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Full Length Article

Differences in Gene Expression Profiles between Equine and Bovine Thyroid Epithelial Cells Attacked by FMDV

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Abstract

Horses (equine) are common livestock, which are unusually insensitive to the Foot-and-mouth disease virus (FMDV). Similarities and differences between bovine and equine attacked by FMDV may uncover the reasons for this insensitivity. In the current study, ETYECs (equine thyroid epithelial cells) underwent apoptosis earlier and at a higher rate than did BTYECs (bovine thyroid epithelial cells) after FMDV infection. Further analysis and verification of gene expression profiles showed significant differences in the PI3K-Akt signaling pathway and Toll-like receptor pathway between BTYECs and ETYECs. Importantly, FMDV activated the PI3K-Akt-Bim and PI3K-Akt-Bad/Bcl2 axes in ETYECs and BTYECs, respectively. The BTYECs became insensitive when expression patterns of TLR3 and MyD88 were changed and following changes in IRF7. Related changes in Bad, Bcl2 and Bim may indicate a complementary relationship between the PI3K-Akt signaling pathway and Toll-like receptor pathway. In summary, FMDV triggers apoptosis and IFNα/β anti-viral effects in ETYECs, but has anti-apoptotic effects and inhibits interferons in BTYECs making them suitable for production of FMDV. These results provide new insights into antiviral mechanisms.

Keywords: Equine; Foot-and-mouth disease virus; Pathogenesis; Signal pathway

Introduction

Foot-and-mouth disease (FMD) virus causes large economic losses when outbreaks. For example, in Taiwan, the FMD epidemic in 1997 resulted in a direct financial cost of about $379 million, in addition to related losses of trade and jobs. More seriously, the clinical symptoms and distribution of FMDV infection are gradually expanding. Many countries in the world continued to have foot-and-mouth disease outbreaks, such as Egypt, Nigeria, Cameroon, Bangladesh, Jordan and other places (Najafi *et al*. 2020). In the Middle East in 2018, researchers isolated virus from the throat and heart tissue of 2 young wild pigs with only clinical symptoms, which has never been found before (Karniely *et al*. 2020).

Only cloven-hoofed animals are infected by FMDV, and non-hoofed animals are not susceptible. However, the underlying mechanisms for such phenomenon remain unclear. A number of investigations on FMDV, such as enhancing the immune responses of livestock through various vaccines, correlation between host and pathogen, or pathways associated with FMDV, have been launched. However, little attention is given to the antivirus mechanism of horses though equine cells, which are the most suitable model for host-FMDV study.

In 1940, researchers reported that a three-day-old foal showed typical vesicular lesions in its buccal mucous membrane and coronary bands, which resembled FMD lesions. This finding raises the question of horse susceptibility to FMDV. Equine cells may be utilized in different immune mechanisms to clear invasive pathogens. Despite the information obtained regarding FMDV, the following aspects remain ambiguous: whether the equine cells, similar to cattle cells, employ integrin as its receptor; whether the infection results in apoptosis prevent the pathogen from further proliferation; and whether other mechanisms regulate gene expression in the equine cells. Here, by using gene expression profiles, we explore the underlying mechanisms behind the antivirus ability of equine cells. By doing so, we expect to find out the antiviral mechanism in cells of equine and further use these for the development of disease-resistant transgenic cattle.

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More significant in the long term is the establishment of FMD-virus-resistant cattle groups which can avoid the enormous economic losses caused by FMD.

Apoptosis induced by viruses is important for host defense because it facilitates the viruses removal and/or functions as a mechanism for viral replication (Kaminskyy and Zhivotovsky 2010). Most of the viruses generate anti-apoptotic factors that facilitate cell survival during the early stages of infection. For example, the nonstructural protein 1 of the influenza A virus induces PI3K activity to prevent premature apoptosis, thus supporting viral replication. Infection induced by Epstein–Barr virus and myxoma virus may be associated with reduced apoptosis (Markus and Wolfgang 2005), meanwhile human immunodeficiency virus 1 may be associated with enhanced apoptosis. The FMDV 2C protein is mainly associated with the endoplasmic reticulum. This protein appears as speckles and can induce apoptosis in BHK-21 cells. Two days post-infection, apoptosis can be detected in most of the tongues and hooves of infected swine, suggesting that FMDV infection may induce apoptotic cell death in vivo (Ku *et al*. 2005). Also, when infected with FMDV, bone marrow derived dendritic cells were induced to underwent apoptosis in a dose-dependent manner (Jin *et al.* 2007). However, it is not known whether apoptosis occurs in FMDV-attacked ETYECs.

