Mechanisms of Enterovirus 71 Antagonizing Type I Interferon Response

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Abstract

Enterovirus 71 (EV71) is a typical positive strand RNA virus that usually causes hand, foot and mouth disease (HFMD). Acute EV71 infection may result in severe neural syndromes and even death especially among young children. To date, no effective treatment is available for EV71 infection.

Type I interferon (IFN), as the first line of host immune response, is critical in mediating viral clearance, host defense, and adaptive immunity development upon virus invasion. Recominant IFNs are also applied to treat and control several clinical viral infections. In case of EV71, previous studies showed that conventional IFNs fail to treat EV71 infection in mice. It suggests that virus has developed mechanisms to escape from type I IFN response. Uncover the underling mechanisms would help us to develop strategies to combate EV71 infection.

In this thesis, I firstly characterized the viral kinetics of EV71 in infected human rhabdomyosarcoma cells and analyzed the correlation between the cellular type I IFN level and viral replication. The induction of IFN-β was observed during viral infection. However, viral replication was not restricted and quickly led to a peak post infection. These results indicated that EV71 infection sensed by host cells could activate the type I interferon production, while virus developed unkown mechanisms to antagonize the IFN response. Secondly, by analyzing with RT-PCR, the mRNA level of IFN stimulated genes (ISGs) were demonstrated to be repressed in EV71 infected cells after treated with recombinant IFN-α when compared with mock infected control. Thirdly, subsequent

analysis showed that EV71 inhibited the type I IFN response by blocking the IFN-mediated phosphorylation of STAT1 and STAT2. Fourthly, EV71 was demonstrated to inhibit Jak/STAT signaling by promoting internalization and degradation of type I IFN receptor subunit IFNAR1. Fifthly, 2A protease encoded by EV71 was identified as an antagonist of the type I IFNs and ecotopic expression of $2A^{pro}$ led to repress the expression of IFNAR1, led to inhibition of the phosphorylation of Jak1, Tyk2, STAT1 and STAT2. Finally, the site-direct mutation indicated that the protease activity of $2A^{pro}$ was essential in this process, although the $2A^{pro}$ could not directly cleave IFNAR1 both *in vitro* and *in vivo*.

Taken together, this study for the first time proved that EV71 was able to inhibit the cellular type I IFN response by reducing IFNAR1 level. Moreover, 2A protease encoded by EV71 was demonstrated to function as the antagonist to the IFN signaling. These data provides new knowledge of EV71 to block the host innate immune response, and may facilitate development of new antiviral therapies for EV71 or other picornaviruses infections.

摘要

腸病毒 71 型 (EV71) 是一種單鏈的核糖核酸病毒。病毒感染易引發手足口病,急性感染對兒童來說更可能引起腦炎、肺水腫及肺出血進而導致死亡。目前,針對 EV71 感染尚沒有有效的治療方式。I 型幹擾素是機體細胞對病毒感染或各種生物誘生作用反應產生並分泌的一類具有多種生物活性的糖蛋白, 可啟動別的基因亞群進行轉錄, 從而具有抗病毒和免疫調節作用。因此重組的 I 型幹擾素常用於臨床的抗病毒治療,但之前研究結果表明在小鼠和細胞模型中,常用的幹擾素並不能有效的治療和抑制 EV71 病毒感染。這表明 EV71 病毒可能通過某些機制來逃逸 I 型幹擾素引起的免疫反應,而揭示 EV71 病毒的逃逸機制將有助於將來的抗病毒治療。 本論文首先對 EV71 病毒的感染動力學進行了詳盡的描述，並且分析了病毒感染過程中 I 型幹擾素與病毒複製的關係。研究發現病毒感染可以引起幹擾素 β 的產生,但是病毒的複製並沒有因此受到抑制反而在感染後很快達到複製高峰。這說明病毒感染雖然被細胞的免疫系統識別但是同時通過某些機制逃逸 I 型幹擾素引起的免疫反應。隨後我們對幹擾素通路下遊的基因亞群(ISGs)進行了分析。與對照相比,EV71 感染可以顯著抑制內源或者外源幹擾素激活的 ISGs 轉錄。通過對 I 型幹擾素通路信號元件的進一步分析,發現 EV71 病毒通過抑制 STAT1 和 STAT2 的磷酸化而抑制幹擾素引起的免疫反應。隨後的實驗證明 EV71 對 Jak/STAT 信號通路的抑制是通過加速幹擾素受體 IFNAR1 的內吞和降解來實現的。為進一步瞭解病毒的逃逸機制,我們對 11 個病毒蛋白進行了相關分析,發現病毒編碼的 2A 蛋白可以

拮抗 I 型幹擾素通路。與病毒感染相似，2A 蛋白也是通過抑制 I 型幹擾素受體 IFNAR1 進而抑制幹擾素通路中 JAK1 和 Tyk2 的磷酸化繼而抑制下遊 STAT1 和 STAT2 的磷酸化, 最後抑制通路下遊抗病毒基因群轉錄的啟動。最後,體外和細胞內的實驗結果進一步證明了 2A 對細胞內 IFNAR1 蛋白水準的抑制並不是通過直接的剪切作用但是仍然依賴于 2A 的蛋白酶活性。綜上所述,本論文闡述了 EV71 病毒抑制 I 型幹擾素通路的具體機制,併發現病毒編碼的 2A 蛋白通過抑制細胞內 I 型幹擾素的相關受體水準來達到逃逸幹擾素免疫反應的作用。

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Publications

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Journal Articles:

1. Lina Yi, **Jing Lu**, Hsiang-fu Kung, Ming-Liang He. The virology and developments toward control of human enterovirus 71. *Critical Reviews in Microbiology*. 2011, 1-15.

2. **Jing Lu**, Yaqing He, Lina Yi, Hong Zan, Hsiang-fu Kung, Ming-Liang He. The viral kinetics of Enterovirus 71 in human rhabdomyosarcoma cells. *World Journal of Gastroenterology* (Accepted).

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Table of Contents

Abstract ii

摘要 iv

Acknowledgement vi

Publications vii

List of Tables and Figures xii

List of Abbreviation xiv

Chapter 1 Introduction 1

1.1 EV71 2

1.1.1 Epidemiology of EV71 2

1.1.1.1 Early epidemic activity 2

1.1.1.2 EV71 in the Asia-Pacific region 3

1.1.2 Classification and molecular epidemiology 6

1.1.3 Genome, lifecycle and kinetics of EV71 9

1.1.4 Clinical features of EV71 infection 13

1.1.5 Pathogenesis 16

1.1.6 Controls of human enterovirus 71 18

1.1.6.1 Vaccines 18

1.1.6.2 Antiviral drugs 20

1.2 Type I interferon 22

1.2.1 Types of interferons 22

1.2.2 The production of type I interferon 24

1.2.2.1 Toll-like Receptors 26

1.2.2.2 TLR signaling pathway in virus infection 28

1.2.2.3 RIG-1 like Receptors 29

1.2.2.4 RIG-1/MDA5 Signaling 30
1.2.2.5 Transcriptional activation of type I IFNs 31
1.2.3 Type I IFN signaling pathway 32
1.2.3.1 Type I IFN receptors 32
1.2.3.2 JAK/STAT signaling 33
1.2.4 Interferon induced antiviral proteins 35
1.2.4.1 dsRNA-dependent protein kinase R 35
1.2.4.2 2'-5' OAS and RNaseL 36
1.2.4.3 The Mx proteins 37
1.2.4.4 ISG15, ISG54 and ISG56 38
1.2.5 Type I IFN induced antiviral status 40
1.2.5.1 IFN induced cell apoptosis and cell cycle arrest 40
1.2.5.2 Immunomodulation 41
1.3 Viral evasion of type I IFN response 42
1.3.1 Viral inhibition of IFN production 43
1.3.1.1 Interfere with PRR activation 43
1.3.1.2 Inactivation of the IFN transcriptional factors 45
1.3.2 Viral inhibition of IFN signaling 48
1.3.3 Antagonists encoded by members of picornavirus family 51
1.3.4 Thesis hypothesis and objective 53
Chapter 2 Materials and Methods 54
2.1 Cells and Virus propagation 55
2.2 Viral infection and cytopathic effects assay 55
2.3 MTT assay 56
2.4 Plasmids 56
2.5 Transfection and cell treatment 59

