

Human TANK-binding kinase 1 is required for early autophagy induction upon herpes simplex virus 1 infection



To the Editor:

Mutations disrupting the Toll-like receptor 3 (TLR3)–dependent–interferon pathway can underlie herpes simplex encephalitis (HSE) of childhood caused by herpes simplex virus 1 (HSV1) infection. These otherwise healthy patients with HSE carry germline mutations in the TLR3–interferon circuit, including TIR domain–containing adapter–inducing IFN- β (TRIF) and TANK-binding kinase 1 (TBK1).^{1,2} Their dermal fibroblasts show impaired interferon production after HSV1 infection and polyinosinic-polycytidylic acid (poly[I:C]) stimulation. A number of these genes (TLR3, TRIF, and TBK1) have also been implicated in the process of autophagy.

On the other hand, HSV1 is known to antagonize the antiviral interferon pathway and the autophagy machinery in part through TBK1. Specifically, TBK1 is targeted by the virus-encoded proteins ICP34.5, ICP27, VP24, and UL46, compromising antiviral interferon signaling.^{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

Here we study the role of autophagy in HSV1 infection using dermal fibroblasts from healthy controls and HSE patients with autosomal dominant 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 after multiple stimuli: cyclic diguanylate monophosphate (c-di-GMP), HSV1 60mer–double-stranded DNA (dsDNA; 60mer-dsDNA), and HSV1 infection. After rapamycin, LC3B (microtubule-associated protein 1 light chain-3B) 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 by means of TLR3, poly(I:C) was used to stimulate fibroblasts, leading to a 12-fold (media, 7.1%; poly[I:C], 86.5%) increase in LC3B puncta in control fibroblasts. TRIF^{-/-} fibroblasts were unable to induce LC3B puncta, implicating TRIF in poly(I:C)-induced autophagy. However, TBK1^{+/-} fibroblasts 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 interferon production (Fig 1, A and C).² Although the role of the dsRNA TLR3 pathway in regulating autophagy has been documented in other cell lines, its involvement in infection remains elusive. In addition to TLR3–interferon signaling, TBK1 is also involved in the HSV1

DNA recognition pathway through stimulator of interferon genes (STING)–TBK1–interferon regulatory factor 3 (IRF3), which serves to activate type I interferons, and STING-dependent autophagy.^{6,7}

Fibroblasts were transfected with c-di-GMP and 60mer-dsDNA, which are known to stimulate STING-induced autophagy and interferon production, to evaluate induction of autophagy through this pathway.^{6,7} Although 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 with the c-di-GMP control. Significant induction of LC3B puncta after 60mer-dsDNA transfection was also observed in control and TRIF^{-/-} fibroblasts by 3-fold (L, 29.0%; 60mer-dsDNA, 84.8%) and 2-fold (L, 32.4%; 60mer-dsDNA, 67.6%), respectively. However, c-di-GMP and 60mer-dsDNA stimulation did not 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 (noninfected, 0.2 AU; infected multiplicity of infection [MOI] 5, 1.8 AU) increase in LC3BII:I ratio and a 2-fold (noninfected, 1.0 AU; infected MOI 5, 0.5 AU) reduction in p62 protein, indicating activation of autophagy, as assessed by means of Western blotting (Fig 1, F). TRIF^{-/-} fibroblasts also showed a 6-fold (noninfected, 0.2 AU; infected MOI 5, 1.2 AU) LC3BII:I increase after infection at MOI 5. However, TBK1^{+/-} fibroblasts showed no change in LC3BII:I or p62 after HSV1 infection, suggesting impaired HSV1-induced autophagy. Depletion of endogenous TBK1 using small interfering RNA (siRNA) in control fibroblasts showed similar impairment of HSV1-induced autophagy (Fig 1, G).

Using immunofluorescence imaging, we found that HSV1 infection triggers 2 LC3B phenotypes in control fibroblasts: perinuclear LC3B puncta in infected cells and cytoplasmic LC3B puncta in antigen-negative plaque-neighboring (“antigen-negative”) cells (Fig 2, A). Although the former occurs later in infection and is likely the phenomenon termed nuclear envelope-derived autophagy because it also stained with LC3A (Fig 2, A),⁸ cytoplasmic LC3B formed early in infection (up to 3 hours after infection; Fig 2, B). Strikingly, TBK1^{+/-} fibroblasts did not 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 and this article’s Methods section in the Online Repository at www.jacionline.org). Although 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.

Antigen-negative LC3B puncta have been reported in trigeminal neurons of HSV1-infected mice but were shown to be cGAMP independent and interferon dependent.⁹ In contrast, we find this phenomenon to be TBK1 dependent and IFN- β