Materials and Methods

Cells, virus, and antibodies

Asia 1/JS/2005, O/CHA/1999 and A/CHA/2009 FMDV strains were supplied by the National FMDV Reference Laboratory of China. The tested titers of these viruses were about 10^7–10^8 PFU/mL in BHK21 cells. BHK21 cells were purchased from ACCA.

A Kazakh horse of about three to five years old and a yellow cow of about two to three years old were euthanized. Tissues from their thyroids were treated as described previously. The isolated epithelial cells from the individual animals were planted into Petri dish with DMEM/F12 culture medium added with 10 ng/mL of EG (Life Technologies) and 10% newborn calf serum (Hyclone). The plated cells were placed on 12- and 24-well cell culture plate (Corning Costar) at concentrations ranging from 2.5×10^5 cells/cm^2 to 5×10^5 cells/cm^2.

The following primary antibodies purchased from Millipore were used: anti-Bad. Anti-MyD88, anti-Bim, anti-IRF7, anti-Akt1, anti-Akt3, and anti-Bcl2 were obtained from Abcam; anti-TLR3, anti-TLR9 were purchased from Santa Cruz.

Gene expression profiles

Total RNA was isolated using an RNase kit (Qiagen) from three replicate samples of controls and infected BTYECs and ETYECs by Asia 1/JS/2005, O/CHA/1999 and A/CHA/2009 FMDV strains at 2, 4, 8, 12, 24, 36, and 48 hpi. The microarray experiments were performed at Shanghai Bio Corporation. Microarray data analysis was conducted using the SBC Analysis system (http://sas.ebioservice.com/index.jsp).

Plasmids, siRNA, and drug treatment

Total RNA from infected calf/equine TYECs were used as template to synthesize cDNA. The PCR products were sub-cloned into IRES2-EGFP vector and hence pIRES2-EGFP-b-MyD88 and pIRES2-EGFP-e-TLR3 were produced.

The siRNAs of b-MyD88 and e-TLR3 were synthesized by TAKARA. The cells were pre-incubated with the indicated inhibitors Akt-X. After 24 h, the medium was replaced, and BTYECs were infected by FMDV. BTYECs were planted at a common density (10^5 cells/well) and were transfected using Lipofectamine 2000 in conformity to the manufacturer's instructions. After 24 h, the cells were infected and collected for experiments. Supernatants were also collected to test viral titers and measure cytokine production.

FMDV virus infection and replication

The virus infection process were processed as previously described (Wang *et al.* 2016). To be brief, BTYECs and ETYECs were infected with FMDV for 1 h at 1MOI. After the inoculum was removed away, the cells were washed with PBS and replaced with culture medium containing newborn calf serum. The cells were collected with TRIzol reagent at 2, 4, 8, 12, 24, 36, and 48 hpi. The Reed–Muench method was used to test viral titers.

Quantitative real-time PCR

Total RNA was isolated and was reversed to cDNA with PrimeScript RT Reagent Kit. The SYBR ExScript qRT-PCR kit (Takara) was used to perform Quantitative real-time PCR on ABI7500 instrument (Applied Biosystems, USA). The comparative CT method was used to analyze data.

Western blot analysis

Protein samples from infected ETYECs and BTYECs were ran in polyacrylamide gels (10%) and then transferred to polyvinylidene (Millipore) followed with blocking, antibody incubation. Signals were showed in ECL Western Blotting Analysis System.

Immunofluorescence analysis

TYECs at the concentration of 2×10^4 were plated on 12-well plates (Corning Costar) and then were infected with FMDV. The cells were fixed with paraformaldehyde (4%)

at room temperature for 10 min and followed with blocking/permeabilizing, and incubation with different primary antibody as well as Alexa Fluor 488-conjugated secondary antibodies.

Flow cytometric determination of apoptosis using annexin-V/PI

Apoptosis was detected using an annexin-V/PI binding kit (Santa Cruz) and analyzed with a flow cytometer (FACS Calibur, BD Biosciences).

