Infection of endotheliotropic human cytomegalovirus of trabecular meshwork cells

Running title: CMV infection of trabecular meshwork cells

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Abstract

Purpose: Human cytomegalovirus (HCMV) infections can cause endotheliitis which is associated with an elevation of the intraocular pressure (IOP). However, the mechanism of the IOP elevation has not been determined. The purpose of this study was to determine whether HCMV strains which are capable of infecting corneal endothelial cells can also replicate, induce anti-viral responses, and can reorganize the actin cytoskeleton in trabecular meshwork cells.

Study design: Experimental study design

Methods: Cultured primary human trabecular meshwork cells (HTMCs) were infected with the Towne or TB40/E strains of HCMV. TB40/E is trophic for vascular endothelial and corneal endothelial cells. Real-time PCR, western blot, and fluorescent immunostaining have been used to determine whether HCMV-infected HTMCs will support the expression of viral mRNA and protein, allow viral replication, and elicit anti-viral host responses. We also determined whether lytic replication was present after an HCMV infection.

Results: HCMV infection led to the expression of viral mRNA and proteins of IE1, glycoprotein B(gB), and pp65. TB40/E infection induced interferon-β, a sign of host anti-viral immune response and MCP-1. Together with the induction of the regulators of actin cytoskeleton, myosin phosphatase Rho interacting protein (MPRIP) and

monocyte chemotactic protein-1 (MCP-1), TB40/E induced a high level of expression of viral proteins, including IE1, gB, and pp65 as well as actin stress fiber formation, and achieved pathogenically high viral titers.

Conclusions: Human trabecular meshwork cells support the replication of endotheliotropic TB40/E strain of HCMV which indicates that this strain may have high virulence for trabecular meshwork.

Introduction

Human cytomegalovirus (HCMV) infection can lead to endotheliitis which is associated with an elevation of the intraocular pressure (IOP) and endothelial cell loss [1]. Its chronic and relapsing nature resembles the clinical characteristic of Fuchs iridocyclitis and Posner-Schlossman syndrome. These disease entities are characterized by chronic and relapsing elevations of the IOP, and HCMV infection has been recognized to be an important causative pathogen for these relapses and elevations.

In HCMV endotheliitis, the IOP elevation and endothelial inflammation frequently recurs during the long course of the disease process. The IOP elevation can last for months or years, and the eye can become refractory to the IOP lowering medications. Because of this, these patients often require filtering surgery. The findings strongly suggest that the HCMV may actively proliferate in the trabecular meshwork and corneal endothelial cells.

The IOP elevation during the long disease course of HCMV endotheliitis will damage the trabecular meshwork cells which can then lead to a severe reduction of aqueous outflow. However, there is no definitive proof that surgically-obtained trabecular

meshwork tissue was permissive for HCMV infection. Because filtering surgery is generally performed after years of IOP elevation, the trabecular meshwork cells were already damaged. This may explain the absence of HCMV proteins in the trabecular meshwork specimens.

When HCMV infects permissive cells, latent infection can be established. During the latency phase, detection of viral protein antigen is very difficult unless the specimen is collected during the reactivation phase. We have shown that corneal endothelial cells can be infected by the Towne strain, the TB40/E strain, and a clinically-isolated strain of HCMV [2, 3]. However, the efficiency of infection measured by $TCID_{50}$ is markedly different depending on the strain, and vascular endotheliotropic TB40/E was also tropic for corneal endothelium. We have hypothesized that endotheliotropic HCMV strain will infect trabecular meshwork cells more efficiently than non-tropic strains of HCMV.

Thus, the purpose of this study was to determine whether endotheliotropic strain of HCMV can infect trabecular meshwork cells and reorganize the actin stress fibers. In addition, we examined whether HCMV infection will induce strong immune responses which may explain the increased outflow resistance of the trabecular

meshwork in eyes with HCMV infection.

Materials and Methods

All protocols and methods adhered to the tenets of the Declaration of Helsinki, and the study was approved by the Ethics Committee of Tottori University.

Cells and virus

Human trabecular meshwork cells (HTMC) were isolated from trabecular tissues stripped form cadaver eyes donated for research purposes. (ScienCell, Carlsbad, CA) The HTMCs were propagated to confluence on 6- or 96-well plates in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum.

A standard laboratory strain, Towne, and an endotheliotropic HCMV strain, TB40/E, were used. The Towne strain is a representative laboratory strain together with AD169, and is one of most widely used HCMV strain. The Towne strain has been used for vaccine development. [4]

The TB40/E strain was created by transfecting human foreskin fibroblast cells (HFF)

transfected by a TB40-BAC4 clone and propagated by standard procedures as described in detail [2]. The viral titers were designated by the 50% tissue culture infection dose ($TCID_{50}$) method using human foreskin fibroblast cells infected with serially diluted supernatants.

Real-time RT-PCR

Total RNA was isolated from HTMCs at the indicated time points after infection by the two HCMV strains and reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). The cDNAs were amplified and quantified by the LightCycler (Roche, Mannheim, Germany) using the QuantiTect SYBR Green PCR kit. The sequences of the real-time PCR primer pairs were:

IE-1:

forward 5'- CCTCCAAGGTGCCACGGCCCGA-3',

reverse 5'- CATCCTCCCATCATATTA-3'.

UL83 (pp65):

forward 5'- GTCAGCGTTCGTGTTTCCCA-3',

reverse 5'- GGGACACAACACCGTAAAGC-3'.

Glycoprotein B (gB):

forward 5'- GGGACACAACACCGTAAAGC-3',

reverse 5'- ATGATGCCCTCRTCCARGTC-3'.

Interferon-β (IFN-β):

forward 5'-CATTACCTGAAGGCCAAGGA -3',

reverse 5'-CAATTGTCCAGTCCCAGAGG-3'

Myosin phosphatase Rho interacting protein (MPRIP):

forward 5'- CTCTCCACACACGAGCTGAC-3',

reverse 5'- TCTTCTGGTGCGTTTCTTCC-3'

Monocyte chemotactic protein-1 (MCP-1):

forward 5'- AGGTGACTGGGGCATTGAT-3',

reverse 5'- GCCTCCAGCATGAAAGTCTC-3'

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH):

forward 5'- AGCCACATCGCTCAGACAC-3',

reverse 5'- GCCCAATACGACCAAATCC-3'.

To measure the degree of viral DNA replication, HTMCs were infected with either the TB40/E or the Towne strain, and viral DNA was extracted from the cells at the indicated time points with the QiaAmp DNA mini kit (Qiagen, Hilden, Germany). The

extracted DNA was amplified with the LightCycler (Roche, Base, Switzerland) and normalized to known dilutions of synthesized pp65 DNA fragments.