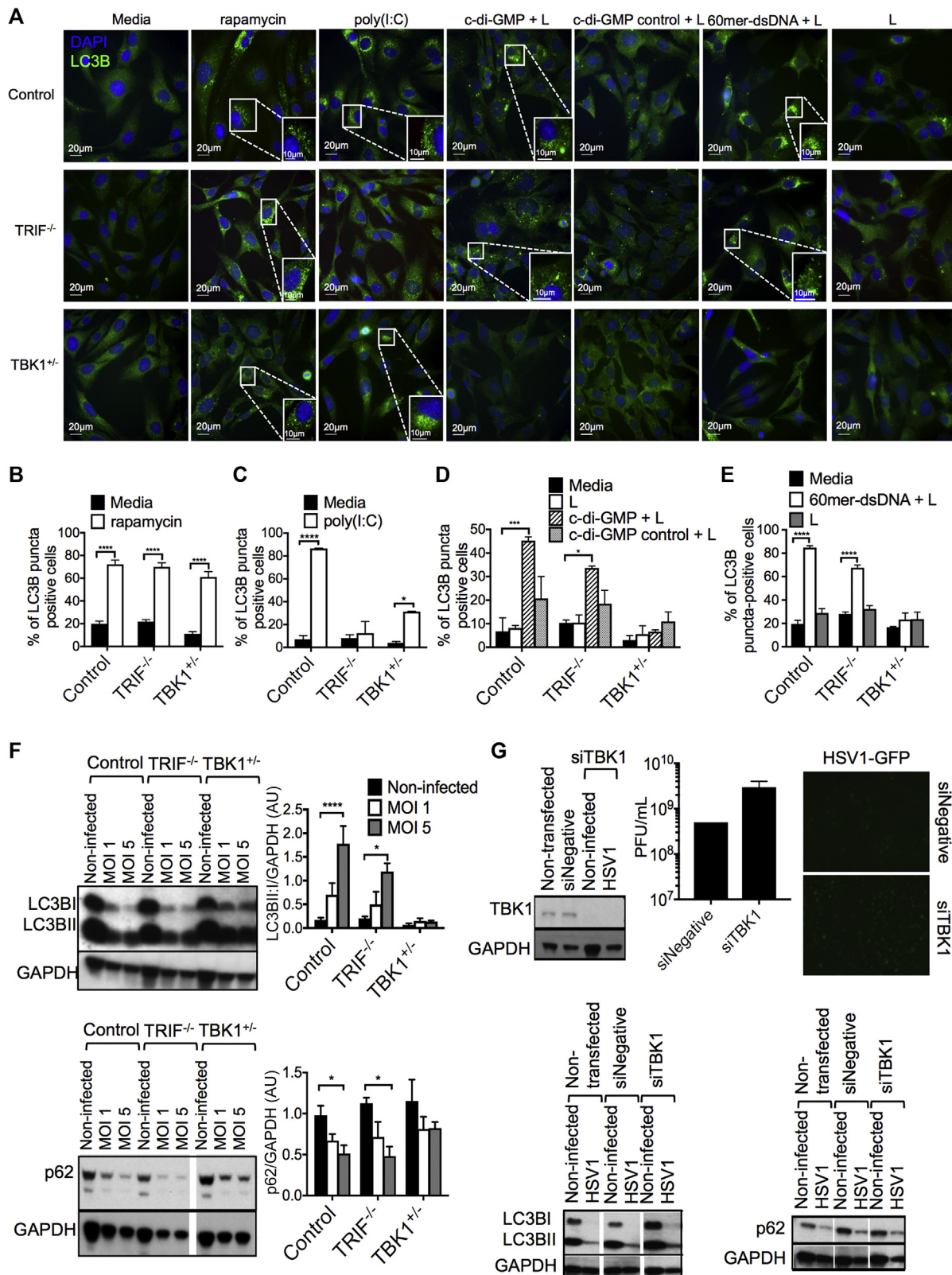


FIG 1. TBK1^{+/-} fibroblasts show impaired cytosolic dsDNA- and HSV1-induced autophagy. **A**, Immunofluorescence images of LC3B puncta (green) and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue) in fibroblasts. **B-E**, Quantification of LC3B puncta-positive fibroblasts stimulated with rapamycin (Fig 1, B), poly(I:C) (Fig 1, C), c-di-GMP or c-di-GMP control (Fig 1, D), and 60mer-dsDNA (Fig 1, E). **F**, Immunoblots and densitometric graphs of HSV1-infected fibroblasts. **G**, Immunoblot confirming TBK1 siRNA knockdown in control fibroblasts and immunoblots for LC3B, p62, and GAPDH in HSV1-infected TBK1 knockdown fibroblasts. Viral titer and immunofluorescence images of infected TBK1 knockdown fibroblasts. AU, Arbitrary units; GFP, green fluorescent protein; L, lipofectamine. All experiments were performed at least 3 times (means $\pm$ SEM). * $P < .05$, *** $P < .001$, and **** $P < .0001$.