Results

Infected ETYECs exhibit a higher rate of apoptosis than BTYECs

In the present study, ETYEC and BTYEC apoptosis was analyzed to determine whether FMDV induced apoptosis as this could function as a protective mechanism for virus clearance. Fig. 1 shows ETYEC and BTYEC apoptosis. The apoptotic rate of the infected equine epithelium was higher and occurred at an earlier stage, from 2 hpi (hour post-infection) to 12 hpi, in ETYECs than in BTYECs. These findings indicate that ETYECs triggered apoptosis at an earlier stage in order to resist viral infection.

Expression profiling of ETYECs and BTYECs during different post-infection time points reveals distinct biological phases

Suckling mice, cell cultures of calf kidneys, cell lines derived from baby hamster kidneys (BHK21), and Chinese hamster ovaries (CHO) are used as laboratory systems for growth and assay experiments on FMDV, however, these do not explain the difference in sensitivity to FMDV between equine and bovine. In 1966, Snowdon showed that high titers of FMDV could be obtained from primary monolayers of calf thyroid epithelia (TYE); in some instances, these titers were 100–1,000-fold more sensitive than established models. Thus, TYE can be used as an effective tool for the study of FMDV *in vitro*.

ETYECs and BTYECs were infected with three types of FMDV and samples were collected at seven time-points (2, 4, 8, 12, 24, 36, and 48 hpi) to construct gene expression profiles in order to determine key anti-viral attributes in ETYECs. Genes in which expression was upregulated or downregulated 2 fold in at least 1-time point were selected after Lowess sub grid normalization and filtering of missing values. In ETYEC samples, a total of 25,923; 25,923; and 30,560 genes were selected in type Asia 1/JS/2005, O/CHA/1999 and A/CHA/2009, respectively. In BTYEC samples, a total of 26,773; 26,773; and 26,773 genes were selected in type Asia 1/JS/2005, O/CHA/1999 and A/CHA/2009, respectively.

Among the genes in which expression changed, significant differences were noted in the virus-related process between ETYECs and BTYECs. According to the KEGG website, these genes were mapped using the Pathway Explorer software on pathways derived from the KEGG database and PI3K-Akt signaling and Toll-like receptor pathways were noted as related to antivirus processes in both changes in TLR3, TLR9, MyD88, IRF7, Akt3, Bcl-2- associated death promoter protein (Bad), Bcl2, and Bim. The biggest difference was the fact that, in ETYECs, the virus influenced the PI3K-Akt-Bim pro-apoptotic axis, while in BTYECs the PI3K-Akt-Bad-Bcl2-axis was activated. TLR3-IRF3/7-IFN α1/β1 and TLR9-Myd88-IRF3/7-IFN α1/β1 patterns were also different between BTYECs and ETYECs (Fig. 2). RT-PCR and Western blot analysis showed significant expression. The expression profiles showed minimal differences in genes among the different virus Noticeably, TLR3 was not detected in uninfected BTYECs or ETYECs, but was present in both FMDV-infected BTEYCs and ETYECs, though the time points for TLR3 expression were inconsistent between ETYECs and BTEYEs. Expression of TLR3 in ETYECs lasted from 2 hpi to 36 hpi, but was found only at 4, 8, and 48 hpi in BTEYEs. MyD88 was present in uninfected ETYECs, but not in BTYECs; MyD88 was only induced in BTYECs after infection (Fig. 3, 4). Thus, the TLR pathway, TLR3 and Myd88 were chosen for further study. The expression profiles showed minimal differences in genes among the different virus types; therefore, type A/CHA/2009 was used to infect the cells in subsequent experiments.

Replication of FMDV was prevented by both TLR3 overexpression and interference

TLR3 is involved in the recognition of viral double-stranded RNA, and is generated by most viruses during infection and is a replication intermediate for ssRNA viruses. In the present study, TLR3 was considered to be a candidate molecule for studying certain molecular characteristics since FMDV RNA undergoes a double stranded "replicative form".