Western blot

HTMCs were infected with the TB40/E strain or the Towne strain at a selected multiplicity of infection (MOI). Cell lysates were harvested at 1, 2, and 3 days post infection (PI), and 10 µg of protein/lane were electrophoresed. The transferred membrane were stained with antibodies for HCMV IE1(NEA-9221, Perkin Elmer, Boston, MA), pp65 (CA003, EastCoast Bio, North Berwick, Maine, USA), or UL44 (CH167, Santa Cruz, Santa Cruz, CA) [5]. The proteins were made visible with horseradish peroxidase-conjugated secondary antibody and chemiluminescence substrate as described [2].

Immunocytochemistry

HTMCs were infected with HCMV at MOI of 5 and stained for HCMV proteins with mouse anti-HCMV monoclonal antibody against IE-1 (MAB8129, Millipore, Darmstadt, Germany) or pp65 (ab31624, Abcam, Cambridge, UK), or glycoprotein B (gB; ab6499, Abcam, Cambridge, UK). IE1, pp65, and gB were made visible by incubation with Alexa 488 conjugated-secondary antibody and stained for actin

cytoskeleton using phalloidin. Images were photographed with a confocal microscope (LSM710, Zeiss, Germany).

Electron microscopy (EM)

HTMCs were infected with either the TB40/E strain or the Towne strain of HCMV and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After post fixation with 1% OsO4 in 0.1 M sodium cacodylate buffer, cells were stained with 1% uranyl acetate. Cells were dehydrated, embedded in Epon 812, and sectioned with a microtome. The sections were stained with 4% uranyl acetate and 0.4% lead citrate and were examined by transmission electron microscopy (TEM).

Statistical analyses

Data are presented as the means ± standard error of the means (SEMs). Statistical analyses were performed by *t* test, Mann Whitney U test, or ANOVA with post hoc test.

Results

When HTMCs were infected with the Towne strain of HCMV at an MOI of 5, an induction of the viral immediate early protein, 1E1, was detected at 6 h, and there

was a gradual increas to 24 h post infection (Fig. 1a right). The transcriptions of the delayed early proteins, gB, and leaky late protein, pp65, were also increased at 24 h PI (Fig.1b, 1c, right).

When HTMCs were infected with the TB40/E strain, IE1 was induced at 6 h. The induction kinetics of gB and pp65 were similar to that following the standard laboratory Towne strain infection, however the induction levels were significantly higher (Fig. 1).

HCMV-infected HTMCs were examined for IE1, pp65, and DNA polymerase processivity subunit UL44 by western blot analysis (Fig. 2). Consistent with the gene transcription outcomes, the IE1 protein was promptly induced at 1-day PI with both strains. Notably, TB40/E induced the expression of pp65 as early as 1-day PI at low MOI. In contrast, Towne infection required 3 days at high MOI for the induction. With TB40/E infection, the UL44 induction was also earlier at 2 days PI compared to 3 days PI with the Towne strain.

We next determined whether HCMV infection will induce the antiviral responses of the host cells, regulation of cytoskeleton, and MCP-1 as an IOP-related cytokine.

198 [14] When HTMCs were infected with the TB40/E strain, there was a significant
199 induction of interferon-β (Fig. 3). In contrast, infection by the Towne strain did not
200 induce interferon-β.
201
202 We then examined whether HCMV infection will induce the regulatory factors for
203 actin cytoskeleton (Fig. 4). After infection by the TB40/E strain of HCMV, the
204 induction of the myosin phosphatase Rho interacting protein (MPRIP) was
205 significantly increased in the HTMCs. Again, Towne infection did not induce MPRIP
206 appreciably. Both strains induced MCP-1 in response to HCMV infection, however a
207 prolonged induction was observed after TB40/E infection at 24 h PI (Fig. 5).
208
209 The location of the viral protein expression and actin stress fiber formation after
210 HCMV infection was determined by immunostaining (Fig. 6). Intense IE1 staining
211 of the nuclei of HTMCs was detected at 2 day PI with nuclear F-actin [6]. The
212 expression of IE1 moved to the assembly compartment in the cytoplasm at 3 days
213 PI. (Fig. 6a, 6b; upper panel) The expression of pp65 was also detected in the nuclei
214 at 2 days PI (Fig 6c.), and it was localized to the viral assembly compartments in the
215 cytoplasm at 3 days PI. (Fig. 6a, 6b; lower panel) The expression of gB was delayed
216 and its expression was observed at 3 days PI. (Fig. 6a, 6b; middle panel)

When HTMCs were infected by TB40/E, an owl's eye appearance was observed in the nucleus with IE staining earlier than after Towne infection (Fig. 6a; white arrow), and it was detected as early as 1-day PI. (Fig. 6a; upper left) The level of expression increased in the cytoplasm at 3 days PI.

The expressions of pp65 and gB were observed in the nuclei at 2 days PI, and they were also localized in the viral assembly compartment and distinct stress fibers of actin were formed.

TB40/E infection induced significant remodeling of the actin cytoskeleton (Fig. 6c) Thus, the TB40/E infection was more robust with an earlier expression of viral proteins and actin stress fiber formation.

The replication of viral DNA in HTMCs was assessed after infection with the TB40/E strain or the Towne strain (Fig. 7). Both strains induced significant increases of viral genome at 2 days PI at MOI of 5, and the increase continued, and both strains supported an efficient replication at MOI 1. Interestingly, TB40/E infection induced 5 times more viral replication than the Towne strain at day 3 PI.

HCMV infected HTMC were examined by TEM. Infection by either the TB40/E strain or the Towne strain led to many viral particles and electron-dense particles (dense bodies) in the cytoplasm of the HTMCs (Fig. 8).

Then, we compared the increase in the level of the TB40/E and Towne strains in HTMC using titration by $TCID_{50}$. HTMCs were infected with TB40/E or Towne at MOI of 1 and 5, and titrated (Fig. 9). TB40/E led to a continuous increase until day 11 PI and reached 10^7 $TCID_{50}$. In contrast, the increase of the Towne strain reached a plateau at day 5 PI, and the maximum viral titer remained 10 times lower than that of TB40/E.

Discussion

Earlier studies have shown that infection of the anterior segment of the eye by HCMV can cause endotheliitis or iritis. Both diseases are characterized by an elevation of the IOP which is not observed for HCMV retinitis. Thus, anterior segment infections by HCMV may belong to a unified spectrum of a disease. Under these conditions, an IOP elevation can be caused by an increase in the outflow resistance in which the trabecular meshwork cells play a major role.

Steroid treatment is well known to induce steroid-induced glaucoma and impair the outflow function of the trabecular meshwork. When the trabecular meshwork cells were exposed to dexamethasone in vitro, there is a remodeling of the actin fibers and an induction of contraction [7]. Thus, stress fiber formation and actin remodeling were suggested as possible mechanisms of the secondary glaucoma. Our results showed that HCMV can replicate in HTMCs and reorganize the actin cytoskeleton of the cells [2]. This may explain why there was an elevation of the IOP associated with HCMV infection.