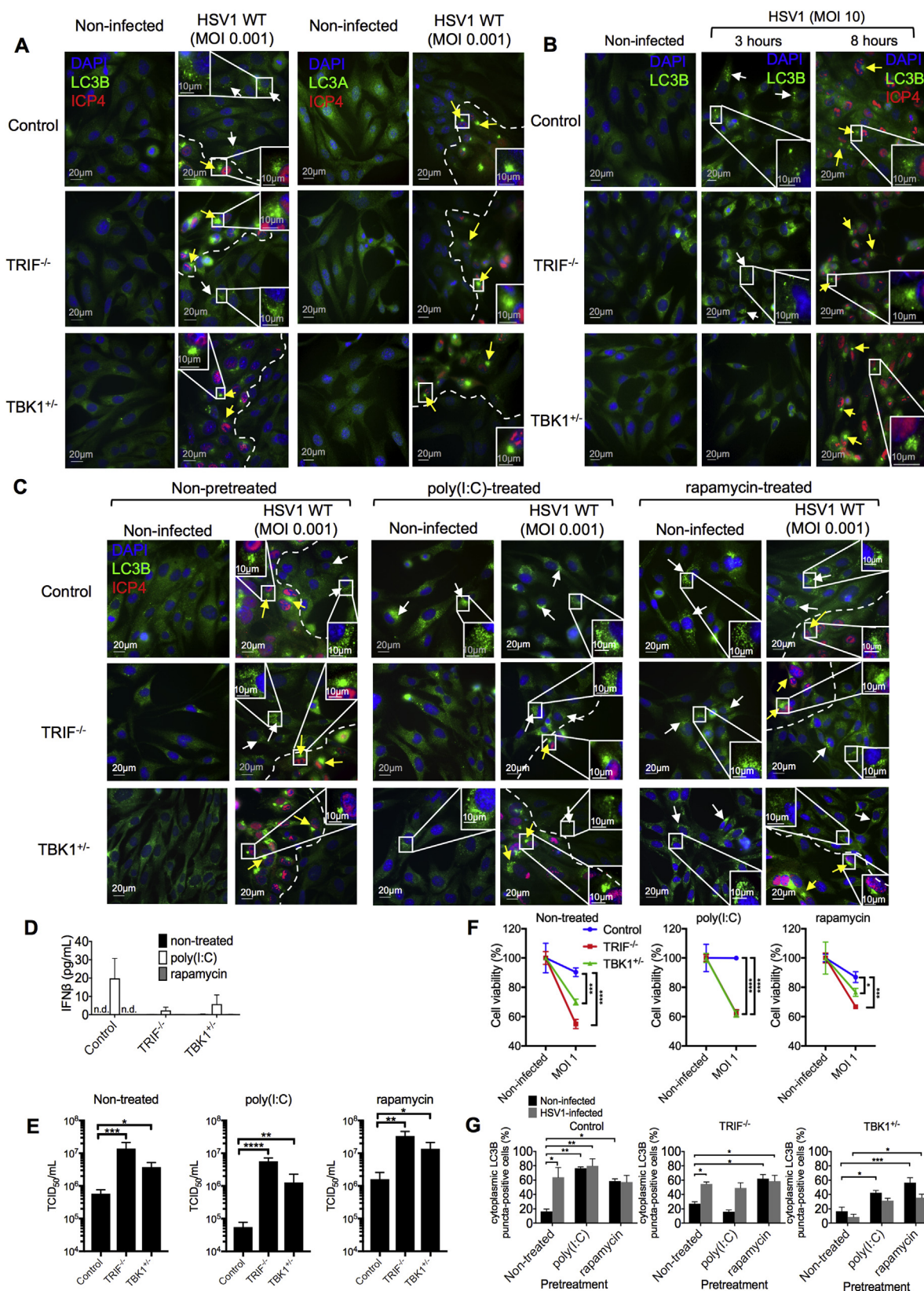


FIG 2. TBK1^{+/-} fibroblasts lack cytoplasmic LC3B puncta induced early in HSV1 infection. **A-C**, Immunofluorescence images of fibroblasts stained for HSV1 ICP4 (red), LC3B or LC3A (green), and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). White arrows indicate cytoplasmic LC3B, and yellow arrows indicate perinuclear LC3A/B. Dashed lines mark the plaque boundary. **D-G**, IFN- β production (Fig 2, D), viral titer (Fig 2, E), cell viability (Fig 2, F), and cytoplasmic LC3B puncta-positive fibroblasts (Fig 2, G) were quantified. *n.d.*, Not detectable. N = 3 (means $\pm$ SEMs). **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

independent because TRIF^{-/-} fibroblasts, shown to have undetectable IRF3 phosphorylation and interferons after HSV1 infection (see Figs E3, A, and E4 in this article's Online Repository at www.jacionline.org),¹ were able to induce this phenotype. Furthermore, interferon treatment was able to induce autophagy in TBK1^{+/-} fibroblasts, ruling out the role of interferon 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 levels after HSV1 infection in contrast to control and TRIF^{-/-} fibroblasts, suggesting autophagy induction by HSV1 may be 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 (see Fig E6 in this article's Online Repository at www.jacionline.org).

These results show that the 2 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 nuclear envelope-derived autophagy, 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. After pretreatment with poly(I:C), HSV1 replication was significantly reduced in control fibroblasts (nontreated: 5.8×10^5 , poly[I:C] treated: 5.5×10^4 , $P = .006$), which can be attributed to production of IFN- β (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-E and G). Interestingly, poly(I:C)-induced LC3B puncta in TBK1^{+/-} fibroblasts was detectable after HSV1 infection. However, this pre-enhanced autophagy and IFN- β production in TBK1^{+/-} fibroblasts did not improve cell viability or viral replication in contrast to control fibroblasts (Fig 2, C-G). TRIF^{-/-} fibroblasts did not induce autophagy or interferons after poly(I:C) treatment¹ and hence were not protected against HSV1 infection (Fig 2, D). Notably, however, cytoplasmic LC3B puncta was present following HSV1 infection of TRIF^{-/-} fibroblasts, confirming that formation of cytoplasmic LC3B puncta is interferon independent (Fig 2, C, D, and G, and see Fig E3, A, and E4).

Rapamycin pretreatment led to induction of autophagy in control, TRIF^{-/-}, and TBK1^{+/-} fibroblasts, as expected (Fig 2, C and G). In control and TRIF^{-/-} fibroblasts, upregulating autophagy with rapamycin before HSV1 infection resulted in the same proportion of cytoplasmic LC3B puncta after infection (nontreated infected vs rapamycin infected; Fig 2, C and G). In contrast, rapamycin-pretreated TBK1^{+/-} fibroblasts showed a 4-fold (nontreated infected: 8.7% vs rapamycin infected: 35.5%) increase of cytoplasmic LC3B puncta in antigen-negative fibroblasts after 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 (nontreated: 70.0% vs rapamycin treated: 76.7%, $P = .04$) and TRIF^{-/-} cells (nontreated: 55.0% vs rapamycin treated: 66.8%, $P = .003$; Fig 2, E-G). Taken together, this shows that rapamycin-induced autophagy selectively increased the level 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 IFN- β because rapamycin did not induce IFN- β (Fig 2, D).

In conclusion, we show that in addition to its antiviral role in interferon production through TLR3 and STING,^{1,2,6} TBK1 induces autophagy following 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 interferon independent. TBK1^{+/-} fibroblasts derived from a patient with HSE 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 virus-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 might serve to control inflammation and has potential implications for patients with HSE.