2.6 RNA extraction and cDNA synthesis 59
2.7 Protein expression and purification 60
2.8 Direct cleavage assays of $2A^{pro}$ 61
2.9 Real-Time Quantitative PCR 61
2.10 Western Blotting analysis 64
2.11 Immunoprecipitation 65
2.12 Immunofluorescence 65
2.13 Statistical Analysis 66
Chapter 3 Results 67
3.1 Viral Kinetics 68
3.1.1 Cytopathic effects and the kinetic of cell viability 68
3.2 EV71 antagonize type I IFN response 78
3.2.1 EV71 infection activated Type I IFN production but inhibited ISGs activation 78
3.2.2 EV71 antagonized the antiviral effect of IFN-α treatment 81
3.2.3 Phosphorylation of STAT1 and STAT2 was inhibited by EV71 84
3.2.4 EV71 Blocked IFN-mediated Jak/STAT signaling by reducing IFNAR1 protein level 88
3.2.5 EV71 promote internalization of IFNAR1 92
3.2.6 Identification 2A as an antagonist of IFN signaling 97
3.2.7 2A Blocks Type I IFN-mediated phosphorylation of STAT proteins and Janus kinases by reducing IFNAR1 99
3.2.8 Inhibition of IFN signaling is depend on the protease activity of $2A^{pro}$ 101
3.2.9 $2A^{pro}$ can not directly cleave IFNAR 103
Chapter 4 Discussion 106
4.1 EV71 viral kinetics in RD cells 107
4.2 EV71 antagonizes type I IFN response 110
4.3 Future studies and perspective: 115

References 119

List of Tables and Figures

Table 1.1.2	Enteroviral species and types	8
Table 2.4	Primer used for cloning	57
Table 2.9	Primers for Quantitative Real-time PCR Analysis	63
Figure 1.1.1.2	The reported HFMD cases and related death in China from 2007 to 2010	5
Figure 1.1.3.1	Schematic of the picornavirus genome, the polyprotein products and their main functions	11
Figure 1.1.3.2	Summary of the picornavirus life cycle	12
Figure 1.2.2	Overview of signaling pathways leading to IFN-β induction	25
Figure 1.2.3.2	Signaling pathway activated by IFN- α/β	34
Figure 3.1.1	Cytopathic effects and kinetics of cell viability upon EV71 infection	69
Figure 3.1.2	The kinetics of EV71 Replication	71
Figure 3.1.3	The kinetics of virus VP1 protein synthesis	73
Figure 3.1.4	The kinetics of EV71 virus package	75
Figure 3.1.5	The kinetics of EV71 virus secretion	77
Figure 3.2.1	EV 71 infection induced IFN-beta response while antagonized the induction of ISGs	80
Figure 3.2.2	EV71 antagonized the antiviral effect of IFN-α treatment	82
Figure 3.2.3	EV 71 infection inhibited IFN induced STAT phosphorylation	85
Figure 3.2.4	EV71 Blocked IFN-mediated Jak/STAT signaling by reducing IFNAR1 protein level	90
Figure 3.2.5	EV71 promote internalization of IFNAR1	94

Figure 3.2.6	Inhibition of IFN-induced MxA activation by EV71 proteins....	98
Figure 3.2.7	2A Blocks Type I IFN-mediated phosphorylation of STAT proteins and Janus kinases by reducing IFNAR1.....	100
Figure 3.2.8	Protease activity is required for 2A inhibiting IFN signaling.....	102
Figure 3.2.9	$2A^{pro}$ can not directly cleave IFNAR.....	104
Figure 4.2	Hypothetical model for regulation of IFNAR1 degradation.....	118

List of Abbreviation

AFP: acute flaccid paralysis

AP-1: activator protein-1

BDV: Borna disease virus

BVDV: Bovine viral diarrhea virus

CA16: Coxsackievirus A16

CARDs: caspase activation and recruitment domains

CDK: cyclin-dependent kinase

CMV: cytomegalovirus

CNS: central nervous system

CPE: cellular pathogenesis

CpG: deoxycytidyl-phosphate-deoxyguanosine

CREB: cAMP response element binding

CSFV: Classical swine fever virus

CTLs: cytotoxic T lymphocytes

DCs: dendritic cells

EBV: Epstein-Barr virus

eIF2α: eukaryotic translational initiation factor 2

eIF4G: eukaryotic translation initiation factor 4 gamma

EMCV: encephalomyocarditis virus

EV71: enteroviruses 71

GCN5: general control of amino acid synthesis 5

GTP: guanine trisphosphate

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HFMD: hand, foot, and mouth disease

HHV-8: Human herpes virus 8

HIV: Human immunodeficiency virus

HMG: high mobility group

HPV: Human papillomavirus

IFNs: interferons

IKKi: inducible IκB kinase

IL-29: interleukin-29

IPS-1: interferon-β promoter stimulator 1

IRES: internal ribosome entry site

IRF3: interferon regulatory factor 3

ISGs: interferon-stimulated genes

JAK: Janus kinases

LPS: lipopolysaccharide

LRRs: leucine-rich repeats

MAVS: mitochondria-associated protein

MAVS: mitochondrial antiviral signaling protein

MIF: migration inhibitory factor

MRI: magnetic resonance image

NK cell: natural killer cell

p.i.: post infection

PAMPs: pathogen-associated molecular patterns

pDCs: plasmacytoid dendritic cells

PFU: plaque-forming unit

PKR: protein kinase R

PRDs: positive regulatory domains

PRR: pattern recognition receptor

qRT-PCR: quantitative real-time PCR

RABV: Rabies virus

RD: rhabdomyosarcoma

SOCS: suppressor of cytokine signaling

STATs: signal transduction and activator of transcription

TBK1: TANK binding kinase 1

TIR: toll/interleukin 1 receptorhomology

TLRs: toll-like receptors

UPR: unfolded protein response

UTRs: untranslated regions

VACV: Vaccinia virus

VLP: virus-like particle

WNV: West Nile virus

Chapter 1 Introduction

1.1 EV71

Enteroviruse 71 (EV71) is a non-enveloped, single-stranded, positive-sense RNA virus from the enterovirus genus in the family of Picornaviridae [1]. EV71 infection was a major cause of outbreaks of hand, foot, and mouth disease (HFMD), most frequently affecting infants and young children [2,3]. As an typical neurotropic virus, EV71 has a propensity to cause neurological disease during acute infection [4,5] and the neurological complications, which occasionally cause permanent paralysis and even death [6,7]. In order to have a general idea on EV71 and viral infection, six bodies of literature are reviewed in this chapter.

1.1.1 Epidemiology of EV71

1.1.1.1 Early epidemic activity

EV71 was first identified in 1969 in California in an infant suffering from encephalitis [8]. In the next three years, this type of virus was continuously isolated from 23 further cases of severe neurological disease in this area [9]. Thereafter, occurrences of EV71 infections were occasionally reported outside of the USA. For example, in Australia, the EV71 was first identified between 1972 and 1973 when an epidemic of aseptic meningitis in Melbourne occurred [10]. Although the first isolation of EV71 virus was in the early 1970s, the real recognition of EV71's neurological toxicity was in the late 1970s when large outbreaks of EV71 infections were occurred in Bulgarian and Hungary. During the epidemic, EV71 was identified as the cause of many disastrous neurological diseases such as aseptic meningitis, encephalitis, and acute cerebellar ataxia [11,12]. In Bulgarian, the first outbreak of EV71 was reported between May and

September in 1975. EV71 infection resulted in 545 cases (77%) of aseptic meningitis, 149 cases (21%) of AFP (acute flaccid paralysis), and even worse, 44 cases of deaths were reported [13]. Children under 5 years of age accounted for most of paralytic cases (93%). In many cases of bulbar disease, the rapid fatal was reported since the death occurred between 10 and 30 hours after illness onset. Three years later after the first outbreak in Bulgarian, the second large EV71 epidemic occurred in Hungary and viral infection result in 1550 cases (826 aseptic meningitis, 724 encephalitis) and 47 deaths [12].