In eyes with open angle glaucoma, IL-4, IL-6, IL-8, IL-16, CCL2(MCP-1), TGF-β1, CCL13, CCL15, CCL22, CCL24, CXCL13, CXCL16, and TNF-α were found to be significantly elevated in the aqueous humor. [8]-[13]. Of these, the elevation of MCP-1 is consistent, and MCP-1 is a representative cytokine whose level is significantly correlated with the elevation of the IOP. [14] MCP-1 is well known to attract mononuclear cells and also induce collagen synthesis by TGF-β1 induction [15]. It also induces the production of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 (TIMP-1). All of these changes may explain the induction of fibrotic change of outflow system leading to the IOP elevation.

Very recently, the AD169 strain of HCMV was reported to be able to infect HTMCs. [16] AD169 infection induced TGFβ1 but its induction required at least 3 days, and the induction levels remained within 10-20% of that of mock infection. Moreover, AD169 infection did not induce any mRNA of cytoskeleton-related molecules including fibronectin, α-SMA, and collagen type 1A. This suggests less fibrogenic ability of AD169 which is in marked contrast to dexamethasone treatment to HTMCs, which induced fibronectin and α-SMA.

We have shown that HCMV can proliferate in primary corneal endothelial cells, and TB40/E is tropic for corneal endothelium as well as vascular endothelium [2]. This led us to hypothesize that endotheliotropic strains can cause more relevant disease characteristics. The results showed that the endothelial cell tropic TB40/E proliferate very efficiently in trabecular meshwork cells with marked actin cytoskeleton stress fiber formation. This implicates the endothelial type of HCMV infection to be the cause of the higher virulence.

In HCMV endotheliitis, the copy numbers of the DNA of HCMV in the aqueous humor determines the frequency of recurrences, degree of IOP elevation, and endothelial

cell loss [17]. These findings indicate that the copy number of the viral genome determines the course of the disease and refractoriness. The adaptive Towne strain also proliferated in HTMCs, however the $TCID_{50}$ remained 10 times lower than that of TB40/E for HTMCs and corneal endothelial infections. The TB40/E strain proliferated well in corneal endothelial cells and required 4 days to reach a plateau with high tissue culture infective dose (TCID) titers. Thus, the trabecular meshwork cells supported both the endotheliotropic and adaptive strains however the TB40/E strain was more efficient in proliferation and had a higher virulence in HTMCs.

Contraction and reorganization of the actin stress fibers presumably play important roles in the IOP regulation through the conventional outflow channels. Rho kinase inhibitors reorganize or disrupt F-actin fibers through Rho GTPase proteins, and they were recently made available for use on patients to reduce the IOP by targeting the outflow through Schlemm's canal. HCMV diverts the actin fiber transport system of the host to organize nuclear egress, viral assembly, and egress from the cytoplasm. Thus, HCMV infection disrupts or reorganizes the actin fiber cytoskeleton. For example, US-28 protein of HCMV activates the RhoA-mediated motility and the ROCK pathway [18, 19]. In the intracellular viral assembly complex of the host, RhoB is diverted to reorganize or disrupt actin stress fibers leading to efficient viral

312 production [20].

313

314 Adaptive HCMV strains, including the Towne and AD169 strains, proliferate well in
315 fibroblasts, however macrophages, dendritic cells, vascular endothelial cells, and
316 epithelial cell do not allow efficient proliferation. These strains are deficient by as
317 many as 20 genes [21], including pUL128, pUL130, and ppUL131A, which mediate
318 viral entry into endothelial cells or epithelial cell lineage [22, 23]. However, whether
319 these factors and other viral genes determine the pathogenicity of HCMV has not
320 been determined.

321

322 We observed the induction of IE1 transcript and gene products in the HTMCs. The
323 induction of IE1 determines the latency or reactivation of HCMV in the infected cells.
324 A strong induction of IE1 by the TB40/E strain indicates a facilitated activation of viral
325 gene induction (Figure 1, 2). However, IE1 expression is not a reliable marker of viral
326 infection because it can be induced without viral replication. IE1 is induced even in
327 non-permissive animal cells because of its very potent promotor activity.

328

329 We showed a strong induction of the pp65 protein which strongly inhibits antiviral
330 interferon responses, and it can serve as the major target of the host acquired

331 immune system. The HCMV proteins, including pp65, provoke strong acquired
332 immunity of $CD8^+$ T cells through MHC class I recognition pathway. Because of the
333 high prevalence of HCMV infection in elderly subject, [24] up to 30% of $CD8^+$ T cells
334 in their bodies responsive to HCMV antigen [25]. This substantial expansion of $CD8^+$
335 T cells that are memory inflated is often facilitated by antigen presentation by non-
336 hematopoietic cells including endothelial cells [26, 3]. This immunostimulatory effect
337 of infected cells is characterized by potent interferon responses which we showed for
338 TB40/E infection.
339
340 gB mediates the adhesion of HCMV to the host heparan sulfate proteoglycan. Thus,
341 neutralization of gB by antibody generation is an important strategy of the host to
342 prevent cell-to-cell spread or cell fusion after infection. Collectively, the strong
343 expression of pp65 and gB by the trabecular meshwork cells and the reactivity of the
344 host acquired immunity may determine the clinical characteristics of the disease.
345
346 There are some limitations for our study. Our analysis was based on an in vitro
347 infection model of cultured trabecular meshwork cells. This may not reflect the in vivo
348 role of trabecular meshwork including the conventional outflow of aqueous humor. In
349 addition, the inflammatory cells recruited by CMV-induced inflammation in vivo, may

cause remodeling of trabecular meshwork. However, our finding that the endotheliotropic strain of HCMV is pathogenic in both the corneal endothelium and trabecular meshwork is important information to understand the disease etiology.

In conclusion, HCMV can infect trabecular meshwork cells and promote the reorganization of the actin cytoskeleton. This then presumably leads to the IOP elevation in the host. Understanding the viral and host aspects of HCMV infection may provide important clues to develop efficacious treatment of refractory anterior segment infection by HCMV.

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Figure legends

Figure 1. Induction of the mRNA of the genes of human cytomegalovirus (HCMV) in human trabecular meshwork cells (HTMC) after HCMV infection

HTMCs were infected with the Towne strain or the TB40/E strain of HCMV, and the induction of the mRNA of IE1(a), pp65 (b), and glycoprotein B (gB)(c) was determined by RT-real-time PCR. Viral genes, IE1, pp65, and gB, were sequentially induced after infection by the Towne strain or the TB40/E strain of HCMV. TB40/E induced significantly higher amounts of IE1, pp65, and gB.

*: $P = 0.05$, **: $P = 0.05$; n = 8

Figure 2. Induction kinetics of viral gene proteins in HTMCs after HCMV infection.