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Increased surface expression of NOTCH on memory T cells in peripheral blood from patients with asthma



To the Editor:

Asthma is a highly prevalent chronic inflammatory lung disease in humans and comprises a range of disease subtypes that share similar clinical characteristics, including episodes of bronchoconstriction, airway hyperreactivity, mucus hyperproduction, and airway inflammation.¹ Although many patients with asthma respond well to standard treatment with glucocorticoids and β_2 -agonists, a fraction of patients do not achieve disease control. The underlying mechanisms driving asthma require further investigation, particularly in patients with uncontrolled asthma who display the highest morbidity and symptom burden.

The most common type of asthma is allergic asthma, which often presents as T_H2 -cell-mediated eosinophilic inflammation.¹ T_H2 cells typically produce the cytokines IL-4, IL-5, and IL-13, which play a key role in asthma immunopathogenesis by inducing heavy chain class switch in B cells to IgE, eosinophilia, and goblet cell hyperplasia, respectively. The NOTCH pathway, which is an evolutionary conserved signaling pathway that regulates cell-cell interactions, can directly induce transcription of IL-4 and GATA-3, the key transcription factor in T_H2 cells.² NOTCH signaling is required for differentiation into many T-cell subsets.² Importantly, house-dust mite-driven allergic airway inflammation is strongly reduced in mice deficient for the downstream nuclear NOTCH effector Recombination Signal Binding Protein For Immunoglobulin Kappa J Region.³ Moreover, blocking NOTCH signaling reduced airway inflammation in mice in a therapeutic setting.^{4,5} On the basis of these findings, we questioned whether NOTCH signaling is increased in T cells from patients with asthma.

We used multicolor flow cytometry to quantify expression of NOTCH1 and NOTCH2 on T cells present in peripheral blood samples from 59 patients with asthma (inclusion and exclusion criteria are detailed in Table E1 in this article's Online Repository at www.jacionline.org) and from 28 age/sex-matched healthy controls (HCs). The study was approved by the Medical Ethical Committee of the Erasmus MC Rotterdam; written informed consent was obtained from every participant before study inclusion.

Patients were categorized into 3 groups, on the basis of asthma control questionnaire (ACQ) score: controlled asthma (CA; ACQ score, <0.75), partially controlled asthma (PA; ACQ score, 0.75 - 1.50), and uncontrolled asthma (UA; ACQ score, >1.5)⁶ (Table I). We found that fractional exhaled nitric oxide and lung function (FEV_1) were not different between the 3 asthma subgroups. Although numbers of eosinophils ($FSC^{high}SSC^{high}CD45^+CD33^-CD16^-Siglec-8^+$ cells) were increased in peripheral blood of patients with asthma compared with HCs, they did not differ across patient subgroups (Table I).

PBMCs were stained with fluorochrome-labeled antibodies to distinguish $CD4^+$ and $CD8^+$ T cells, naive and memory T cells, as well as T_H subsets on the basis of chemokine receptors (see Table E2 in this article's Online Repository at www.jacionline.org; Fig 1, A). Using the gating strategy outlined in Figure 1, A, we measured NOTCH expression on T-cell subsets and found that the proportions of positive cells were generally low (Fig 1, B and C). Nevertheless, we detected a significant increase in the proportions of NOTCH1⁺ cells, but not in the proportions of NOTCH2⁺ cells within the total $CD4^+$ T-cell population from patients with asthma, compared with HCs (Fig 1, C). The proportions of NOTCH1⁺ and NOTCH2⁺ cells within the $CD4^+CD3^+$ fraction (containing $CD8^+$ and $\gamma\delta$ T cells) were increased in patients with asthma compared with HCs, particularly in the PA/UA subgroups (Fig 1, C).

Both the naive and the memory $CD4^+$ T-cell compartments contained NOTCH1⁺ cells (Fig 1, C). Importantly, compared with HCs, frequencies of NOTCH1⁺ cells were higher in naive and memory $CD4^+$ T-cell fractions from the total group of patients with asthma and from the PA/UA subgroups (Fig 1, C). In contrast, proportions of NOTCH2⁺ cells were higher in memory than in naive $CD4$ T-cell fractions. Hereby, in the memory—but not in the naive— $CD4^+$ T-cell compartment, proportions of NOTCH2⁺ cells were significantly increased in

TABLE I. Characteristics of participants

Characteristic	HC	CA	PA	UA	P value
N	28	17	17	25	
Age (y)	31.0 (22-50)*	32.5 (19-48)	34.1 (18-56)	31.0 (18-51)	NS
Sex (M/F)	8/20	4/13	3/14	1/24	(HC-UA)†
BMI	23.4 (18.9-31.0)	24.6 (19.0-30.6)	26.5 (18.6-37.7)	27.7 (20.6-36.6)	(HC-UA)‡; (CA-UA)†
ACQ score		0.43 (0-0.67)	1.03 (0.75-1.42)	2.40 (1.5-4.17)	(CA-PA; CA-UA; PA-UA)‡
FEV_1 (% pred)		97.4 (66-122)	95.7 (73-119)	94.4 (60-120)	NS
FENO (ppb)		19.6 (7-47)	25.5 (6-70)	37.2 (9-152)	NS
Eosinophils (%)§	0.46 (0.025-2.31)	1.91 (0.033-9.22)	1.70 (0.073-6.62)	2.75 (0.034-9.66)	(HC-CA)†; (HC-UA)‡

BMI, Body mass index; F, female; FENO, fractional exhaled nitric oxide; M, male; NS, not significant; ppb, parts per billion.