1.1.1.2 EV71 in the Asia-Pacific region

In the Asia-Pacific region, the EV71 infection was more severe than the other areas. The initial cases of EV71 infection were reported during HFMD epidemics in Japan and Australia in 1970s [10,14,15]. Thereafter, small epidemics were recorded in Hong Kong (1985) and Australia (1986), in which several cases of AFP were identified [16,17]. However, since late 1990s the densely populated Asia Pacific region has become the hotspot for EV71 epidemics: Taiwan, Singapore, Malaysia, China, Vietnam, and Australia have experienced recurrent various sizes of EV71 epidemics. In 1998, a large EV71 epidemic occurred in Taiwan [18,19,20]. A total of 130,000 cases of HFMD and herpangina caused by both EV71 and CA16 infections were reported. Virological and histopathological studies indentified that EV71 was responsible for ~70% of HFMD cases during this epidemic. Besides, cases with acute EV71 infection were also identified, which initially manifested as brainstem encephalitis , and further developed into acute pulmonary oedema or other neurological diseases. It is recorded that during the 1998 Taiwan EV71 epidemic, EV71 infection resulted in 405 cases of

severe neurological disease of which 78 deaths were included [21,22,23]. In China, EV71-associated HFMD epidemics were initially occurred in Hubei Province during the winter of 1987 [24]. No cases of AFP or aseptic meningitis were identified during this epidemic. Local outbreaks have been continuously reported since late 1990s. Recently, HFMD caused by EV71 is becoming an emerging infectious disease, which poses a major threat to China. The data from health ministry showed that the infected cases and deaths caused by EV71 were increased year by year from 2007 to 2010 (Fig 1.1.1.2). In 2008, the outbreak of HFMD caused nationwide anxiety, around 490,000 infections and 126 deaths were reported. The HFMD caused by EV71 was mainly occurred in rural areas and nearly 80% of cases were reported over 10 provinces and autonomous regions, including Henan, Shandong, Jiangsu, Guangxi, Anhui, Guangdong, Hebei, Hubei, Hunan and Zhejiang (http://news.xinhuanet.com/english/2009-04/10/content_11165126.htm). Compared with outbreak in 2008, the EV71 epidemicseemed to come earlier and the number of HFMD disease cases reached a peak from May to July in 2009, and recurrence was reported in many provinces which underwent the 2008 outbreaks. There were 1,155,525 HFMD cases among which 353 deaths were recorded in 2009 in China, while the percentage of EV71 infections among HFMD is not very sure because the virological technique is not well defined. In 2010, the largest EV71-caused HFMD epidemic occurred in China. According to the most recently data reported by Chinese government, a total of ~1,700,000 cases of HFMD and herpangina were identified with more than 900 deaths (http://www.moh.gov.cn/publicfiles/business/htmlfiles/zwgkzt/pyq/list.htm).

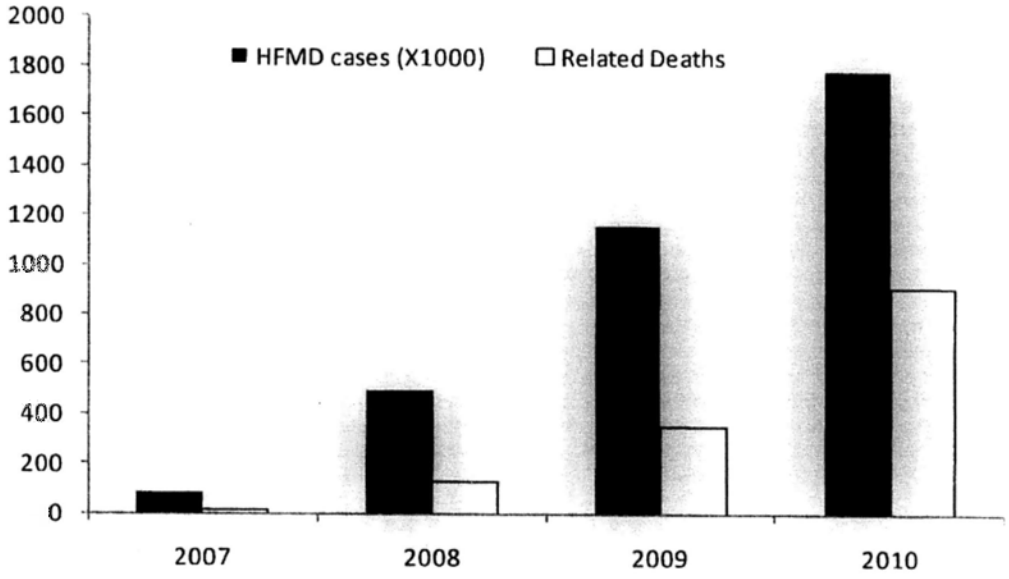


Figure 1.1.1.2 The reported HFMD cases and related death in China from 2007 to 2010.

1.1.2 Classification and molecular epidemiology

EV71 belongs to the enterovirus genus in the family of Picornaviridae. The Picornaviridae family contains 12 genera of RNA viruses including Aphthovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Teschovirus, Tremovirus, Avihepatovirus, Senecavirus, and Sapelovirus [1]. The first seven genera are currently recognized as human pathogens. For Enteroviruses, the species mainly include poliovirus, echovirus, coxsackievirus A and B, and other different serum types of enterovirus [1]. Based on their molecular and biological properties, the Enteroviruses can be further classified into at least 90 subtypes and separated into mainly ten species (Table 1.1.2) [25].

In previous studies, EV71 was mainly divided into three genotypes (A, B, and C) and different genotypes of EV71 circulated at different regions. The phylogenetic analysis of EV71 is according to the sequence of capsid protein VP1 which is conserved in recombination and has a high degree of diversity [26]. Using this region for analysis, each group of EV71 has at least 15% divergence from the others [27]. In group A, there is only one member, the prototype BrCr strain, which was first identified in California, USA, in 1970. Thereafter, this strain was not reported outside the USA until 2008 when it was isolated in Anhui province of central China [28]. Genotypes B can be further sub-divided into genogroups B1–B5 [29,30]. The B group, in particular B3 and B4, were identified as the major causes of the EV71 epidemic in Malaysia (1997) and Singapore (1999) [30,31]. Genotypes C contains genogroups C1–C5 [32,33]. This genotype virus was mainly circulates in Asia-Pacific region and was recognized as the predominant one in recent large epidemic in Taiwan, mainland China and Vietnam

[34,35,36]. The low-level circulation of subgroup C1 viruses was initially reported sporadically in the mid-1980s [27,37]. The large outbreak of subgroup C2 viruses was reported in Taiwan (1998), and Australia (1999) [31,34,38]. Subgroup C3 caused the outbreak in Japan (1994) and in Korea (2000) [31,39,40]. Subgroup C4 has been reported in Japan, Vietnam, and Taiwan. Since 2000, the C4 genogroup EV71 was isolated from sporadic infections in mainland China which indicated it became the predominant circulating subtype in mainland China [34,35,36]. Subgroup C5 has been reported in southern Vietnam and Taiwan [35,41].

Enterovirus species	Types
Human enterovirus A	Human Coxsackievirus A2–8, 10, 12, 14, 16. Human enterovirus 71, 76, 89–92.
Human enterovirus B	Human Coxsackievirus A9, B1–6. Human echovirus 1–9, 11–21, 24–27, 29–33. Human enterovirus 69, 73–75, 77–88, 93, 97, 98, 101, 106, 107.
Human enterovirus C	Human poliovirus 1–3. Human Coxsackievirus A1, 11, 13, 15, 17–22, 24. Human enterovirus 95, 96, 99, 102, 104, 105, 109.
Human enterovirus D	Human enterovirus 68, 70, 94.
Human rhinovirus A, B, C	
Porcine enterovirus B	
Bovine enterovirus	
Simian enterovirus A	

Table 1.1.2 Enteroviral species and types [42].

1.1.3 Genome, lifecycle and kinetics of EV71

The genome of EV71 consists of a single open reading frame (ORF) flanked by 5'/3'-untranslated regions (UTR) and a polyadenylated tail. This ORF encodes a polyprotein of approximately 2200 amino acids which can be subdivided into P1, P2 and P3 regions. P1 is further divided into four structural proteins (VP1-VP4) and the other two regions contribute to seven proteins (2A-C, 3A-D) function in viral replication and pathogenesis (Fig 1.1.3.1) [43]. Only little information about EV71 life cycle was reported, while most of the knowledge was speculated according to studies on other enteroviruses (Fig 1.1.3.2). The cell entry of EV71 has been explored recently, and the process involves viral surface attachment, receptor binding and finally uptake through endocytic pathways [44]. Once the viral genomic RNA is uncoated and successfully released into the host cell cytoplasm, a cap-independent pattern of translation would occur immediatly through the recruitment of host translational machinery at the internal ribosome entry site (IRES) located in the 5' UTR of the viral RNA. After translation, the viral polyprotein can be subsequently cleaved into four structural (VP1, VP2, VP3, and VP4) and seven non-structural (2A, 2B, 2C, 3A, 3B, 3C, and 3D) proteins, in which $2A^{pro}$ and $3C^{pro}$ function as proteases in this process. [5]. For virus RNA replication, a complementary minus-strand RNA is synthesised in the cytoplasm and then this minus-strand RNA can serve as a template for new plus-strand RNA molecules. These events typically take place in virus-induced membrane complexes within host cells. Finally, the newly synthesized virus RNA may go into another round of translation and replication, or is packaged into capsid proteins to produce infectious viral particles (see review in Ref [45]).