HTMCs were infected with the Towne strain or the TB40/E strain at MOI of 1 or 5, and the extracted viral proteins were assayed by western blot analyses. The proteins of IE1, pp65, and UL44 were sequentially expressed after the HCMV infection. Infection by the TB40/E strain led to stronger inductions of pp65 and UL44.

Figure 3. Induction of interferon response in HTMCs after infection by the TB40/E strain of HMCV.

The extracted mRNA at 6 h post infection was evaluated for interferon using real-time RT-PCR for interferon-β. TB40/E infection induced significantly higher levels of interferon-β than Towne. *: $P = 0.016$, **: $P = 0.0001$; n = 5.

Figure 4. Induction of myosin phosphatase Rho interacting protein (MPRIP) in HTMCs after infection by the TB40/E strain of HMCV.

HTMCs were infected with the TB40/E strain or the Towne strain. The level of mRNA induction of myosin phosphatase Rho interacting protein (MPRIP) was evaluated using RT-PCR. TB40/E infection induced significantly higher levels of MPRIP at 12 h PI than Towne. *$P = 0.0001$; n = 5.

Figure 5. Induction of MCP-1 in HTMCs after infection by HMCV

HTMCs were infected with the TB40/E strain or the Towne strain. The level of mRNA induction of MCP-1 was evaluated using RT-PCR. TB40/E infection prolonged the induction of higher levels of MCP-1 at 24 h, while induction of MCP-1 by Towne was observed at 6 h PI and was transient. *: $P = 0.04$, **: $P < 0.001$; n = 5.

Figure 6. Induction of viral gene proteins and remodeling of actin cytoskeleton after HCMV infection.

HTMCs were infected with the TB40/E strain (a) or the Towne strain (b) of HCMV at MOI of 5 and immunostained for IE1, pp65, and glycoprotein B (Green). TB40/E infection induced significant remodeling of the actin cytoskeleton (c, pp65, green). Actin was stained using phalloidin (red). Nucleus was labelled by DAPI (blue). *: viral assembly compartment.

Figure 7. Viral genome replication for HCMV in human trabecular meshwork cells (HTMCs).

HTMCs were infected with the HCMV and examined for viral genomic copy equivalent using real-time PCR. Both the TB40/E strain and Towne strain induced significant increases of the viral genome. TB40/E infection induced 5 times more viral replication than Towne at day 3 post infection. n = 6

Figure 8. Production of infectious virions after HCMV infection of human trabecular meshwork cells (HTMCs)

HTMCs were infected with the TB40/E strain or the Towne strain of HCMV at MOI of 0.1 and examined by electron microscopy. Viral particles (arrow) and dense bodies (arrowhead) can be seen in the cytoplasm. Bars indicate 500 nm (TB40/E) and 200 nm (Towne).

Figure 9. Efficient growth of HCMV in HTMCs.

HTMCs were infected with TB40/E or Towne at MOI of 1 and 5 and were titrated for tissue culture infectious dose ($TCID_{50}$). Both strains had efficient growth in HTMCs. The maximum viral titer was significantly higher for the TB40/E strain. n = 4.

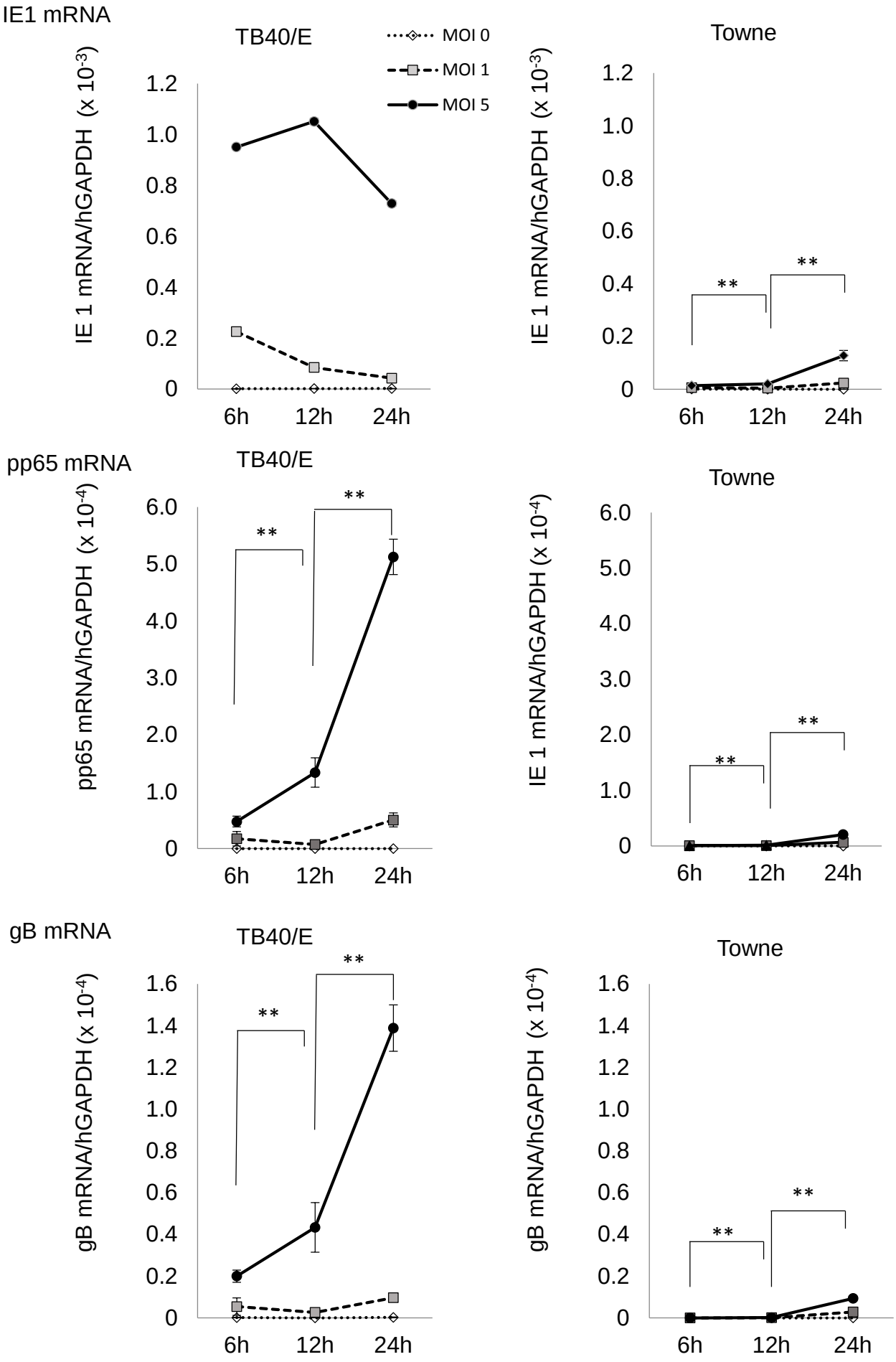

IE1 mRNA
TB40/E
MOI 0
MOI 1
MOI 5
IE 1 mRNA/hGAPDH (x 10-3)
1.2
1.0
0.8
0.6
0.4
0.2
0
6h
12h
24h
Towne
IE 1 mRNA/hGAPDH (x 10-3)
**
**
pp65 mRNA
TB40/E
pp65 mRNA/hGAPDH (x 10-4)
6.0
5.0
4.0
3.0
2.0
1.0
0
**
**
Towne
IE 1 mRNA/hGAPDH (x 10-4)
**
**
gB mRNA
TB40/E
gB mRNA/hGAPDH (x 10-4)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
Towne
gB mRNA/hGAPDH (x 10-4)
**
**