Differences in TLR functions between ETYECs and BTYECs may be important for viral replication and/or inhibition. No obvious differences in TLR3 expression between carrier and non-carrier cattle may indicate that TLR3 mRNA is not affected by FMDV infection, that TLR3 is not required, or that TLR3 is required only transiently to generate effective antiviral responses to FMDV infection. In the further studies, TLR3 overexpression and interference influenced the levels of IRF7, IFNα1, IFNβ1, Bad, Bcl2 and Bim, but not IRF3 and MyD88. Virus titer testing showed that FMDV failed to replicate in BTYECs (Fig. 5). The current study supports the previous finding that TLR3 is activated instantaneously in cattle *via* a MyD88-independent pathway and that when this is interrupted FMDV replication is terminated.

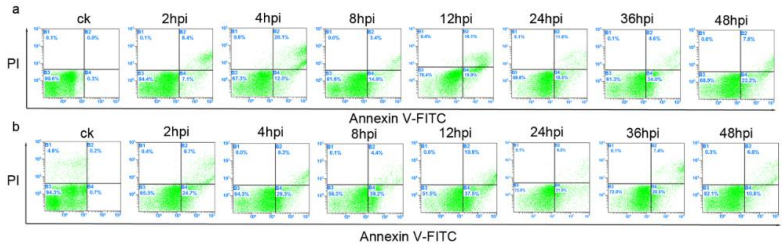

Fig. 1: Apoptosis of FMDV-infected ETYEC and BTYEC

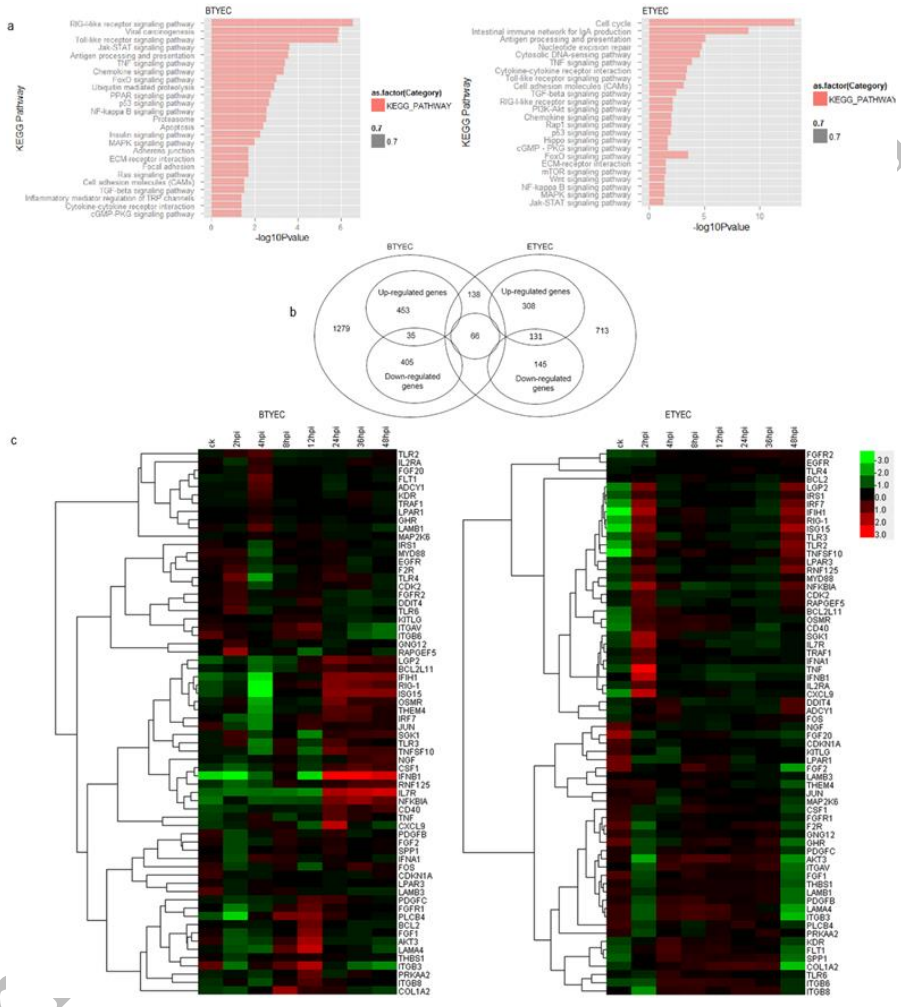

Fig. 2: FMDV infection influenced gene expression in ETYECs and BTYECs. (**a**) Many pathways were activated after FMDV infection in both BTYECs. Only those related pathogens and signaling are listed (+/− 2-fold, $P \leq 0.05$). (**b**) Genes related to various biological functions were enriched (+/− 2-fold, $P \leq 0.05$) through K-means clustering. In ETYECs and BTYECs, 713 and 1279 genes, respectively, that were significantly upregulated or downregulated were selected. Large differences were found in 66 genes, among the 138 genes common to both BTYECs and ETYECs. These genes were mainly mapped using the Pathway Explorer software on pathways derived from the KEGG database. **c**) Heat map of genes for which significant differences in expression were found between BTYECs and ETYECs

