 at indicated MOIs for 24 hours before collecting supernatants and measuring IFNβ by ELISA. Data are represented as mean ± SEM and were analysed by two-way ANOVA; n=3; **P*<0.05 and *****P*<0.0001.

Fig E5 - IFN-induced autophagy in fibroblasts (A) Control, TRIF$^{-/-}$ and TBK1$^{+/-}$ fibroblasts were stimulated with 1X10^5 IU/mL of IFNα-2A for 24 hours before being fixed and stained for endogenous LC3B (green). DAPI (blue) was used as the nuclear stain. The scale bar of each representative image is 20 µm. Inset represents the magnified view of the indicated area and has a scale bar of 10 µm. White arrows indicate LC3B puncta. Images are representative of three independent experiments (n=3)

Figure E6 - Primary fibroblasts showed similar HSV1-induced autophagy phenotypes to SV40-transformed fibroblasts. Primary fibroblasts grown on coverslips were infected with HSV1 (MOI 10) for 3 or 8 hours before being fixed and stained for endogenous LC3B (green) and/or HSV1 ICP4 (red) proteins. DAPI (blue) was used as the nuclear stain. The scale bar of each representative image is 20 µm. Inset represents the magnified view of the indicated area and has a scale bar of 10 µm. White arrows indicate cytoplasmic LC3B, while yellow arrows indicate perinuclear LC3B. Images are representative of three independent experiments (n=3).

METHODS

Cell lines

Human SV40-immortalized dermal fibroblasts from healthy control, TBK1$^{+/-}$ (p.G159A), TRIF$^{-/-}$ patients[E1, E2], and Vero (African green monkey kidney) cells were maintained in 5% CO_2 incubator at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS).

Viral infection and quantification

Human fibroblasts were infected with HSV1-GFP (KOS strain with GFP-tagged capsid protein VP26) or HSV1 (strain 17AR+) at various MOIs and timepoints for immunoblot and immunofluorescence experiments. After 1 hour infection in DMEM supplemented with 2% FBS, the virus was removed and new media added with 1% HSV1 human neutralizing antibody. Viral titres were determined by infecting a confluent monolayer of Vero cells in a 12-well or 96-well plate, and performing plaque assay on them or calculating the 50% end point ($TCID_{50}$/mL)[E3].

Stimulation and treatments

Fibroblasts cells were stimulated with 25 µg/mL of poly(I:C) (GE Healthcare), 10 nM of rapamycin (Calbiochem) or 1X10^{5} IU/mL of IFNα-2A (PBL Assay Science) for 24 hours, or treated with 1µM of TBK1 inhibitor BX795[E4] (Sigma) for 16 hours, or transfected with 8 µg/mL of c-di-GMP (Invivogen) or c-di-GMP control (Invivogen) for 2 hours, or 2 µg/mL of HSV1 dsDNA (60mer sequence: 5′-TAAGACACGATGCGATAAAATCTGTTTGTAAAATTTATTAAGGGTACAAATTGCCCTAGC-3′; Integrated DNA Technology) for 3 hours. C-di-GMP, c-di-GMP control and HSV1 dsDNA were delivered by Lipofectamine® 2000 (L) transfection. For pre-treatment experiments, fibroblast were incubated with either rapamycin or poly(I:C) for 16 hours before infecting with HSV1.

Cell viability assay

Cells were plated in a flat-bottomed 96-well plate in triplicates at a density of 0.18X10^{6} cells/mL in 10% FBS-supplemented DMEM. Fibroblasts were pre-treated for 16 hours before being infected with HSV1 MOI of 1 for 24 hours. The viability of fibroblasts was measured using CellTiter 96® AQueous

Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega) and performed as per manufacturer's instructions. Cell viability was determined by normalizing to non-infected cells of each cell line.