Figure.1

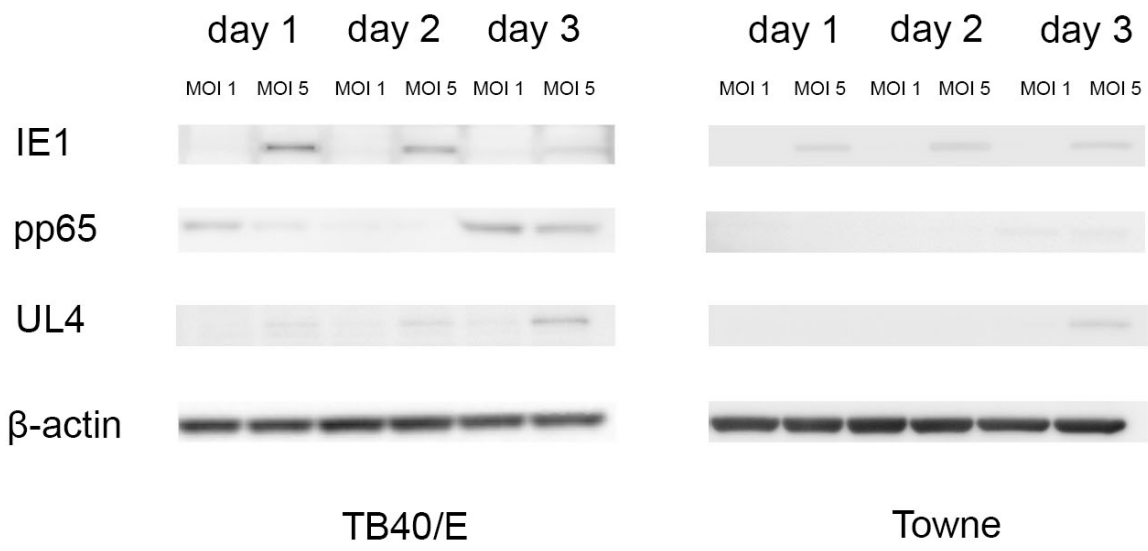

day 1
day 2
day 3
MOI 1
MOI 5
MOI 1
MOI 5
MOI 1
MOI 5
IE1
pp65
UL4
β-actin
TB40/E
day 1
day 2
day 3
MOI 1
MOI 5
MOI 1
MOI 5
MOI 1
MOI 5
Towne