*Continuous variables shown as mean (range).

† $P < .05$.

‡ $P < .01$; only significant comparisons are given. Mann-Whitney U test (or Fisher exact test to compare sex).

§Determined by flow cytometry in total peripheral blood; erythrocytes were lysed using standard osmotic lysis buffer.

METHODS

Cell lines

Human SV40-immortalized dermal fibroblasts from healthy control subjects, TBK1^{+/-} (p.G159A) patients, and TRIF^{-/-} patients^{E1,E2} and Vero (African green monkey kidney) cells were maintained in a 5% CO₂ incubator at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS.

Viral infection and quantification

Human fibroblasts were infected with HSV1–green fluorescent protein (KOS strain with green fluorescent protein–tagged capsid protein VP26) or HSV1 (strain 17AR+) at various MOIs and time points for immunoblot and immunofluorescence experiments. After 1 hour of infection in DMEM supplemented with 2% FBS, the virus was removed, and fresh media added with 1% human serum. Viral titers were determined by infecting a confluent monolayer of Vero cells in a 12- or 96-well plate and performing a plaque assay or calculating the 50% end point (TCID₅₀/mL).^{E3}

Stimulation and treatments

Fibroblast cells were stimulated with 25 µg/mL of poly(I:C; GE Healthcare, Chicago, Ill), 10 nmol/L rapamycin (Calbiochem, San Diego, Calif), or 1 × 10⁵ IU/mL of IFN-α-2A (PBL Assay Science, Piscataway, NJ) for 24 hours; or treated with 1 µmol/L of the TBK1 inhibitor BX795 (Sigma, St Louis, Mo)^{E4} for 16 hours; or transfected with 8 µg/mL c-di-GMP (Invivo Gen, San Diego, Calif) or c-di-GMP control (InvivoGen) for 2 hours; or 2 µg/mL HSV1 dsDNA (60mer sequence: 5'-TAAGACACGATGCGA-TAAAATCTGTTTGTAAAATTATTAAGGGTACAAATTGCCCTAGC-3'; Integrated DNA Technology, Coralville, Iowa) for 3 hours. c-di-GMP, c-di-GMP control, and HSV1 dsDNA were delivered using Lipofectamine 2000. For pretreatment experiments, fibroblasts were incubated with either rapamycin or poly(I:C) for 16 hours before infection with HSV1.

Cell viability assay

Cells were plated in a flat-bottom 96-well plate in triplicate at a density of 0.18 × 10⁶ cells/mL in 10% FBS-supplemented DMEM. Fibroblasts were pretreated for 16 hours before infection with HSV1 (MOI 1) for 24 hours. Fibroblast viability was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) Kit (Promega, Madison, Wis) and performed according to the manufacturer's instructions. Cell viability was determined by normalizing to noninfected cells of each cell line.

RNA interference

Cells were seeded in 10% FBS-supplemented DMEM at 0.05 × 10⁶ cells/well in a flat-bottomed 24-well plate and incubated for 18 hours in a 37°C humidified incubator. Medium was then replaced with fresh 2% FBS-supplemented DMEM, and cells were transfected with 15 nmol/L scramble (siNegative; Ambion, Thermo Fisher Scientific, Waltham, Mass) or 3 pooled TBK1-specific (siTBK1) siRNAs (siRNA ID nos. 134003, 134002, 899; Ambion) at 80% confluency using Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY) and incubated for a further 48 hours in a 37°C incubator.

Immunofluorescence and quantification of puncta-positive cells

Cells were grown to 50% confluency on 13-mm-diameter coverslips and fixed with 100% methanol on ice before washing with 1 × PBS. Blocking was done in 10% FBS-PBS, and cells permeabilized with 0.1% Triton X-100 in

PBS. Staining was performed in a moist chamber using the following antibodies: LC3B (1:500 dilution; Abcam, Cambridge, United Kingdom), cleaved LC3A (1:100 dilution; StraTech, Eden Prairie, Minn), HSV1-immediate early protein ICP4 (clone 10F1; 1:500 dilution; Virusys, Taneytown, Md), and Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (1:750 and 1:1000 dilutions, respectively; Life Technologies). Images were taken with a 63 × oil-immersion lens on a widefield fluorescence microscope (Axio Observer; Zeiss, Oberkochen, Germany). 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 1 × Laemmli buffer supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), 10% β-mercaptoethanol, and 1:1000 benzamide nuclease (Sigma). Lysates were denatured and electrophoresed on 12% tris-glycine or 10% bis-tris gels (Bio-Rad Laboratories, Hercules, Calif). Proteins were transferred onto polyvinylidene difluoride membrane (Invitrogen, Carlsbad, Calif), probed with primary and secondary horseradish peroxidase-conjugated antibodies (1:1,000 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 before lysis for immunoblotting to block LC3BII recycling to LC3BI. The following antibodies were used for immunoblotting: LC3B (Cell Signaling Technology), p62 (MBL International, Woburn, Mass), TBK1 (Cell Signaling Technology), IRF3 (D83B9; Cell Signaling Technology), phosphorylated IRF3 (S396; Cell Signaling Technology), STING (D2P2F; Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–horseradish peroxidase (Santa Cruz Biotechnology, Dallas, Tex). GAPDH was used as a loading control.

ELISA

IFN-β secretion in recovered supernatants of cells infected with HSV1 or pretreated with rapamycin or poly(I:C) for 24 hours was measured with the VeriKine-HS human IFN-β serum ELISA kit (assay range, 1.2–150 pg/mL; PBL Assay Science), according to the manufacturer's instructions.