RNA interference

Cells were seeded in 10% FBS-supplemeted DMEM at $0.05X10^6$ cells/well in a flat-bottomed 24-well plate and incubated for 18 hours in 37°C humidified incubator. Medium was then replaced with fresh 2% FBS-supplemented DMEM and cells were transfected with 15 nM of scramble (siNegative) (Ambion) or pooled three TBK1-specific (siTBK1) small interfering RNAs (siRNAs) (siRNA ID no. 134003, 134002 & 899, Ambion) at 80% confluency using Lipofectamine® RNAiMAX vector (Life Technologies) and incubated for further 48 hours in 37 ^{o}C incubator.

Immunofluorescence and quantification of puncta-positive cells

Cells were grown at 50% confluency on 13 mm diameter coverslips and fixed with 100% methanol on ice, before washing them with 1X phosphate buffered saline (PBS). Blocking was done in 10%-FBS PBS and cells were permeabilized with 0.1% TritonX-100 in PBS. Staining was done in a moist chamber using the following antibodies: LC3B (1:500 dilution, Abcam), cleaved LC3A (1:100 dilution, Stratech), HSV1 immediate early protein ICP4 (clone 10F1) (1:500 dilution, Virusys), Alexa Fluor-488 and -594 conjugated secondary antibodies (1:750 and 1:1000 dilutions respectively, Life Technologies). Images were taken with 63X oil-immersion lens on a widefield fluorescence microscope (Zeiss Axio Observer). The number of LC3B puncta-positive cells was quantified on a minimum number of 100 cells per experiment.

Immunoblotting

Whole-cell lysates of fibroblasts infected with HSV1 for 48 hours were harvested in 1X Laemmli buffer supplemented with protease inhibitor cocktail (Roche), 10% β-mercaptoethanol and 1:1000 of benzonase nuclease (Sigma). Lysates were denatured and electrophoresed on 12% tris-glycine or 10% bis-tris gels (Biorad). Proteins were transferred onto PVDF membrane (Invitrogen), probed with primary and secondary horse-radish peroxidase (HRP)-conjugated antibodies (1:1000 and 1:10, 000 dilution respectively) and subsequently detected using enzyme-chemiluminescent reagents (GE Healthcare). For LC3B immunoblots, bafilomycin A1 (Sigma) was added to culture medium for 6

hours prior to lysis for immunoblotting to block LC3BII recycling to LC3BI. The following antibodies were used for immunoblotting: LC3B (Cell Signaling Technology), p62 (MBL International), TBK1 (Cell Signaling Technology), IRF3 (D83B9) (Cell Signaling Technology), IRF3-phospho (S396) (Cell Signaling Technology), STING (D2P2F) (Cell Signaling Technology), and GAPDH-HRP (Santa Cruz Biotech). GAPDH was used as a loading control.

ELISA

IFNβ secretion in recovered supernatants of cells pre-treated with rapamycin or poly(I:C) for 24 hours was measured using the VeriKine-HS^{TM} human IFNβ serum ELISA kit (assay range: 1.2 – 150 pg/mL) (PBL Assay Science) following the manufacturer's instructions.

Statistical analysis and software

Immunofluorescence images were analysed by Icy software. Densitometric analyses of immunoblots were carried out using ImageJ. Statistical significance was assessed by two-way ANOVA using Prism 7 (Graphpad) software. Number of fluorescent cells or LC3B puncta-positive cells was quantified using ImageJ software. All experiments were performed at least 3 times.