Overexpression of MyD88 prevents FMDV replication, however interference of MyD88 does not prevent FMDV replication in BTEYCs

MyD88 and MyD88-dependent immune activation has been reported for many viral infections, such as Hepatitis C and influenza A. However, studies on the role of MyD88 in FMDV infection have mainly focused on the activation of MyD88-dependent induction of interferon (IFN) approach to enhance the antiviral ability of vaccines. These studies suggested that MyD88 genes might be activated by FMDV infection. However, the outcome of MyD88 expression prior

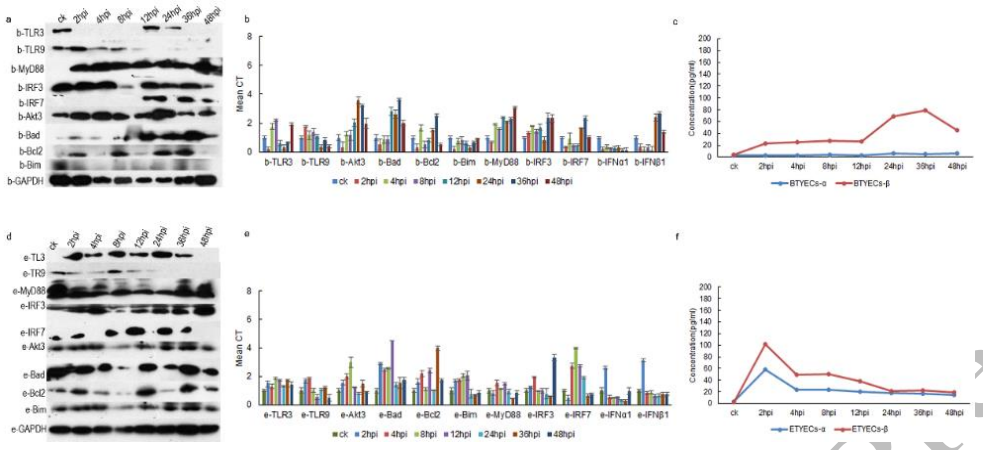

Fig. 3: Verification of selected genes in BTYECs and ETYECs. There were substantial differences in the levels of TLR3, MyD88, IRF7, Akt3, Bcl2, and Bim protein, as well as mRNA between BTYECs (**a**, **d**) and ETYECs (**b**, **c**). IFNα1 and IFNβ1 (**c**, **d**, **e**) were also activated by FMDV infection in both BTYECs and ETYECs

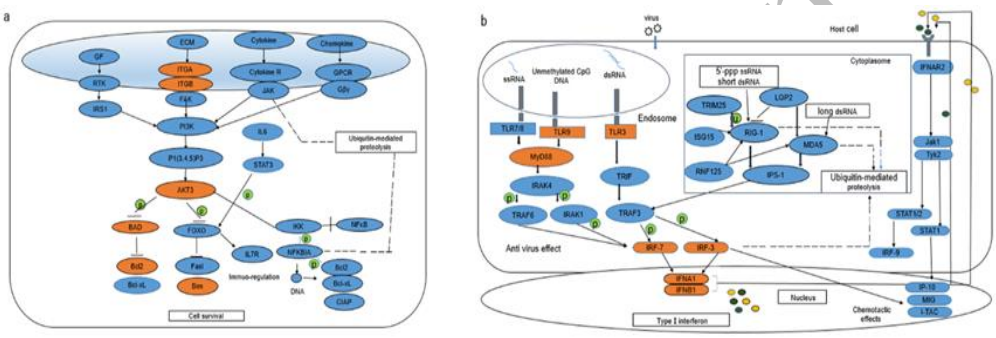

Fig. 4: Discrepancy in the antiviral ability of BTYECs and ETYECs was mainly related to two pathways: the PI3K-Akt3 signaling pathway and TLR signaling pathway