Statistical analysis and software

Immunofluorescence images were analyzed with Icy software. Densitometric analyses of immunoblots were carried out with ImageJ software (National Institutes of Health, Bethesda, Md). Statistical significance was assessed by using 2-way ANOVA or the Student *t* test with Prism 7 software (GraphPad Software, La Jolla, Calif). Numbers of fluorescent cells or LC3B puncta-positive cells were quantified with ImageJ software. All experiments were performed at least 3 times.

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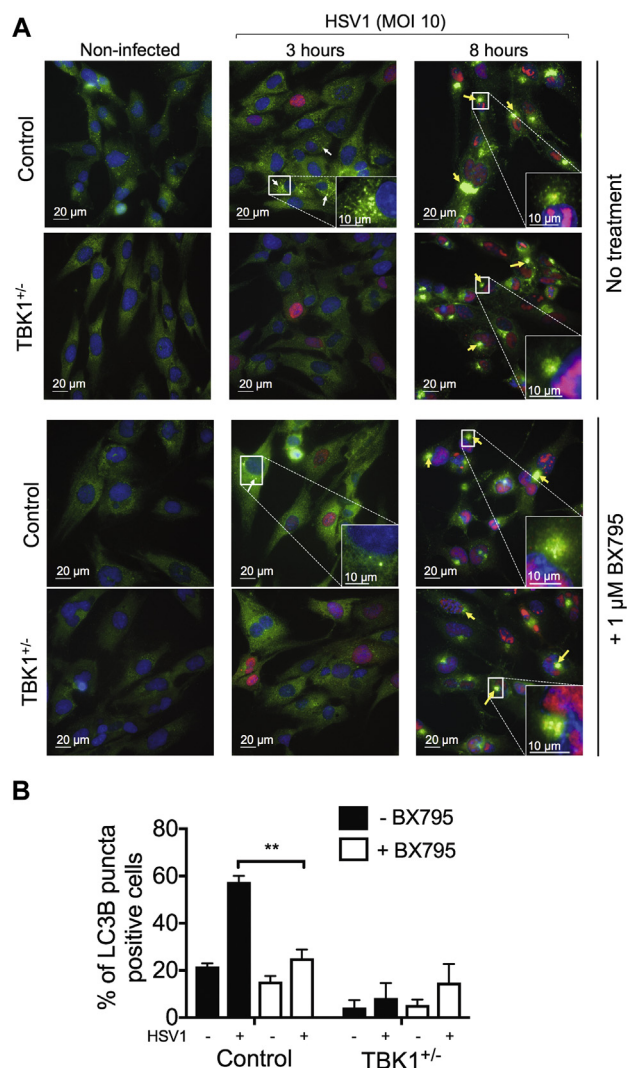


FIG E1. TBK1 inhibition reduced cytoplasmic LC3B puncta formation but did not affect perinuclear LC3B formation after HSV1 infection. **A**, Control and TBK1^{+/-} fibroblasts were pretreated with 1 μ M BX795 for 16 hours before infection with HSV1 (MOI 10) for the indicated length of time. Cells were fixed and stained for LC3B (green), ICP4 (red), or both. 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI; blue) was used for nuclear staining. The scale bar of each representative image is 20 μ m. The inset represents the magnified view of the indicated area and has a scale bar of 10 μ m. White arrows indicate cytoplasmic LC3B, whereas yellow arrows indicate perinuclear LC3B. **B**, The percentage of cells positive for cytoplasmic LC3B puncta in Fig E1, A, was counted on at least 100 cells. Images are representative of 3 independent experiments (n = 3). Data are represented as means $\pm$ SEMs and analyzed by using 2-way ANOVA. ***P* < .01.