REFERENCES

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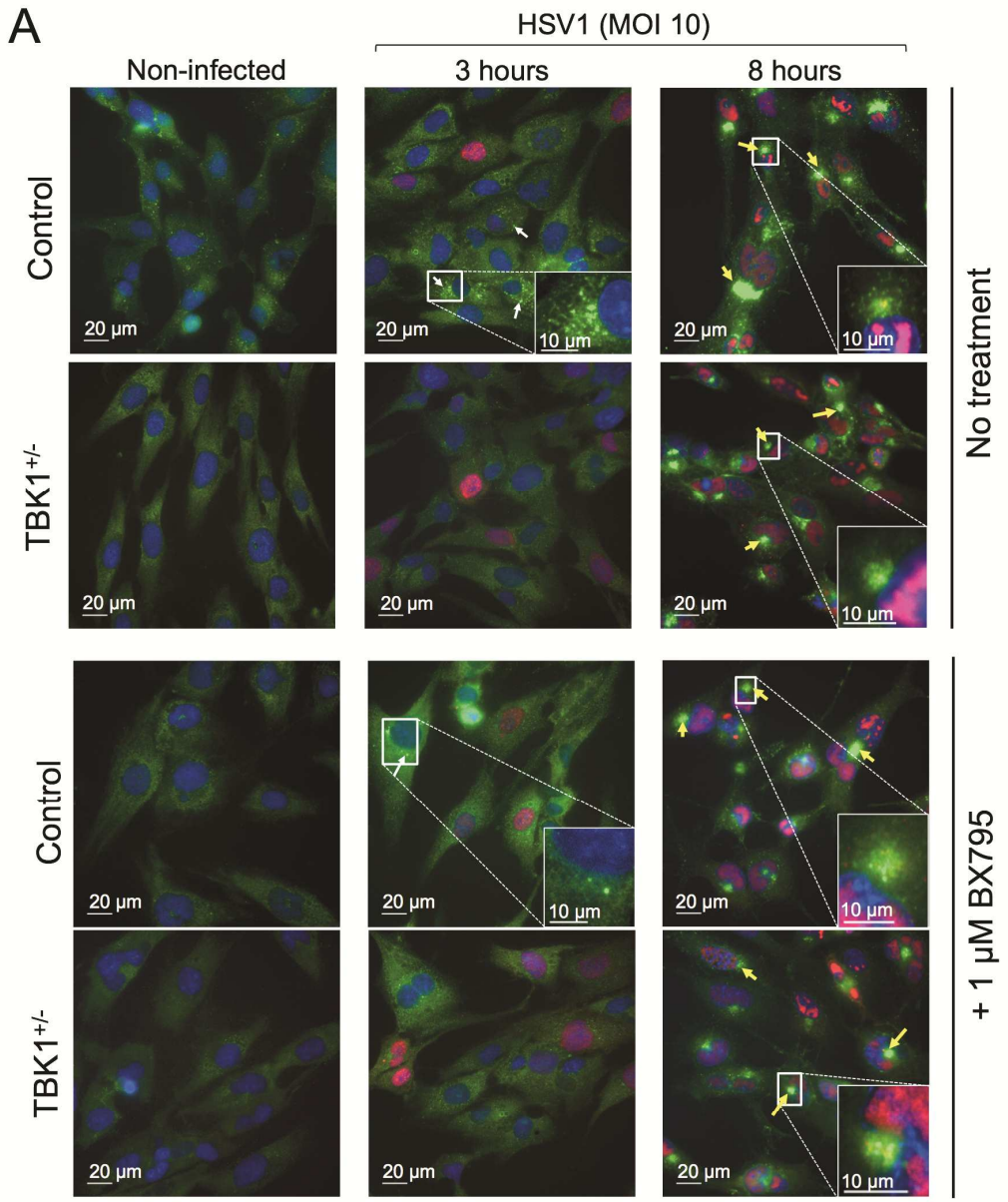
E3. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hyg. 1938;27:493–497.

E4. Clark K, Plater L, Peggie M, Cohen P. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. J Biol Chem. 2009;284:14136–46.

Fig E1


A
HSV1 (MOI 10)
Non-infected
3 hours
8 hours
Control
TBK1+/-
No treatment
+ 1 μM BX795
20 μm
10 μm