Although the disease with rapid courses was observed during EV71 infection, it is still elusive on EV71 viral kinetics. Studies on other viruses demonstrate that the knowledge of virus kinetics is important for clarifying viral pathogenesis and exploring effective treatments. The knowledge of the viral kinetics on human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) has greatly improved the understanding of the cell response to these viruses and mechanisms of related antiviral therapy [46,47,48]. In the *Picornaviruse* family, the kinetics of swine vesicular disease virus (SVDV) and foot-and-mouth disease virus (FMDV) has been described in several studies [49,50]. However, little information is known about EV71 infection. In the part one of results, rhabdomyosarcoma (RD) cells were used as an *in vitro* model and the viral kinetics of EV71, including the kinetics of viral replication, viral protein synthesis, packaging and secretion, were intensively investigated.

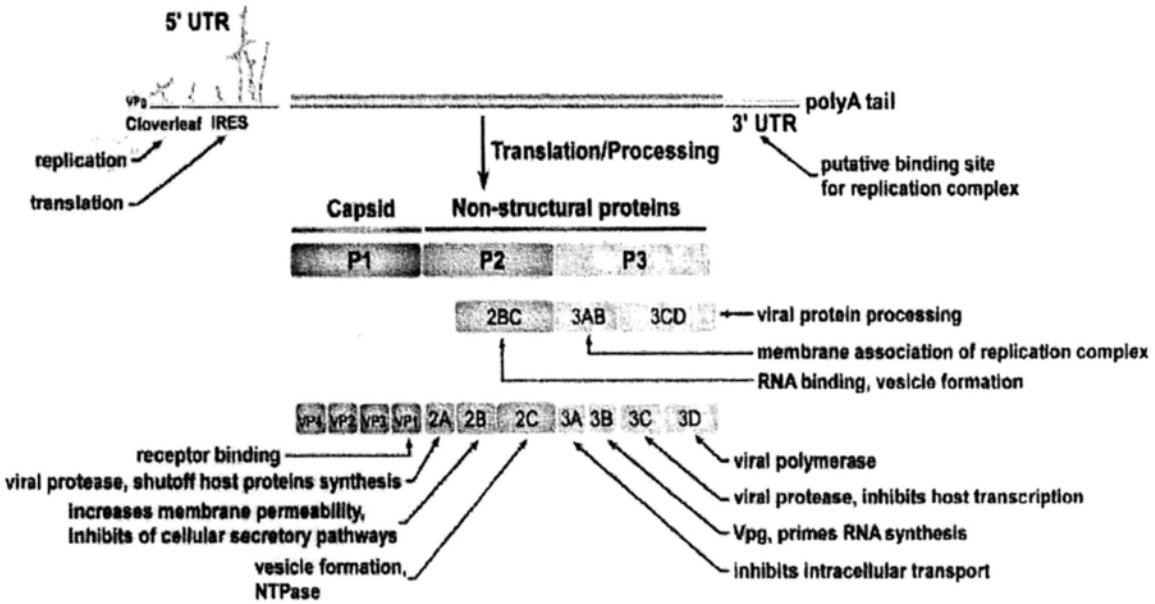


Figure 1.1.3.1 Schematic of the picornavirus genome, the polyprotein products and their main functions. A diagrammatic representation of the enterovirus genome is shown. The 11 mature polypeptides together with the three main cleavage intermediates are presented. The main biological functions are included for each polypeptide [51].

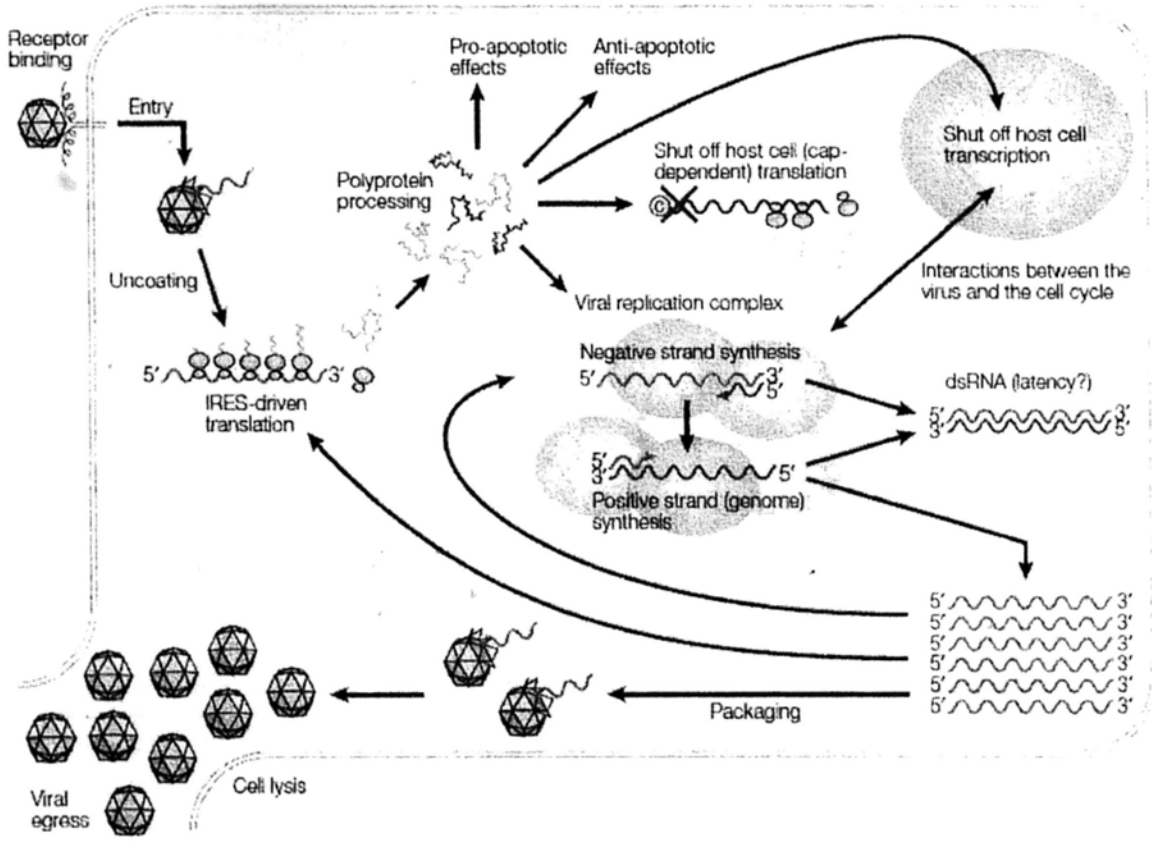


Figure 1.1.3.2 Summary of the picornavirus life cycle. The life cycle of a typical picornavirus is presented, and the main events included are described in the text [52].

1.1.4 Clinical features of EV71 infection

The clinical features of EV71 infection mainly include HFMD, herpangina, neurological disease and neurogenic pulmonary oedema.

HFMD is a common illness in children under 10 year's age. The infection has an incubation period of 3-7 days. The illness is characterized by fever, lymphadenopathy and the development of a vesicular on the palmar and plantar skin, buccal mucosa and tongue and a papulovesicular exanthema on the hands, feet and buttocks. Coxsackievirus A16 (CA16) and EV71 are recognized as the major cause of the epidemics of HFMD, although other coxsackieviruses like CA5, CA9, CA10 can also be the cause of this disease [53]. Clinical observation from HFMD epidemics in Asia-Pacific region indicate EV71 are more common than other viruses, and the clinical features of HFMD caused by EV71 are distinguishable from other virus [54,55]. Different from CA16, the manifestation caused by EV71 is more likely to have a high fever (≥39°C) last for more than 3 days and a higher risk of developing complications and fatalities [56,57].