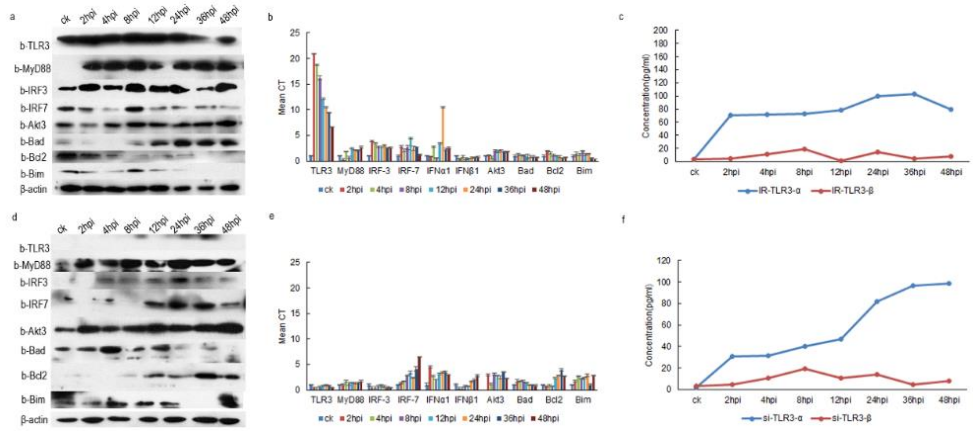

Fig. 5: The role of TLR3 in the IFN response against FMDV infection. Both (**a**–**c**) IRF-7 (IR-TLR3) overexpression and (**d**–**f**) IRF7 (si-TLR3) interference influenced the production of IFNα, IFNβ, and Bim

to viral infection and/or whether MyD88 plays a critical antiviral role in ETYECs have not yet been elucidated. BTYECs were transiently infected with overexpression vector (IRES2-EGFP-b-MyD88) and downstream expression was monitored. Compared to normal FMDV-infected BTYECs, IRF7 and Bcl2 increased while IRF3 decreased at all time- points. IFNα levels increased from 12 to 48 hpi; however, IFNβ levels did not change at all time points. Bim expression increased in FMDV-infected ETYECs increased from 2 to 48 hpi. The virus titer

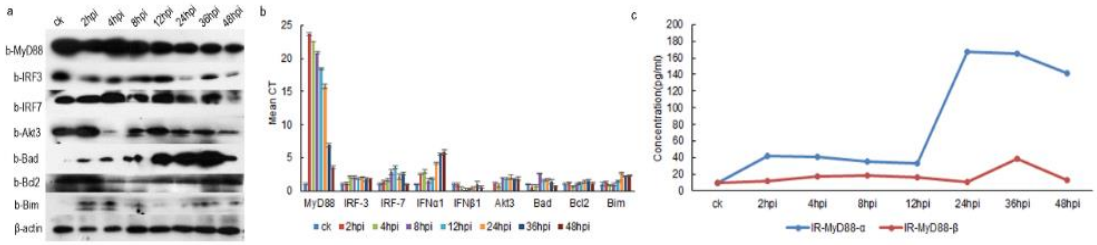

Fig. 6: Overexpression of MyD88 and the IFN response to FMDV infection. (**a**, **b**) mRNA levels were determined by quantitative RT–PCR and Western blot analysis. **c**) Supernatants were collected at 2, 4, 8, 12, 24, 36 and 48 hpi after infection and IFN-α/β levels were measured by ELISA. Overexpression of MyD88 increased IRF-7, Bim, and IFNα expression levels