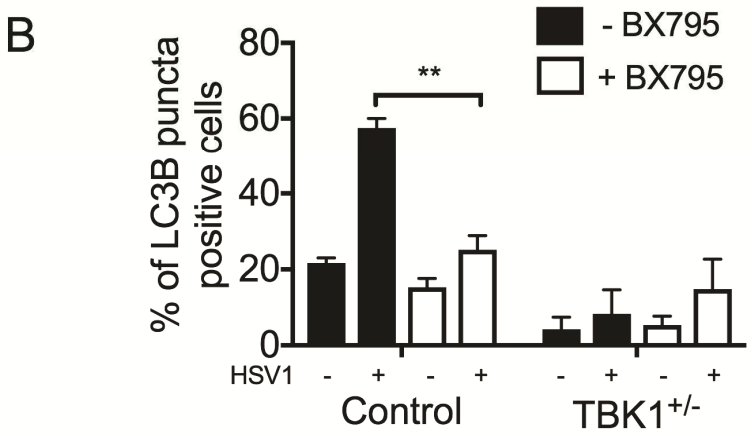

B
% of LC3B puncta positive cells
80
60
40
20
0
**
- BX795
+ BX795
HSV1
-
+
-
+
-
+
-
+
Control
TBK1+/-

Fig E2

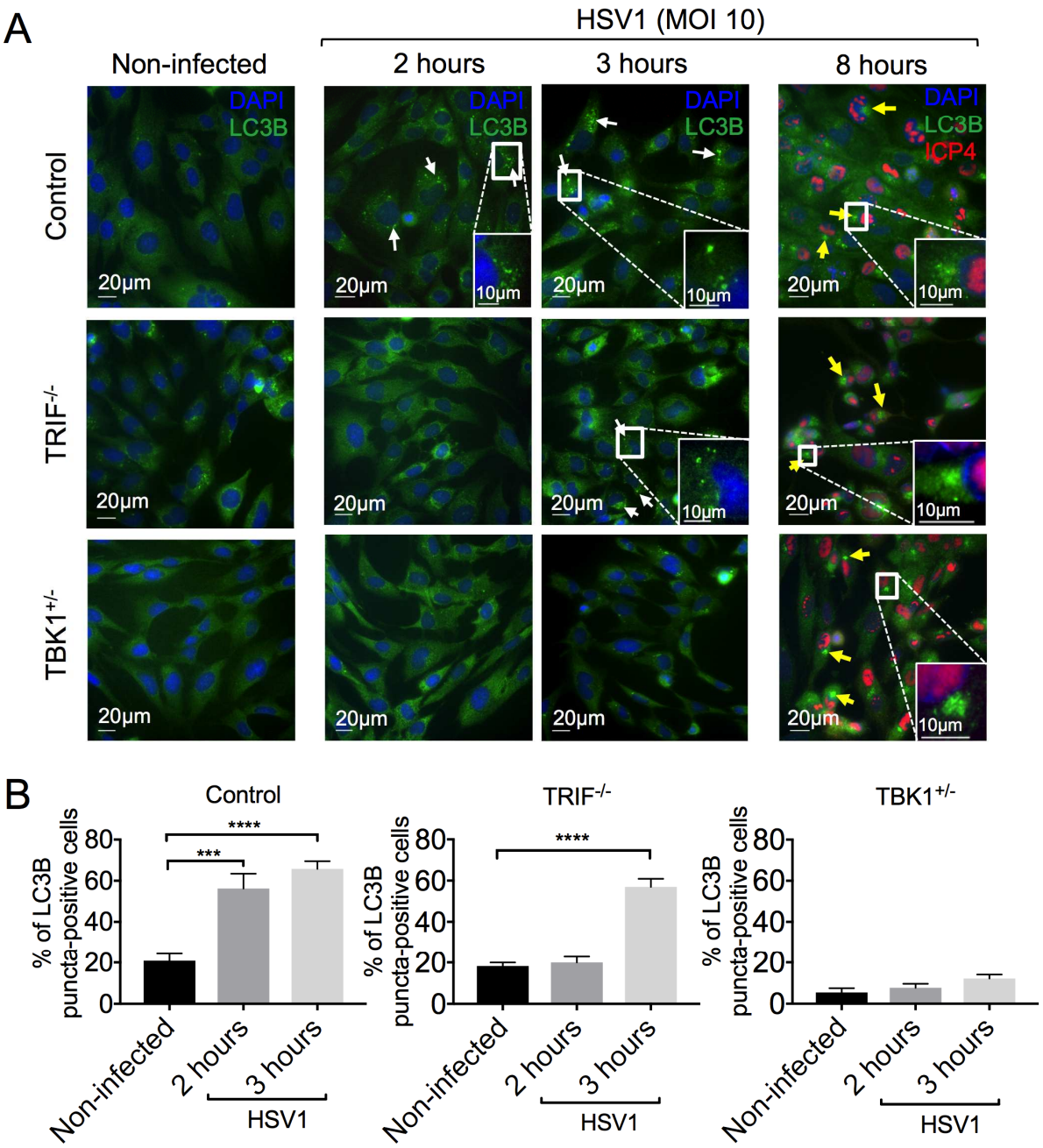

A
HSV1 (MOI 10)
Non-infected
2 hours
3 hours
8 hours
Control
TRIF-/-
TBK1+/-
DAPI
LC3B
ICP4
20μm
10μm
B
Control
TRIF-/-
TBK1+/-
% of LC3B puncta-positive cells
0
20
40
60
80
***
****
Non-infected
2 hours
3 hours
HSV1