Herpangina is another common disease caused by EV71 infection. Clinical observation from EV71 epidemics in Hong Kong, Japan and Taiwan indicate that herpangina caused EV71 infection is characterized by an abrupt onset of fever and sore throat. The disease is associated with the development of raised popular lesions on the mucosa of the anterior pillars of fauces, soft palate and uvula [53]. During the 1998 Taiwanese epidemic, herpangina is diagnosed as the second most common disease after HFMD caused by EV71. Moreover, about 10% of children with EV71-associated neurological disease in Taiwan had an initial or concurrent diagnosis of herpangina [58].

HFMD or herpangina are important diagnostic indicators of EV71 infection, but by themselves are not servere diseases. In few patients, severe central nervous system (CNS) diseases and complications may occur, and children under 5 years of age have the highest incidence of these severe complications. For many years, polioviruses were the most important neurotropic viruses and led to large outbreaks of paralytic disease. To data, EV71 is caused more and more attention since the poliovirus has been eradicated in many regions and the large outbreaks of EV71 and associated neurological disease are frequently reported in Asia-Pacific region in recent years. The clinical observations indicate that EV71 mainly causes neural disorder in virus-infected patients by inducing inflammation in the CNS region including the cerebral cortex, the brain stem, and all levels of the spinal cord [59]. The inflammation may be caused by directly viral infection because the viral particle, viral genome and antigens have been detected in these related CNS tissues by histopathology, immunohistochemistry, and reverse transcriptase PCR [59,60]. Moreover, the inflammation occurred in CNS mainly the brain stem spinal cord could result in encephalitis, aseptic meningitis, and brain stem encephalitis. In the case of EV71 associated with encephalitis, observations from ultrasonographic and magnetic resonance image (MRI) assays revealed CNS inflammation predominantly affects and damage of the grey matter of the spinal cord and the whole medulla oblongata [61,62].

Pulmonary edema or hemorrhage as a complication of systemic EV71 infection has a higher mortality [7,63]. Although the real cause is still elusive, the pulmonary oedema is observed to be closely associated with CNS inflammation. Previous studies on patients who died from EV71-induced pulmonary oedema indicated the inflammation was found in the spinal cord and the brain regions and was not detected in the lung and

heart of patients [7,59]. These results suggest that EV71 associated pulmonary edema is neurogenic origin and is secondary to autonomic dysfunction resulting from infection within brainstem.

AFP is also a notable complication associated with EV71 infection. Sporadic cases of AFP have been associated with these EV71 outbreaks, with varying rates of incidence reported around the world [9,12,16,27,64,65]. Compared with poliovirus associated disease, EV71-associated AFP seems to be milder and have higher rates of recovery. However, the more varied presentation and clinical features of EV71-associated AFP suggests more than one neuropathological mechanism is involved in this process. Furthermore, a clinical study on infected patients have shown that the AFP in EV71 patients was mostly reversible under clinical manifestation, but patients with bilateral AFP and bilateral anterior horn lesion might have less severe outcomes, such as residual motor weakness [66]. So the EV71-induced paralysis syndromes and the paralysis sites are determined by hyper intense lesions in the anterior horn and ventral root in specific spinal cord sites.

1.1.5 Pathogenesis

As mentioned above, EV71 is a potential pathogen for causing a large number of neurological diseases. To date, two possible ways have been suggested for EV71 to induce these severe diseases.

First, EV71 can induce inflammation in all levels of the CNS region in infected patients[67]. The inflammation may be directly caused by viral infection since a large amount of studies have demonstrated the association between EV71 infection in neural tissue and induced inflammation in associated CNS regions. Before inducing the inflammation, the virus either enters the CNS from the blood across the blood-brain barrier or is transmitted to the CNS through peripheral nerves via retrograde axonal transport [68,69,70]. The experiments on EV71-infected mice model suggest that EV71 is highly neurotropic and retrograde axonal transport in neuron cells. It may represent the major transmission route for EV71 into the CNS [71]. Besides neuron cells, EV71 can also infect the immune cells like human T cell line (Jurkat), the macrophage cell line (THP-1), the human peripheral blood mononuclear cells and human dendritic cells (DCs) in culture system [72,73]. EV71 infection have been demonstrated to lead NF-κb (nuclear factor κB) activation that promotes production of many pro-inflammatory factors or cytokines [74,75]. As a result, the secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and macrophage migration inhibitory factor (MIF) are often observed during virus infection [72].

Secondly, neuronal damage can also be caused by EV71-induced cell apoptosis [76,77,78] and autophagy [79]. Studies have shown the hallmark event of early

apoptosis like chromosomal degradation and membrane blebbing was observed in EV71 infected neural cells, and this may serve as an alternative mechanism of organ damage in systemic infections [80]. Several studies have extensively explored the characterizations and mechanisms of EV71 triggered apoptosis [73,80]. In EV71-infected neural cells, the efflux of cytochrome c from the mitochondria to the cytoplasma and subsequent cleavage by caspase-9 has been identified. So the mitochondrial pathway of apoptosis was regard as a main pathway for EV71-induced cell apoptosis. Moreover, studies on EV71-infected glioblastoma cell line (SF268) indicated that the virus induced cell apoptosis is depended on viral protein synthesis while the virus adsorption, internalization, entry, uncoating, and viral RNA replication were not required to trigger apoptosis [81]. Among 11 viral proteins, EV71 2A and 3C protease are the factors that induce apoptosis in infected cells. Caspase-3 is activated in SF268 cells that expressed 3C protease [82]. Moreover, the cleavage on cellular protein by 3C protease could be another apoptosis factor. For example, EV71 3C protease can cleave the cleavage stimulation factor, 3' preRNA, subunit 2, 64kDa (CsF-64), thereby impaires the host cell mRNA polyadenylation and induces apoptotic cell death [83]. Similar, 2A protease of EV71 was also reported to induce cell apoptosis in HeLa cells but the mechanism is still not clear. Previous work on poliovirus indicates that $2A^{pro}$ of poliovirus induces the caspases-independent apoptosis by cleaving eIF4GI (eukaryotic translation initiation factor 4 gamma I) [84]. It is now conceivable that EV71 $2A^{pro}$ induces apoptosis indirectly by activation of other unidentified cellular substrates pertinent to an endogenous cell suicide program. Further experiments are needed to better identified these substrates and delineate the mechanism(s).

1.1.6 Controls of human enterovirus 71

1.1.6.1 Vaccines

Due to the severe disease caused by EV71 infection, more and more studies have been taken to prevent EV71 infection. Currently, the development of effective EV71 vaccine appears to be a top priority. Conventional vaccines are being investigated by different approaches. These include live-attenuated or inactivated whole virus vaccine, DNA vaccine and recombinant protein vaccine [85,86].

Live-attenuated or inactivated whole virus vaccine has achieved good results in several studies on animal model while its protection role and potential side effects on human need to be further investigated. Studies on mice model showed that passive transfer of serum from formalin-inactivated and heat-inactivated virus vaccine immunized adult mice could provide protection against EV71 challenge in neonatal mice [85]. In rhesus monkeys model, the experimentally inactivated vaccine was also demonstrated to induce an immune response and offers protection to against future virus attacks [87]. Two laboratory-adapted EV71 strains including a vero cell-adapted virulent strain (*YN3-4a)* and an attenuated strain (S1-3') possess several desirable features suitable as vaccine candidates against EV71. *YN3-4a* exhibits characteristics that are especially crucial for inactivated viral vaccine such as high viral yield, strong immunogenicity, broad-based antigenic coverage, and passage stability [88]. The stain S1-3' is suitable as a live attenuated antigen. Monkeys inoculated with S1-3' showed a mild neurological symptom (tremor) but survived with lethal challenge. The sera of immunized monkeys also display a broad spectrum of neutralizing activities against different genotypes of EV71 [89]. Currently, another inactivated vaccine has entered

the stage of clinical trials. This vaccine is produced by using an EV71 strain isolated in China.

Subunit vaccines, including DNA vaccine or recombinant protein vaccine, have also shown ability to elicit maternal antibodies and to protect animals against EV71 infection [90,91]. The antigenic diversity of EV71 is caused by variations within structure proteins VP1 to VP3. Particularly, VP1 displays the densest neutralizing epitopes. Two VP1 subunit vaccines of EV71, one administered as a DNA vaccine, and the other as a recombinant protein vaccine, have illustrated protective effects against lethal EV71 challenge in newborn mice [85,90,91,92]. The cytokine profile of immunized mouse showed high levels of IFN-γ and IL-12 in the DNA vaccine groups, and high levels of IL-10 and IFN-γ in the VP1 protein groups, indicating that VP1 DNA vaccine elicits predominantly a Th1 response, while the subunit VP1 recombinant protein elicits a mixed Th1 and Th2 response. Synthetic peptide Scanning experiments revealed a small peptide (residue 208–222 of VP1) to be potent in eliciting a neutralizing antibody titer comparable to that obtained with a whole virion-immune sera. Hydrophobic profile assays showed that this highly conserved sequence is located within the major hydrophilic regions and expected to be exposed at the surface of the protein, hence making it a promising and attractive candidate for synthetic peptide-based EV71 vaccine [93].