Figure.2

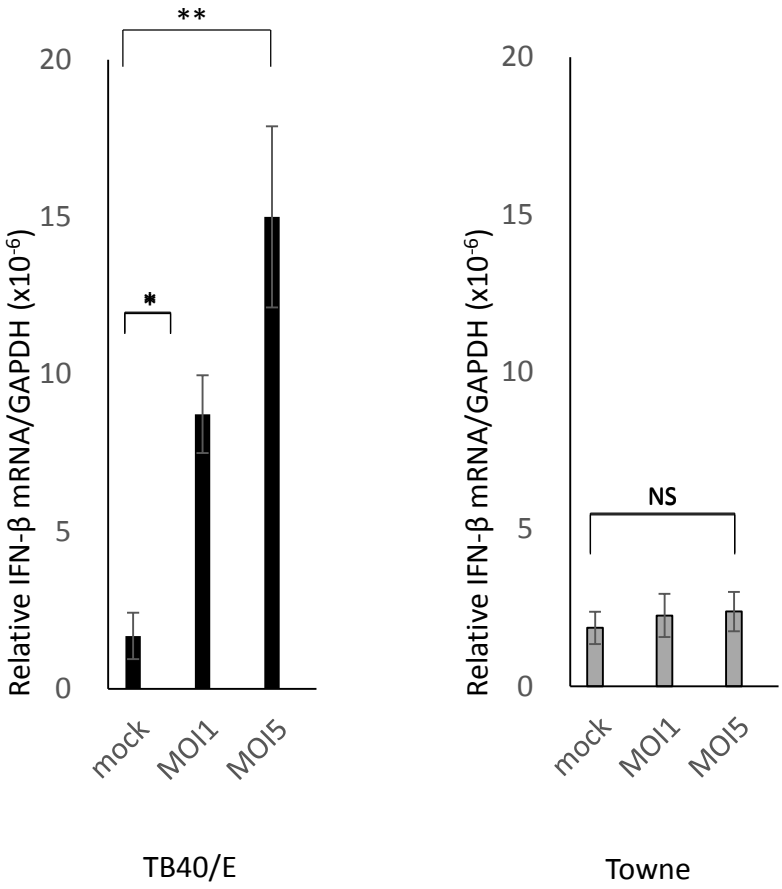

**
*
20
15
10
5
0
Relative IFN-β mRNA/GAPDH (x10-6)
mock
MOI1
MOI5
TB40/E
NS
Towne

Figure.3

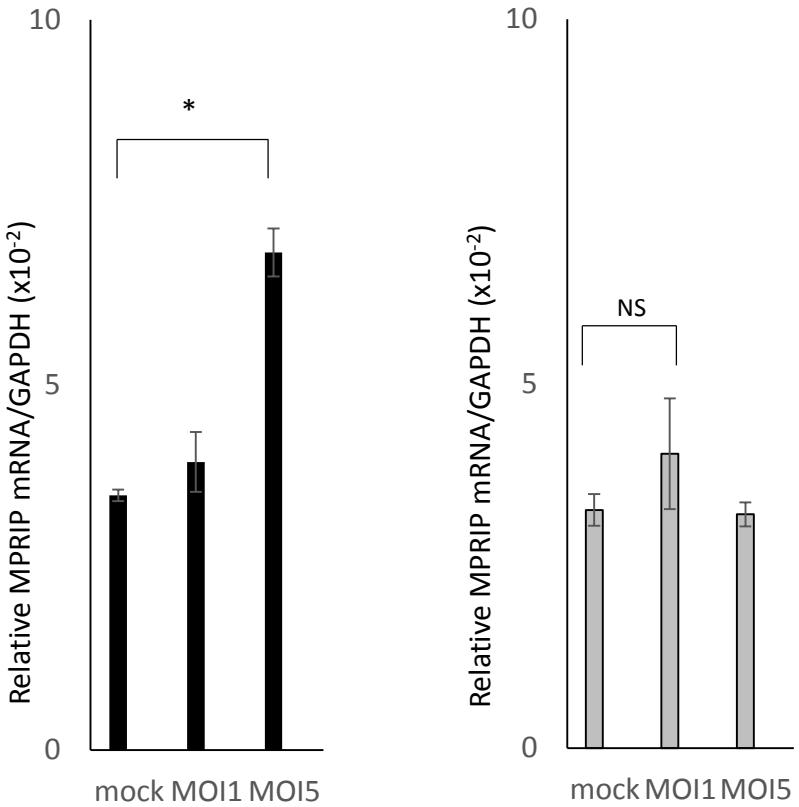

10
5
0
Relative MPRIP mRNA/GAPDH (x10-2)
*
mock MOI1 MOI5
TB40/E
10
5
0
Relative MPRIP mRNA/GAPDH (x10-2)
NS
mock MOI1 MOI5
Towne

Figure.4

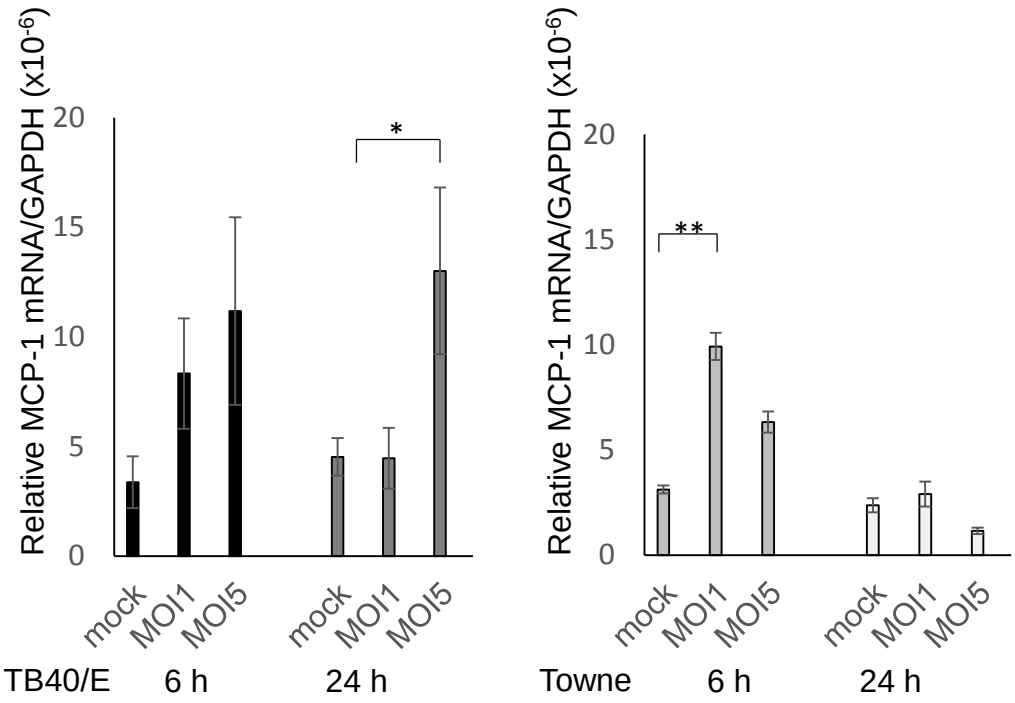
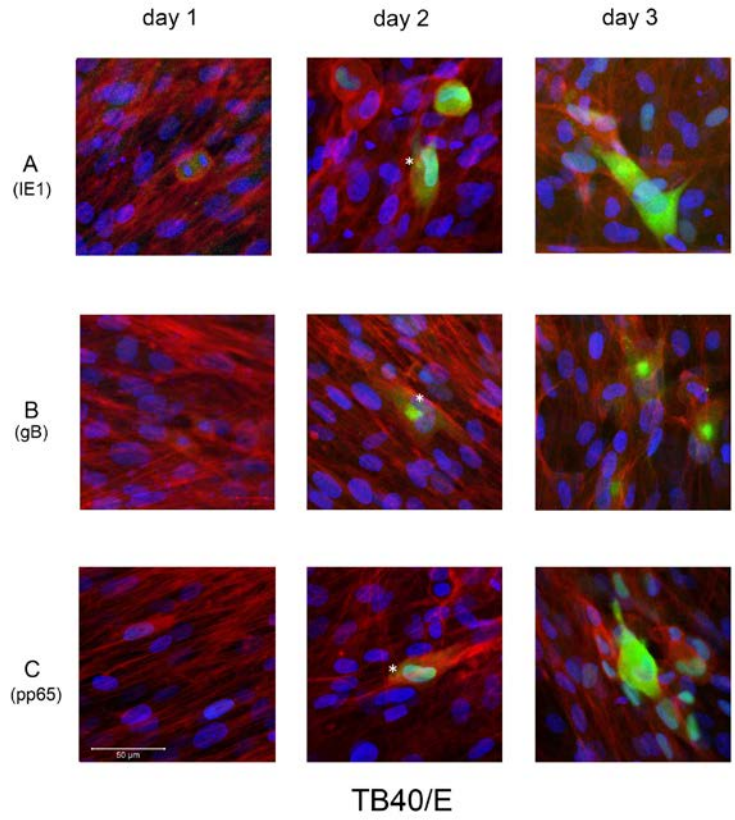

Relative MCP-1 mRNA/GAPDH (x10-6)
20
15
10
5
0
*
mock
MOI1
MOI5
mock
MOI1
MOI5
TB40/E
6 h
24 h
Relative MCP-1 mRNA/GAPDH (x10-6)
20
15
10
5
0
**
mock
MOI1
MOI5
mock
MOI1
MOI5
Towne
6 h
24 h