Fig E3

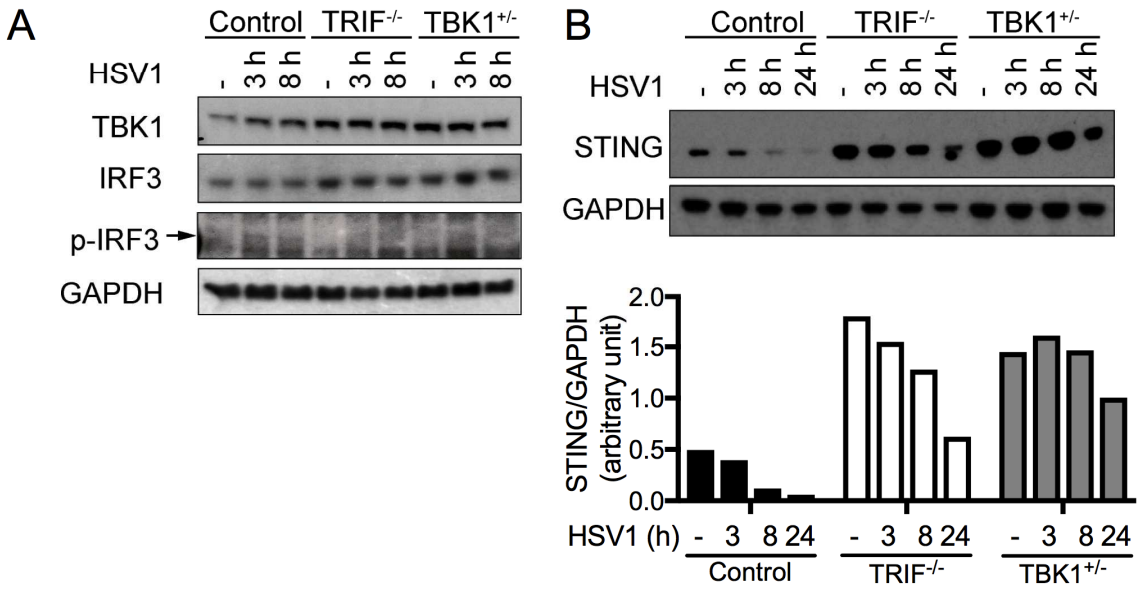


Fig E4

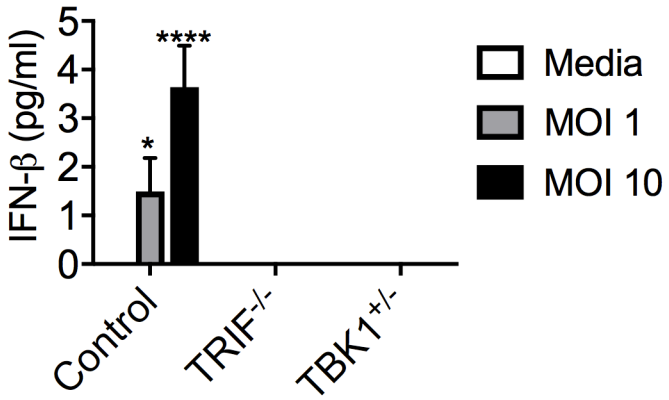