Virus-like particle (VLP) would be another idea option for vaccine. A potent vaccine has been achieved by VLPs [94]. VLPs are empty particles that resembles the authentic virus in appearance, capsid structure and protein composition. Because of high density display of viral antigens/epitopes without viral nucleic acids, VLPs would elicit strong and long-lasting immune responses and mitigate the potential side effects. More

importantly, the immunized the mother mice with VLPs confer effective protection to newborn mice, the survival rate against the lethal viral challenge increase up to 89% [95].

Taken together, all of the above mentioned vaccines have remained in preclinical stage in animal models, except an inactivated one reported by Dong et al, which has been approved to enter the clinical trials at the end of 2010 in China. However, there are still great challenges in EV71 vaccine development. As indicated previously, one of the problems associated with the development of EV71 vaccine is the antigenic coverage. In serological neutralization assays, vaccines using one strain showed little protective effects for other strains with different genotypes. At present, there are three genotypes of EV71. Cross-protection to different genotypes of the EV71 vaccines derived from China is unclear, because the viruses isolated in this region were all within subgroup C4. Lack of appropriate animal models is another problem for testing the efficacy and immunogenicity of vaccines. Transgenic mice expressing the appropriate human receptor molecule(s) would be a possible approach for evaluating candidate vaccines. The recent identification of EV71 receptors paves a way for developing suitable animal models and probably accelerates the development of human vaccines. Moreover, further understanding of the molecular mechanisms underlying EV71 infection would also contribute to the development of effective EV71 vaccines [96,97].

1.1.6.2 Antiviral drugs

Currently, no effective drug is in clinical use against EV71 infection. Nowadays, computer-assisted drug design has been widely used to screen candidate compounds against EV71. Pleconaril, a promising drug candidate for the treatment of human

enterovirus infection, is the result of the successful combination of X-ray crystallography and computer modeling [98,99]. Pleconaril can neutralize most of the enterovirus-induced cytopathic effects *in vitro* and *in vivo* assay, but it failed to show a protective activity against EV71 at concentration tested *in vitro* [100,101,102]. The limited activity of Pleconaril against EV71 was due to subtle differences in the sizes and shapes of the hydrophobic pockets among these viruses. By using Pleconaril as a template for computational drug design, a series of imidazolidinone derivatives (Pyridyl imidazolidinones) are screened out. They have showed potent activities against EV71 infection [103,104,105].

Interferons (IFNs), a series of structurally related glycoproteins, are notable for their most intrinsic ability to interfere with viral replication [106,107]. Previous studies showed that type I interferons represent an essential innate defense mechanism for controlling EV71 infection in mice [108]. To date, there are about twenty different human type I IFNs identified. Although they are highly homologous in the amino acid sequences and share the same receptors, the biologic effect of each IFN is apparently different [109]. Recently, Yi et al have tested activities of seventeen IFN subtypes on anti-EV71. Interestingly, conventional IFN-α2a only showed relative weak antiviral activity. While compared with conventional IFN-α2a, IFN-α14 displayed higher anti-EV71 activity. The concentration required to inhibit the 50% CPE caused by EV71 were 188 U/ml which was 20 times less than that of IFN-α2a [110]. The mechanism led to this difference was still unknown.

1.2 Type I interferon

Type I IFNs are multifunctional cytokines that play a key role in innate immune response to viral infection. In this chapter, the background information related the production and function of type I IFN are introduced.

1.2.1 Types of interferons

Interferons are proteins made and released in response to the presence of pathogens such as viruses, bacteria, or parasites. IFN was firstly identified as a secreted substance that elicited antiviral activity against influenza infection [111], and interferons are named after their ability to "interfere" with viral replication within host cells. IFNs perform its antiviral function through several ways: i) they are quickly produced in response to viral challenge and function to suppress infection via the transcriptional and translational induction of a series of proteins that interfere with different stages in the replicative cycle of viruses; ii) they activate immune cells, such as natural killer cells and macrophages; iii) they increase recognition of infection by up-regulating antigen presentation to T lymphocytes; iv) they increase the ability of uninfected host cells to resist new infection by virus.

To date, approximate ten distinct IFNs have been identified in mammals and seven of these have been described in humans. Based on the cognate receptor for each of the IFN gene products, they are grouped into three classes: Type I IFN, Type II IFN, and Type III IFN. The Type I IFNs are comprised of different subtypes including: multiple IFN-α subtypes (13 in man), one to three IFN-β (one in man) and other genes, such as IFN-ε, IFN-ω, IFN-δ and IFN-τ [112,113]. The IFN-α/β genes are produced directly in

response to viral infection, whereas IFN-ε, IFN-ω, IFN-δ and IFN-τ play less well-defined roles. Thus, in most of times IFN- α/β represents virally induced 'type I IFN', and type I IFN signaling is always referred to IFN- α/β signaling pathway. The IFN-α/β can be produced by most cell types and bind as monomers to the interferon alpha receptor (IFNAR) complex which comprised two subunits of the type I IFN alpha receptor, IFNAR1 and IFNAR2 [114]. Different type I IFNs exhibits differential affinity for its receptor. To date, the recombinant IFN-alfacon-1 which was generated by comparing the most frequent occurring amino acid among endogenous type I IFNs presents the highest affinity for type I IFN receptors [115,116]. Type II IFN has a single member, named IFN-γ which is functionally active as a dimer and binds with high affinity to its cognate receptor subunits, IFNGR1 and IFNGR2 [117,118]. Unlike type I IFNs, IFN-γ is only secreted by mitogenically activated $CD4^+$ T helper1 (Th1) cells, dendritic cells (DC) and natural killer (NK) cells, rather than in direct response to viral infection [119]. Type III IFNs comprise 3 members, IFNλ1, IFNλ2 and IFNλ3, also referred to as interleukin-29 (IL-29), IL-28A, and IL-28B, respectively [120,121]. Type III IFNs are induced directly in response to viral infection and can bind with high affinity to the IFN lambda receptor subunits, IFNLR1 and IFNLR2 [122]. Type III IFNs can also produced in many cells but unlike IFN- α/β, the type III IFN receptor only present in limited tissues. Recent studies indicate that type III IFNs use the similar pathway as type I IFNs to senses viral infection, and elicit an equivalent antiviral response [123]. However, the exact role of the type III IFNs remains unclear and their functions in antiviral infection have not been established yet.

1.2.2 The production of type I interferon

The induction of type I IFN in response to viral infection is tightly regulated. Recent findings suggest that two phases are involved in this process: an early phase and a later, positive-feedback phase [124,125]. In the early phase, viral pathogen-associated molecular patterns (PAMPs) like dsRNA which are generated during the viral infection can be firstly detected by different intracellular sensor (Figure 1.2.2). Most of cells including fibrolasts, conventional dendritic cells and hepatocytes sense viral dsRNA by using two intracellular RNA helicases, retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated protein-5 (MDA5). For plasmacytoid dendritic cells (pDCs), Toll-like receptors (TLRs) are used to detect viral infection. After detected viral components, these receptors can transfer the signaling to following pathways and result in the activation of several transcription factor families. The activated transcription factors including interferon regulatory factor 3 (IRF3), NF-κB, and activator protein-1 (AP-1) can transfer into nuclear and bind to the positive regulatory elements within the IFN-β promoter. As a result, the production of IFN-β is activated in the first wave [126]. In the positive-feedback phase, the initially produced "first-wave" IFN-β binds with the type I IFN receptor and activate IFN- α/β signaling pathway. As a result, the expression of IRF7, the master regulator of IFN gene, is activated. IRF-7 is responsible for a positive feedback loop that is very efficient at inducing IFN-β and most of the IFN-α gene [124,125,127,128]. In next sections, the different cell sensors of viral infection and related signaling pathway to induce type I IFN were introduced.