Figure.5


day 1
day 2
day 3
A
(IE1)
B
(gB)
C
(pp65)
50 µm
TB40/E

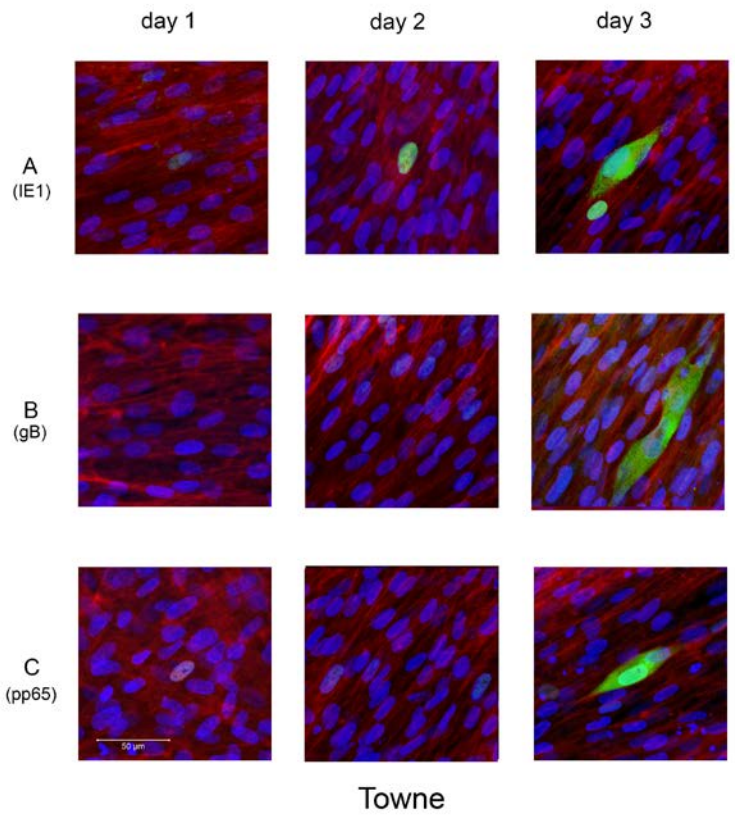
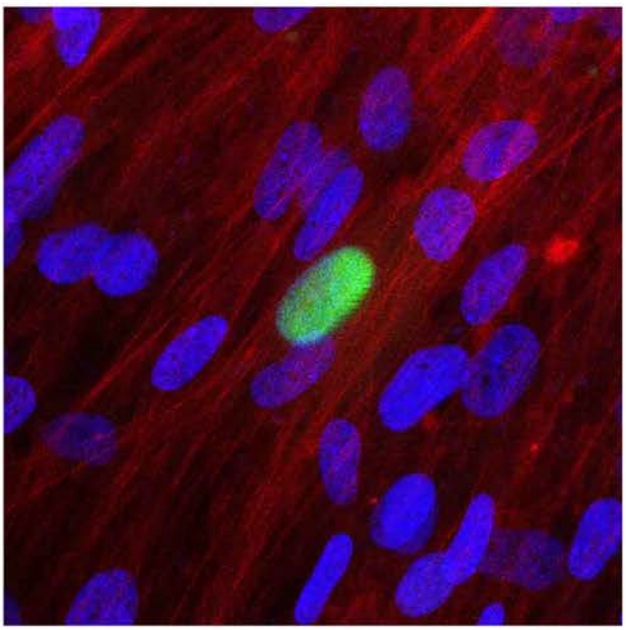
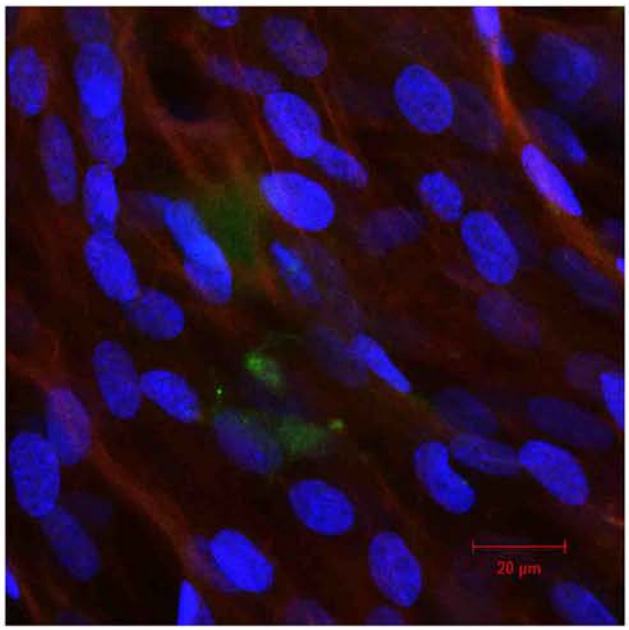

day 1
day 2
day 3
A
(IE1)
B
(gB)
C
(pp65)
50 µm
Towne

Figure.6

c.



TB40/E



Towne

Figure.6

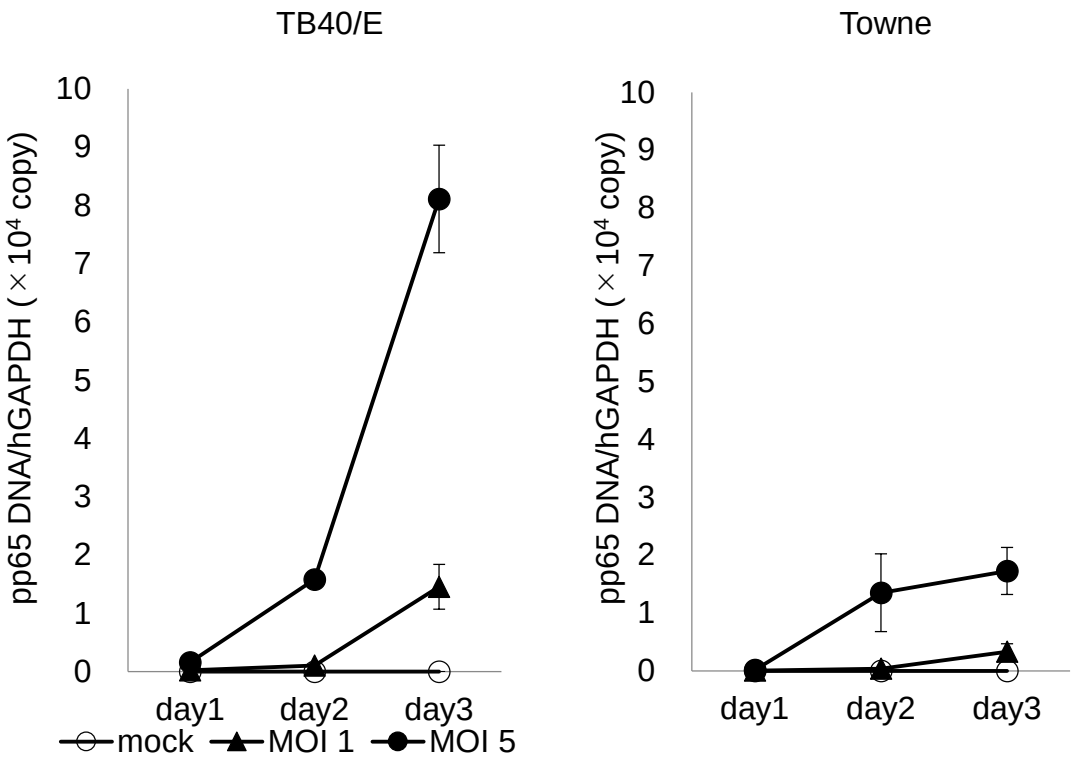
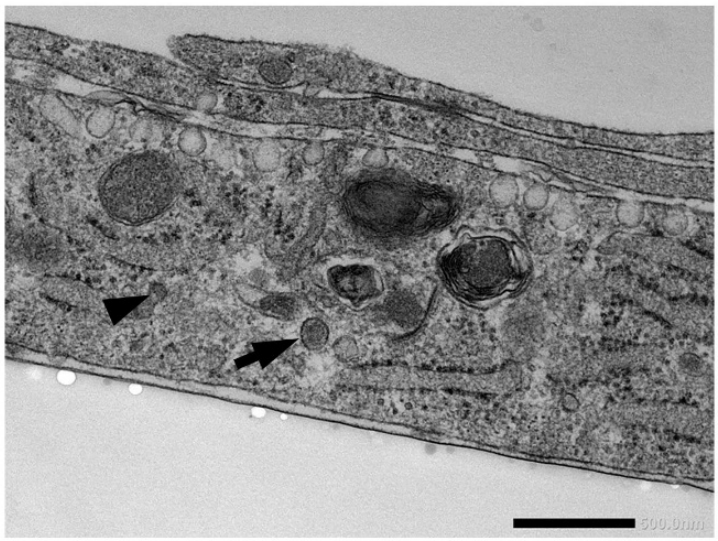
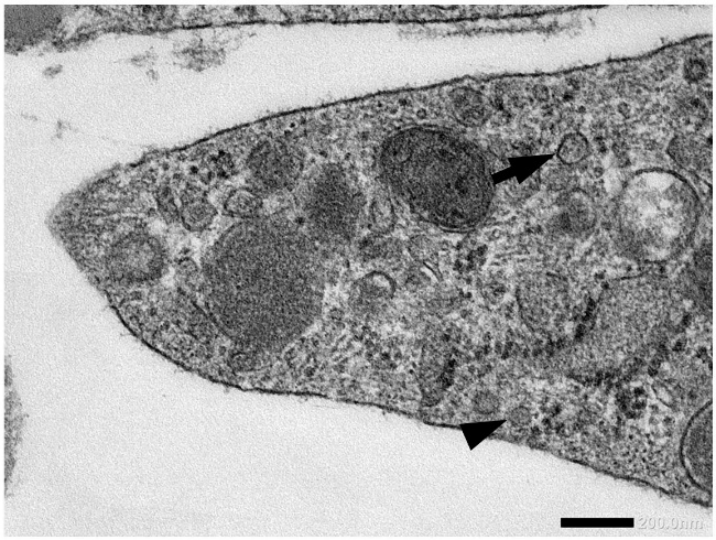