Fig E5

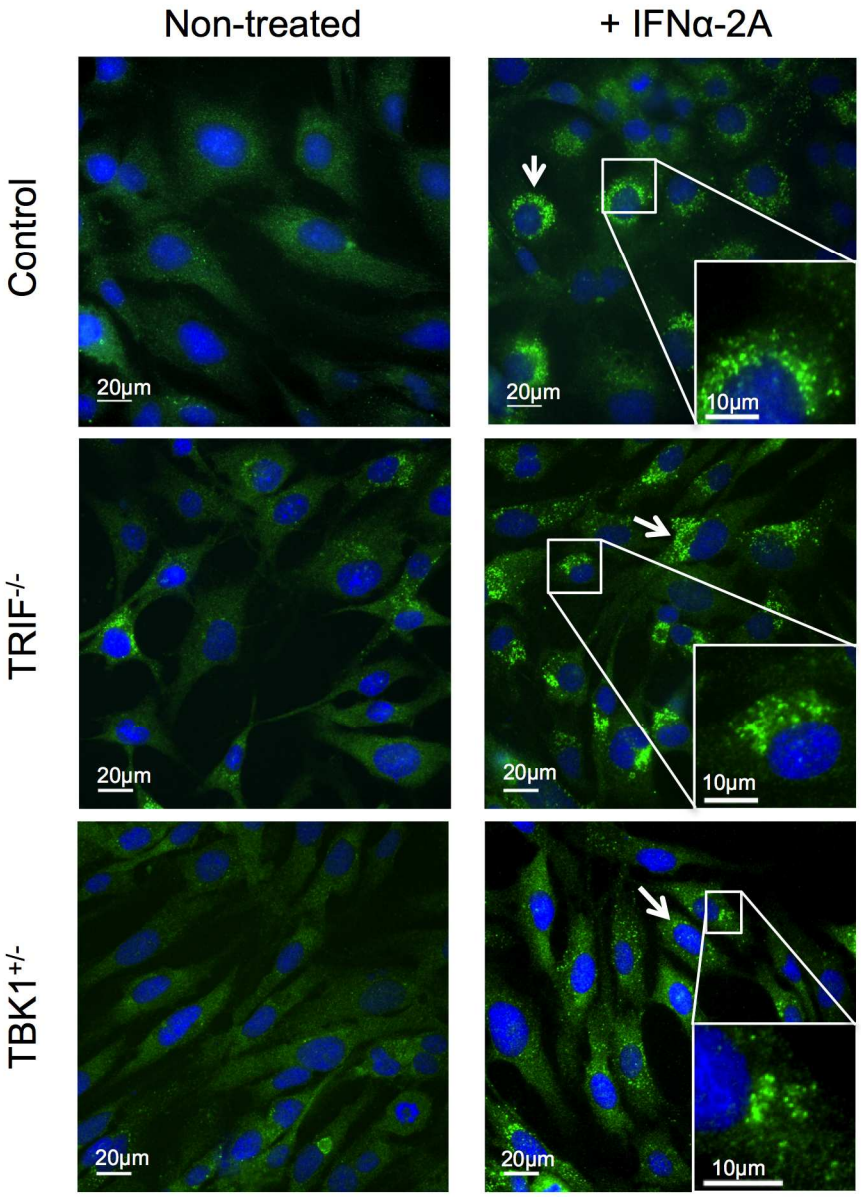
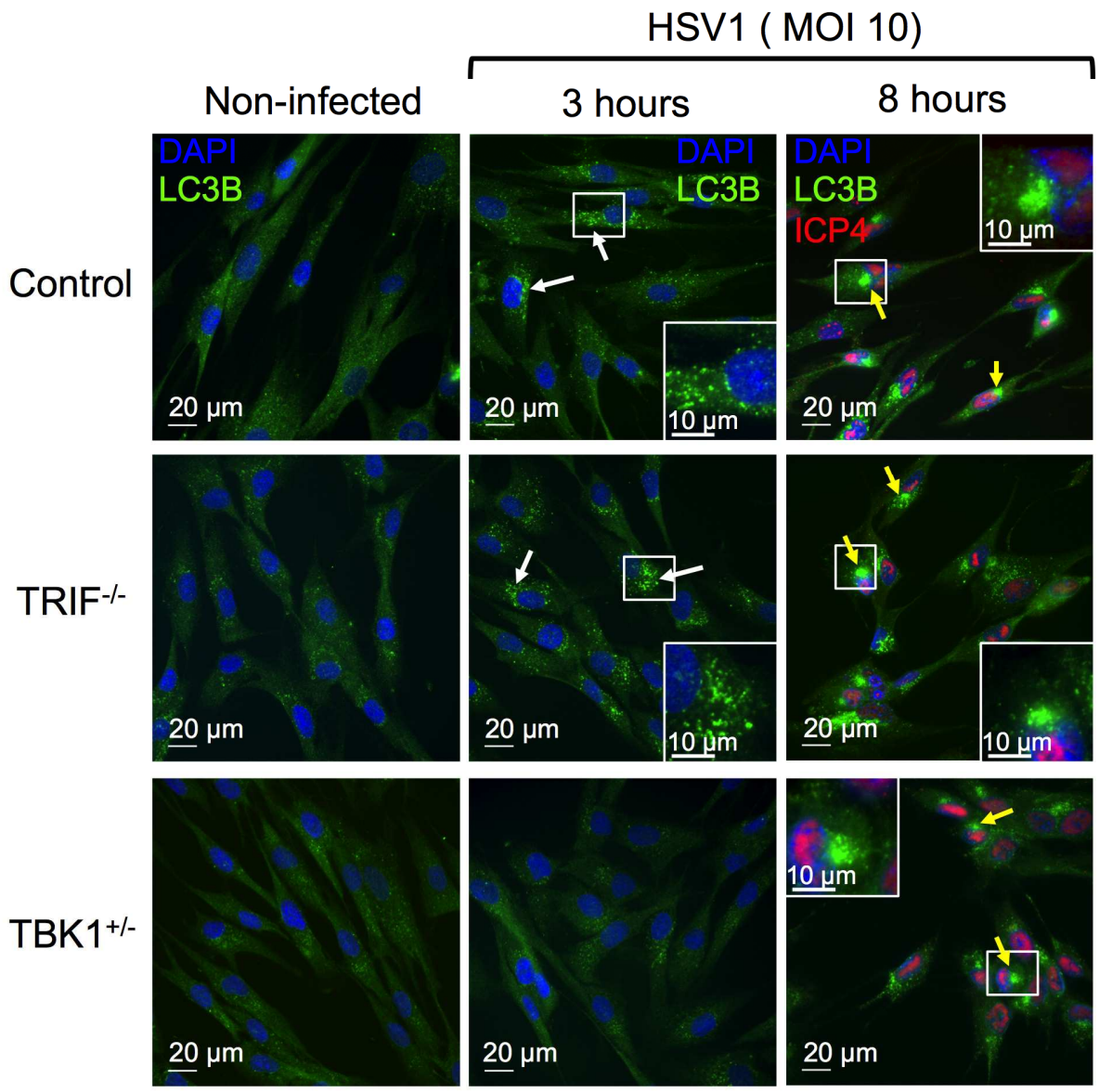


Fig E6


HSV1 (MOI 10)
Non-infected
3 hours
8 hours
Control
TRIF-/-
TBK1+/-
DAPI
LC3B
ICP4
20 µm
10 µm