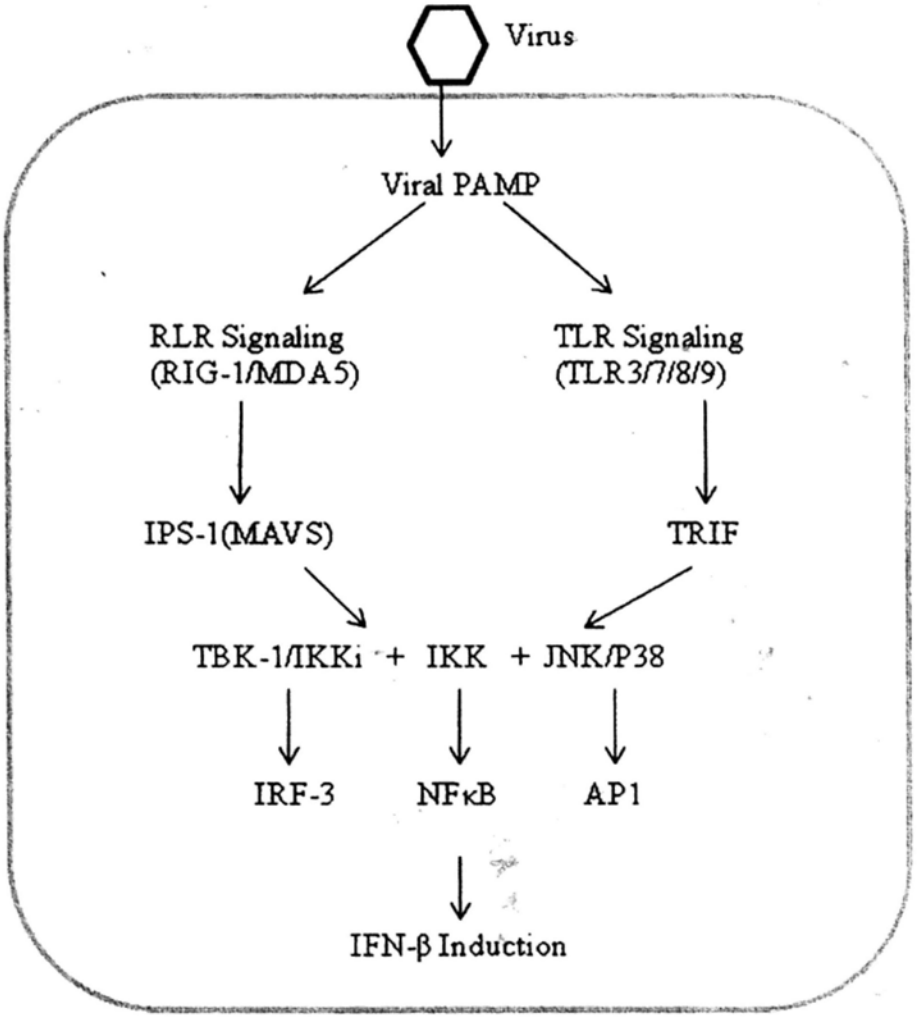


Figure 1.2.2 Overview of signaling pathways leading to IFN-β induction.

1.2.2.1 Toll-like Receptors

Toll-like receptors are a class of pattern recognition receptor (PRR) that recognize PAMPs derived from not only viruses but also other microbes including bacteria, fungi, and protozoa [129]. Once these microbes have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs which activate cellular immune responses. To date, 10 Toll-like receptors in humans have been identified. TLRs are generally expressed on hematopoietic cells and their expression levels differ in varied cell types [130]. The cell locations of TLRs are either on the plasma membrane (TLR1, TLR2, TLR4-6, and TLR10) or on the ER and endosomal vesicles [131].

As a type I transmemebrane protein, each TLR composed of extracellular/luminal domains containing leucine-rich repeats (LRRs) followed by one or two cysteine-rich regions which bind to specific PAMPs [131]. TLRs have short transmembrane domains connected to a cytoplasmic toll/interleukin 1 receptorhomology (TIR) domain, which serves as a scaffold for assembling signaling complexes and recruitment of downstream adaptor and signaling components in response to ligand recognition [132]. Each TLR acts alone or in combination with other TLRs to detect unique PAMPs. For instance, TLR1 forms heterodimer with TLR2 (TLR1/2) and recognizes triacyl lipopeptides [133]. Combining with TLR1 or TLR6, TLR2 recognizes a wide variety of PAMPs, including lipopeptides and lipoproteins of Gram-positive bacteria, peptidoglycan, mycoplasma lipopeptides and fungal zymosan [134]. TLR3 is the receptor for double-stranded RNA [135]. TLR4 was first identified to be involved in recognition lipopolysaccharide (LPS), an endotoxic component of Gram-negative bacteria [136,137]. TLR5 is activated in the presence of bacterial flagellin [138]. TLR7 and

TLR8 recognize single-stranded RNA [139,140], which is produced during viral replication. TLR9 can be activated by unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) motifs [141] existing in bacterial and viral genomes.

1.2.2.2 TLR signaling pathway in virus infection

As mentioned above, TLR3, 7, 8, 9 are functioned in sensing viral infection, and these TLRs are responsible for recognizing viral double-strand (ds) RNA (TLR3), viral single-stranded (ss) RNA (TLR7,8) or double-stranded CpG-rich DNA (TLR9), respectively [142,143]. TLR3 is predominantly located in endosomes and phagosomes while on the cell surface in natural killer (NK) cells [130,144]. Upon activation, TLR3 signals through its adaptor molecule MyD88 which forms a complex with TRAF3 [145]. TRAF3 in turn activates kinases TBK1 and IKKi, triggering the subsequent phosphorylation of IRF3 which transcriptionally activates IFN-β gene expression [146]. TLR7, 8, 9 are highly expressed in human pDCs which are specialized IFN producers and represent a major source of IFN-α in humans [147]. TLR7, 8 and 9 also invoke the MyD88-dependent signaling pathway to induce type I IFN. Different from TLR3, IRF3 is not required for the signaling activation. TLR7, 8 and 9 can recruit MyD88, IRAK4, IRAK1 and TRAF6 to trigger the activation of downstream TAK1 which lead to the activation and subsequently nuclear translocation of NF-κb and AP-1. Moreover, IRF-7 is phosphorylated by IRAK-1 and transcriptionally activates multiple IFN-α genes [148,149]. pDCs differ from the other cell types in their capacity to constitutively express considerable amounts of IRF-7 which is essential in the positive feedback loop for high IFN-α/β production [127,150,151].

1.2.2.3 RIG-1 like Receptors

Beside the TLRs, several cytosolic receptors have been identified as TLR-independent viral sensors that bind viral RNA and trigger the production of type I IFN. The RIG-1 was the first RNA sensor identified capable of detecting viral dsRNA [152]. RIG-1 contains a C-terminal helicase domain that interacts with viral RNA in an ATP-dependent manner, and two N-terminal caspase activation and recruitment domains (CARDs) which is essential for signaling downstream to activate NF-κB and IRF3. Recently, two other members of viral RNA sensors in the RLH family have been described: MDA5 and likely ortholog of mouse D111gp2 (Lgp2) [153,154].

RIG-1 and MDA5 are ubiquitously expressed in most types of cells. These two receptors have similar structures, e.g., they contain two CARD-like domains and a helicase domain. The RIG-1 and MDA5 seem to function in parallel and have a degree of virus specificity [154]. For instance, RIG-I detects VSV, influenza, Japanese encephalitis virus and Sendai virus [155], whereas MDA5 is shown to detect EMCV, Theiler's virus, Mengo virus and polyI:C [156]. In fact, dsRNA was only a partial requirement for RIG-1 signal transduction, and recent studies suggested that dsRNA with 5' triphosphate as well as ubiquitination (lysine 63-linked polyubiquitin chains) via the TRIM25 E3 ubiquitin ligase were required for RIG-1 detection [157,158,159]. Unlike RIG-I and MDA5, Lgp2 only processes the helicase domain and does not contain CARDs, which were known to be important for downstream signaling [153]. As a result, Lgp2 may function as a negative regulator of RIG-I and MDA5 [160].