TB40/E
Towne
pp65 DNA/hGAPDH (×10⁴ copy)
10
9
8
7
6
5
4
3
2
1
0
day1
day2
day3
mock
MOI 1
MOI 5

Figure.7



TB40/E



Towne

Figure.8

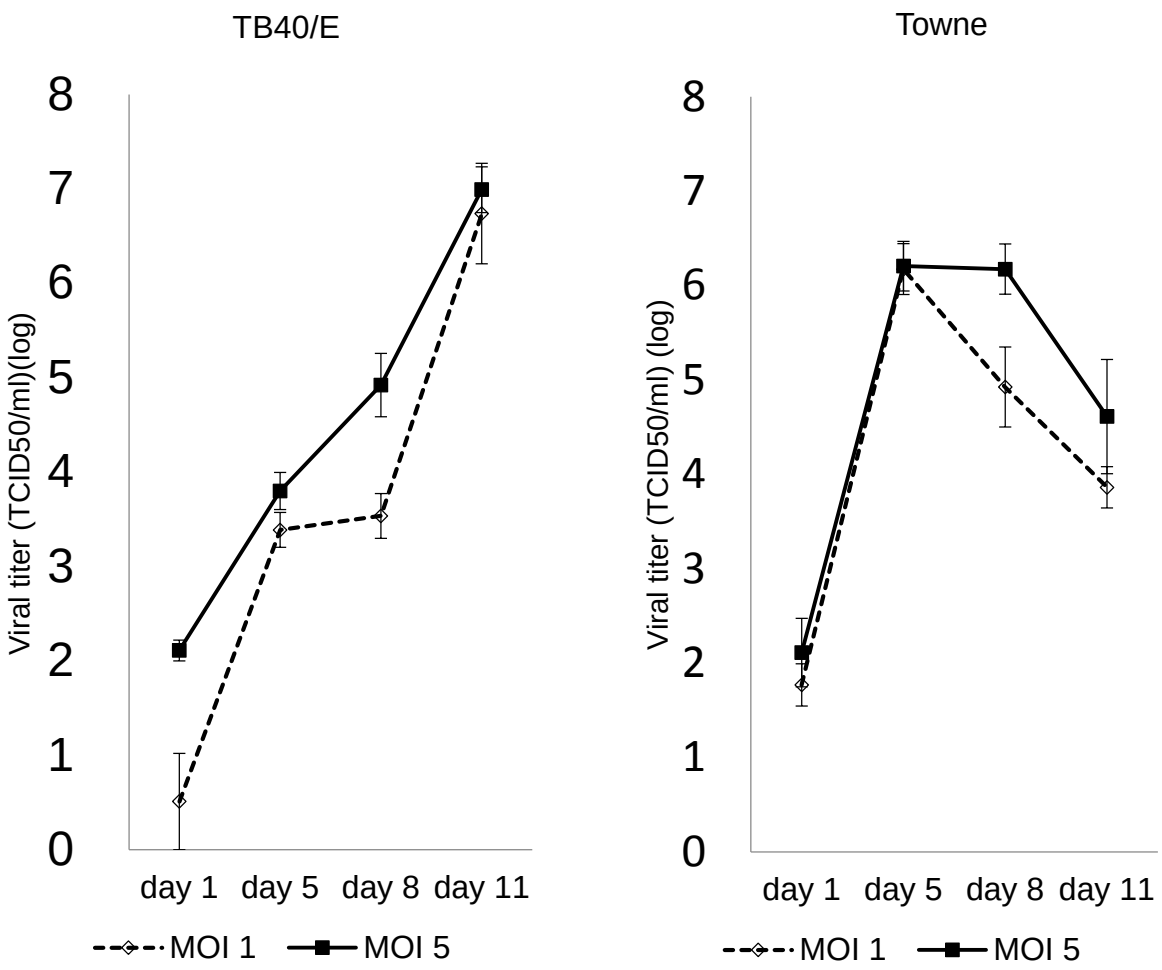

TB40/E
Towne
Viral titer (TCID50/ml)(log)
Viral titer (TCID50/ml) (log)
0
1
2
3
4
5
6
7
8
day 1
day 5
day 8
day 11
MOI 1
MOI 5

Figure.9