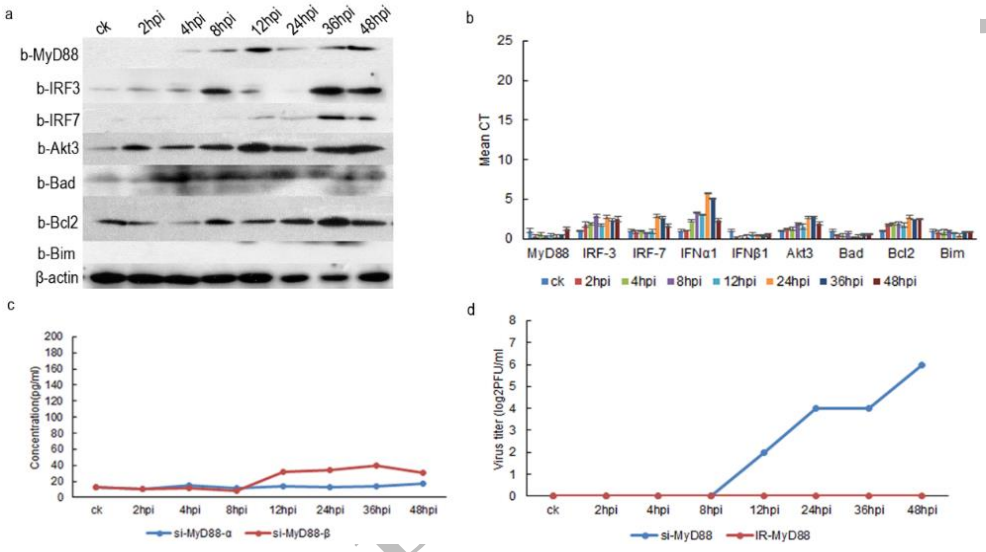

Fig. 7: Interference of MyD88 genes prohibits FMDV replication in BTYECs. (**a**, **b**) mRNA levels were determined by quantitative RT–PCR and Western blot analysis. (**c**) Supernatants were collected at 2, 4, 8, 12, 24, 36 and 48 hpi after infection and IFN-α/β levels were measured by ELISA. (**d**) MyD88 interference did not prevent FMDV infection, whereas MyD88 overexpression prevented viral replication

demonstrated that the life cycle of FMDV was not finished (Fig. 6). These results indicate that MyD88 contributes to antiviral ability and that a relationship may exist between MyD88 and, Bim.

During infection, more than one kind of virus triggered MyD88-dependent mechanisms (Mccarthy *et al.* 2020; Wang *et al.* 2020). To verify whether MyD88 is the key element that determines the fate of invading FMDV in ETYECs, BTYECs were treated with small interfering RNA (si-MyD88). IRF7 and IFNα expression levels decreased along with MyD88 levels. IRF3 levels were not influenced, though Bad and Bcl2 expression levels changed. Levels of Bad decreased at all time-points and Bcl2 continued to be expressed at a lower level than in normal BTYECs. Bim expression was not determined (Fig. 7). IFNβ levels did not change at all time points. The FMDV virus titer did not change.

Overall, these results indicate that IRF7 is essential for the MyD88-dependent induction of IFN-α/β genes through TLR9. Overexpression of MyD88 inhibited viral replication, whereas the absence of MyD88 did not influence virus replication, but the expression of Bim. The above observations indicate that MyD88 contributes to the induction of either IFN-α/β or Bim and to changes in Bad and Bcl2 in ETYECs.

Discussion

FMDV replicates itself by suppressing induction of antiviral molecules at the transcriptional and/or translational levels. FMDV virus may also interfere with secretory pathways to inhibit the releasing of IFNs and other cytokines, which can adversely influence virus replication and dispersion. FMDV can also circumvent premature apoptosis-mediated cell death due to its very short replication cycle, thereby resulting in rapid viral production. Mechanisms in ETYECs that can prevent successful viral replication may enhance host protective responses through multiple steps to ensure ineffective replication.

The IFN signal pathway can be activated by viral infection and are vital in the host's innate antiviral response. In ETYECs, interferon production is regulated through the

TLR9-MyD88-IRF7 and TLR3-IRF7 pathways. Activation of transcription factors, such as members of the nuclear factor kappa B family , IRF7, IRF3 (Sin *et al.* 2012), and MyD88, which bind to specific sequences present at IFN promoter regions, can regulate the expression of type I IFN.