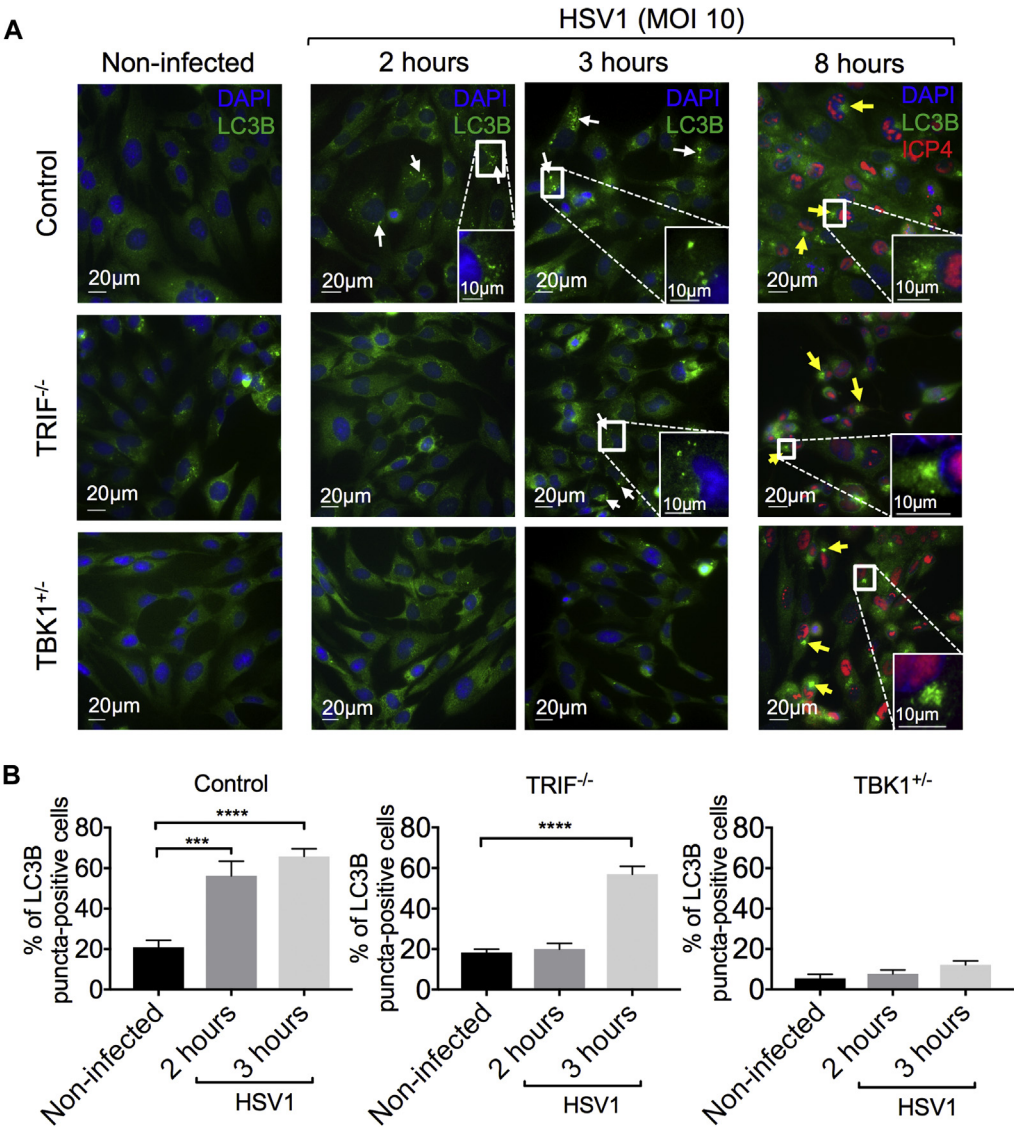


FIG E2. TRIF^{-/-} fibroblasts showed delayed cytoplasmic LC3B puncta formation. **A**, Fibroblasts grown on coverslips were infected with HSV1 (MOI 10) for indicated lengths of time before being fixed and stained for LC3B (green), HSV1 ICP4 (red), or both. 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI; blue) was used as the nuclear stain. The scale bar of each representative image is 20 µm. The inset represents the magnified view of the indicated area and has a scale bar of 10 µm. White arrows indicate cytoplasmic LC3B, whereas yellow arrows indicate perinuclear LC3B. **B**, The percentage of cells positive for cytoplasmic LC3B puncta was counted on at least 100 cells. Images are representative of 3 independent experiments (n = 3). Data are represented as means ± SEMs and were analyzed by using 2-way ANOVA. ****P* < .001 and *****P* < .0001.

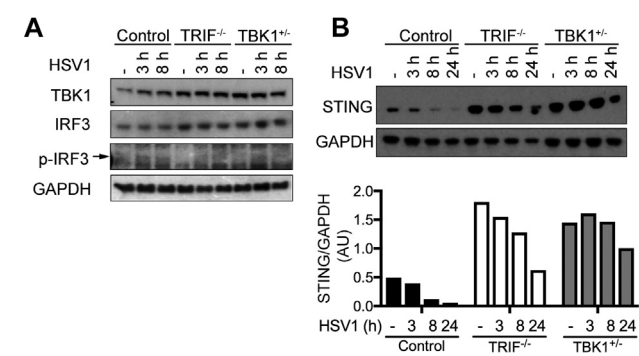


FIG E3. Immunoblots of TBK1, IRF3, phosphorylated IRF3, and STING during HSV1 infection. Control, TRIF^{-/-}, and TBK1^{+/-} fibroblasts were infected with HSV1 (MOI 1) for the indicated lengths of time. Whole-cell lysates were electrophoresed and probed for endogenous TBK1, IRF3, and phosphorylated IRF3 (*p-IRF3*; **A**) and STING (**B**) proteins. GAPDH was used as a loading control. Relative level of STING to GAPDH was measured using densitometry (arbitrary units).

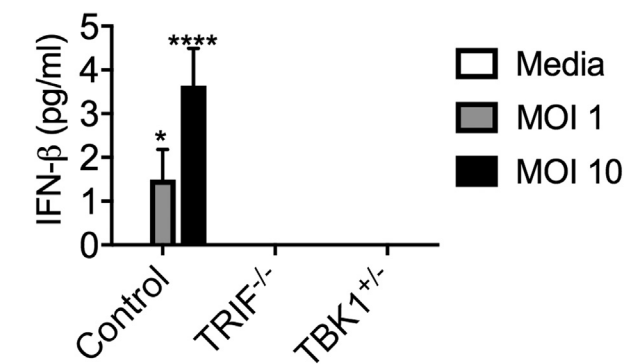


FIG E4. TRIF^{-/-} and TBK1^{+/-} fibroblasts showed impaired IFN-β production after HSV1 infection. Control, TRIF^{-/-}, and TBK1^{+/-} fibroblasts were infected with HSV1 at indicated MOIs for 24 hours before collecting supernatants and measuring IFN-β levels by ELISA. Data are represented as means ± SEMs and were analyzed by using 2-way ANOVA (n = 3). **P* < .05 and *****P* < .0001.