During viral infections and autoimmune diseases, virus-like particle enhanced the germinal center response through TLR/MyD88 signaling in B cells (Tian *et al.* 2018). TLR/MyD88 signaling protects against acute rotavirus infection. All these data indicate that the TLR–MyD88 pathway is essential for host defense. TLR-mediated IFN-α induction requires the formation of a complex consisting of MyD88, TRAF6, and IRF7. In the current study, MyD88 was consistently expressed in ETYECs and when ETYECs were infected, the expression of IRF7 was stimulated immediately. This continuous action ensured the production of IFNα/β. Like the induction of IFN genes by viruses in MEFs, a positive feedback mechanism may also constitute an essential aspect of ETYECs. Expression of IRF7 is greatly induced and robustly amplified by IFN produced from Influenza virus infection (Wu *et al.* 2020), which confirm our results.

Plasmacytoid dendritic cells (pDCs) are the main producers of type I IFNs in response to endosomal TLRs, such as TLR9. Induction of IFN-α/β through the activation of TLR9 constitutes a critical aspect of antiviral activity. CpG-DNA activates innate immunity through TLR9 and stimulation of innate immunity with CpG also protects against a number of viral infections, such as herpes viruses, and birnavirus. Spatiotemporal regulation of MyD88–IRF7 signaling is important for high-level IFN induction in answer to TLR9 activation. IFN inducers have been well-known for their capacity to enhance innate protection against FMDV using CpG. IRF7 is a transcription factor that amplifies IFN-α/β production in response to viral invasion. pDCs can robustly produce IFNα in response to simian immunodeficiency virus infection. Decrease of IRF7 and NF-kB phosphorylation are related to decline of the ability to produce IFNα (Mitchell *et al.* 2020),while deletion of IRF7 completely lead to abolishment of IFNα production after influenza A viruses infection (Hatesuer *et al.* 2017). These results imply that IRF7 has a critical position in the MyD88-dependent pathway. MEFs lacking Myd88 retain the ability to induce IFNα/β mRNAs in response to herpes simplex virus type 1, and vesicular stomatitis virus, thus indicating a MyD88-independent, but IRF7-dependent pathway for IFNα/β gene induction. These results suggest the possibility of TLR3-mediated interferon production in ETYECs.

Members of the Bcl-2 family can be divided into two teams: pro-apoptotic and anti-apoptotic. Anti-apoptotic team included Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and A1, meanwhile pro-apoptotic team owned Bak (Bcl-2 homologous antagonist/killer protein), Bax (Bcl-2-associated X protein), Bim, tBid, Bad, Puma and Noxa (Jullien *et al.* 2020). Induction of apoptosis requires the activation of members of both of these two groups. For example, when infected by HIV, the expression of BCL2, BCLXL in macrophages rose, BIM translocated to the mitochondria, also, the expression of BAD and BAX reduced (Campbell *et al.* 2019). Severe fever with thrombocytopenia syndrome (SFTS) virus infection triggered BAK upregulation and BAX activation, leading to cytosolic release (Li *et al.* 2020). Thus, it is possible that silence of Bim significantly inhibited apoptosis, while overexpression of Bcl-2 remarkably protected cells from apoptosis (Lee and Fairlie 2019).

The organism is a complete system, thus, signal pathways do not function independently. For example, PI3K selectively influences production of type I IFN by adjusting IRF7 in pDCs. Interferon-γ can promote double-stranded RNA-induced TLR3-dependent apoptosis in airway epithelial cells (Gan *et al.* 2016). Stimulation of TLR3 by viral dsRNA may activate PI3K, subsequently protecting dsRNA-stimulated or virus-infected cells from apoptosis, thus further facilitating virus propagation. In addition, the IFN-β production is strongly impaired by inhibitors of PI3K. KSK-CpG induces apoptotic cell death in A20 lymphoma cells partly by increasing TLR9 levels. Thus, in BTYECs, a combination of the antiviral effects of IFNα1/β and the pro-apoptotic role of Bim are possible and feasible.

Conclusion

Numerous genes have been described that are significantly different from ETYECs and BTYECs. These are related to other signal pathways and are not discussed in this study. For example, laboratory of genetics and physiology 2, melanoma differentiation-associated gene 5 inhibit TLR-independent sensing of viral replication via the retinoic acid-inducible Gene-I and are also involved in antiviral action. NF-κB inhibitor alpha is the target of degradation by phosphorylation-dependent ubiquitination and can strictly control the activation of NF-κB. These genes must be studied more extensively. Furthermore, genes in at least three signaling pathways have been found to lead to ubiquitin-mediated proteolysis, and these genes were activated when ETYECs were infected. Thus, further research is needed.

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