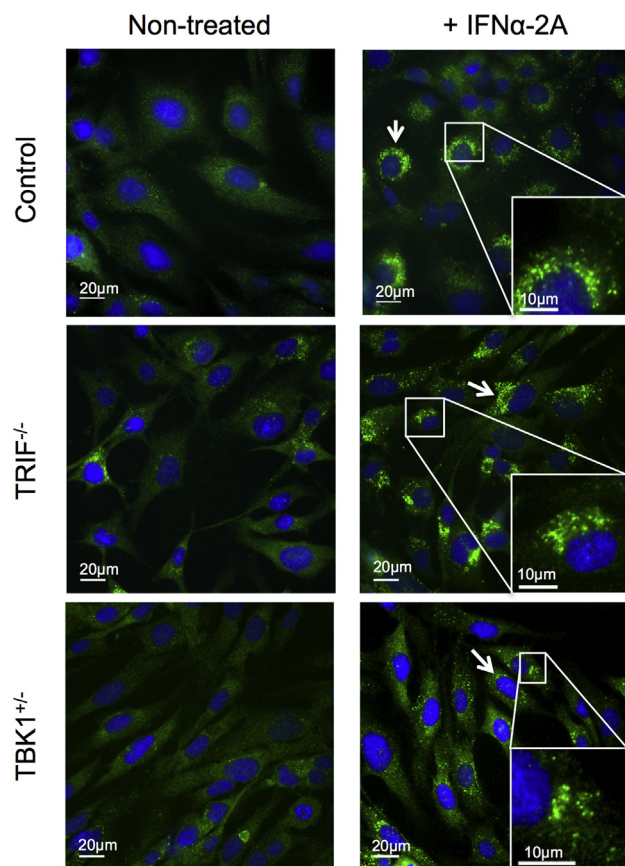


FIG E5. Interferon-induced autophagy in fibroblasts. **A**, Control, TRIF^{-/-}, and TBK1^{+/-} fibroblasts were stimulated with 1×10^5 IU/mL IFN- α -2A for 24 hours before being fixed and stained for LC3B (green). 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI; blue) was used as the nuclear stain. The scale bar of each representative image is 20 μ m. The inset represents the magnified view of the indicated area and has a scale bar of 10 μ m. White arrows indicate LC3B puncta. Images are representative of 3 independent experiments ($n = 3$).

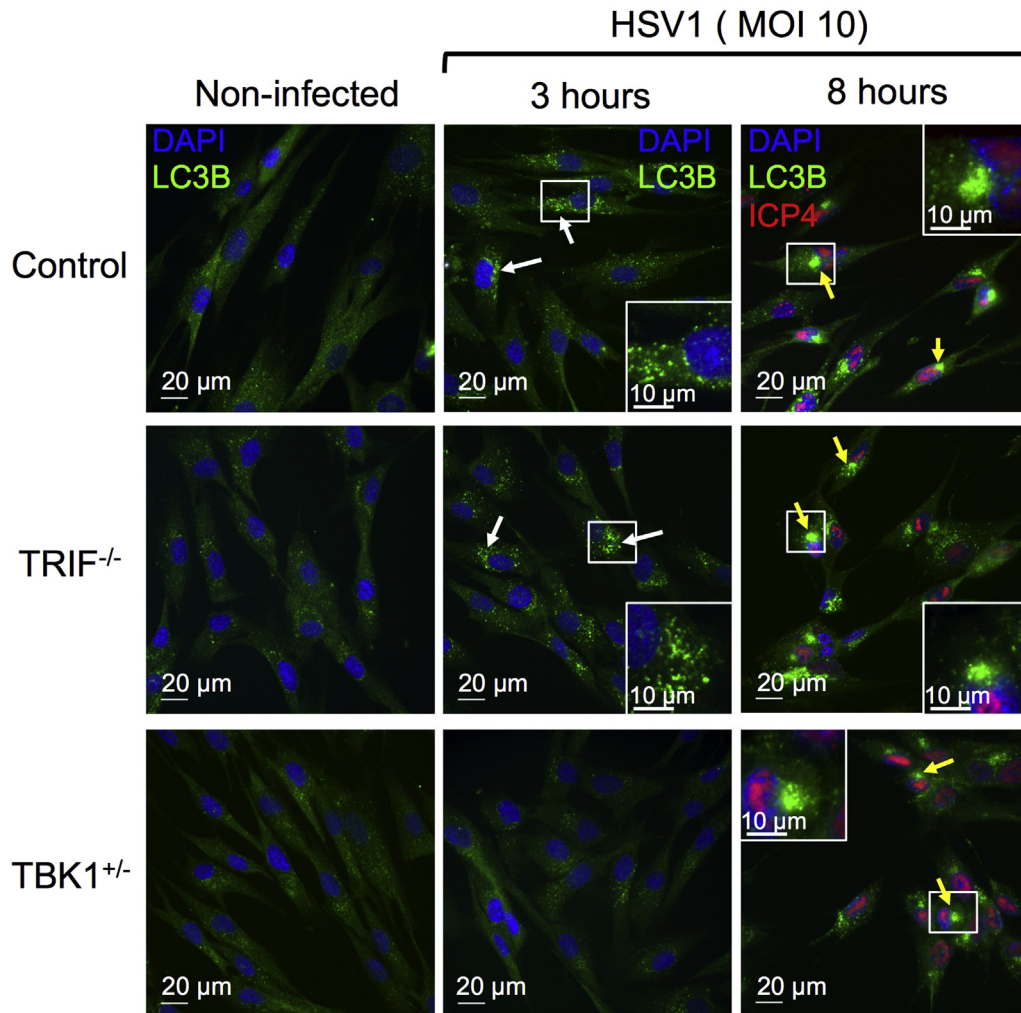


FIG E6. Primary fibroblasts showed similar HSV1-induced autophagy phenotypes to SV40-transformed fibroblasts. Primary fibroblasts grown on coverslips were infected with HSV1 (MOI 10) for 3 or 8 hours before being fixed and stained for LC3B (green), HSV1 ICP4 (red), or both. 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI; blue) was used as the nuclear stain. The scale bar of each representative image is 20 μ m. The inset represents the magnified view of the indicated area and has a scale bar of 10 μ m. White arrows indicate cytoplasmic LC3B, whereas yellow arrows indicate perinuclear LC3B. Images are representative of 3 independent experiments (n = 3).