1.2.2.4 RIG-1/MDA5 Signaling

After binding with specific viral RNA, the CARD domains of RIG-I and MDA5 would have a conformational change that transfers the signal to downstream partners [152,161]. The downstream signaling pathway for RIG-I and MDA5 appears to be shared (Figure 1.2.3). RIG-I and MDA5 both bind to a downstream adaptor called IPS-1 for "interferon-β promoter stimulator 1" or MAVS for mitochondria-associated protein [162,163,164]. After binding, the C-terminal region of IPS-1 can further interact with FADD and RIP1 which are both involved in NF-κB signaling [162]. Also, IPS-1 can activate the essential transcription factor IRF3 via phosphorylation by TANK binding kinase 1 (TBK1) and the inducible IκB kinase (IKKi) which are both known [165,166]. IRF3 belongs to the IFN regulator factor (IRF) family and plays a central role in the activation of IFN-β transcription. The phosphorylated IRF3 forms either homodimeric or heterodimeric complexes and translocates to the nucleus where it recruits the transcriptional coactivatiors p300 and cAMP response element binding (CREB) protein to target the promoters of type I IFNs and other cytokines, thereby activating transcription [167,168]. In addition, activated by dsRNA, the transcription factor NF-κB and AP-1 are recruited and translocate into nuclear to up-regulate type I IFN gene expression [169].

1.2.2.5 Transcriptional activation of type I IFNs

As mentioned above, the transcription of the IFN-β gene requires the recruitment of the transcriptional complex at the enhancer region upstream of the IFN-β gene transcription start site. The enhancer region of the IFN-β gene includes four positive regulatory domains (PRDs I, II, III and IV), In contrast, the promoter regions of IFN-α genes contain PRD I- and PRD III-like elements (PRD-LEs) [170]. Initially high mobility group (HMG)-1 binds to the IFN-β promoter, and results in a conformational change in the promoter. Transcription factors including IRFs, NF-κB, and AP-1 can then bind their respective cis-acting sequences. NF-κB binds PRDII, IRFs recognize PRD-I and PRD-III, and the c-Jun heterodimer binds PRDIV. The binding of transcription factors then leads to assembly of the transcriptional initiation complex including general control of amino acid synthesis 5 (GCN5), CREB-binding protein and finally the RNA polymerase complex to the start site of transcription [171].

1.2.3 Type I IFN signaling pathway

1.2.3.1 Type I IFN receptors

All members of the type I family act on cells via the same cognate receptor that consists of two subunits: IFNAR1 and IFNAR2. IFNAR1 is unique in normal cells, whereas IFNAR2 has three isoforms which is generated from the same gene by alternative splicing and differential usage of exons and polyadenylation. These transcripts encode a long isoform IFNAR2c which consists of an extracellular domain, a transmembrane region and a cytoplasmic domain [172], a short isoform IFNAR2b which lacks a cytoplasmic domain [173] and a soluble IFNAR2a chain. Without cytoplasmic domain, the IFNAR2b cannot transfer the signal into the cell and then performs as a negative regulator. In contrast, soluble IFNAR2a is capable of acting as both activator and inhibitor of IFN signaling, yet more studies are required to elucidate its precise function [174].

Although all tissues and most cell lines express IFNAR1 and both soluble and transmembrane isoforms of IFNAR2, there is some differential expression of IFNAR1 and IFNAR2. Microarray analysis of IFNAR related expression suggests that the expression of IFNAR1 is more widespread and IFNAR2 is more restricted in specific tissues and cells. The binding of type I IFNs to these cognate receptors leads to activation of receptor-associated downstream signaling to protect the host against infection and mount-controlled immune responses. However, deregulated type I IFN associated signaling can lead to toxicity, autoimmunity and even death. Thus, several negative regulators including SOCS-1 (suppressor of cytokine signaling), SHP, and UBP43 are identified to suppress IFN signal via interacting with IFNARs. For example,

the SOCS-1 can bind with conserve region of IFNAR1 to inhibit the activation of downstream molecules. This region is also required for the recruitment of E3 ubiquitin ligases and then is important for ubiquitination and degradation of the receptor [175].

1.2.3.2 JAK/STAT signaling

Binding with the corresponding receptor IFNAR1 and IFNAR2, the secreted IFNs leads to receptor aggregation and activation of receptor-associated kinases: JAKs. The JAK family is comprised of JAK1, 2, 3 and Tyk2 in which Tyk2 and JAK1 interacts with IFNAR1 and IFNAR2 respectively while JAK1 and JAK2 are involved in type II interferon signaling (Fig 1.2.3.2). The dimerization of IFNARs can trigger the cross-phosphorylation of Tyk2 and JAK1 [176,177]. The activated Janus kinases (JAK), JAK1 and Tyk2 then phosphorylate tyrosine residues on STATs (signal transduction and activator of transcription) including STAT1 (STAT1α and STAT1β) and STAT2. These two STATs are phosphorylated and activated by interacting with each other via reciprocal Src-homology (SH2) domains [178,179,180]. Phosphorylated STAT1 and STAT2 form heterodimers and bind with IFN regulatory factor 9 to form the transcription factor ISGF3 (Interferon-stimulated gene factor 3) [181]. The ISGF3 complex translocates into the nucleus and binds a promoter region called ISRE (IFN-stimulated response element) [182]. This interaction initiates activation of hundreds of ISGs (interferon-stimulated genes) that collectively alter the cellular or viral processes and modulate the immune response toward establishing an antiviral state [183,184]. Also, STAT signaling can sometimes be negatively regulated, and the SOCS proteins are well known inhibitors for JAK/STAT signaling. As mentioned above, the SOCS-1 can inhibit the signaling by binding with IFNAR. In addition, SOCS1 and SOCS3

containing SH2 domain which can bind phosphotyrosine residues within the activation loop of the JAK and/or nearby residues and thus inhibit signal transduction [185,186].

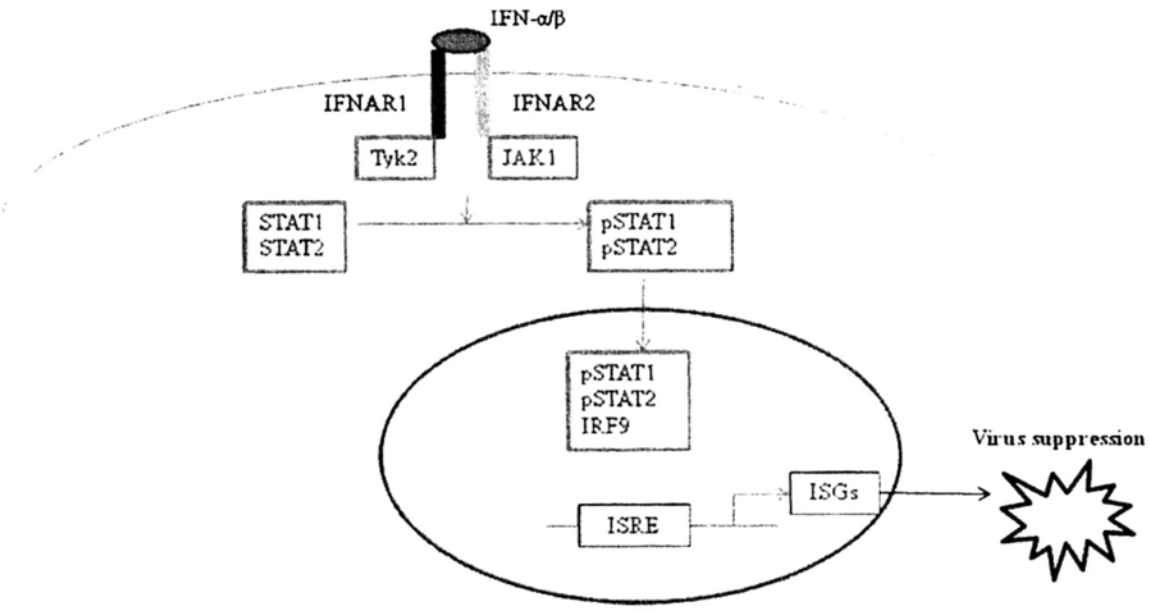


Figure 1.2.3.2 Signaling pathway activated by IFN- α/β.

1.2.4 Interferon induced antiviral proteins

Of the many genes that are up regulated by type I IFN, only a small number proteins that have antiviral activity. To date, three antiviral pathways have been firmly established which include the PKR (protein kinase R), the 2'-5' OAS (2'-5' oligoadenylate synthetases)/RNaseL system, and the Mx (Orthomyxovirus resistance) proteins.

1.2.4.1 dsRNA-dependent protein kinase R

PKR is one of the well characterized ISG products which play an essential role in IFN-inducible antiviral responses. PKR contains an N-terminal dsRNA binding domains and a C-terminal kinase domain that gives it pro-apoptotic function. PKR is synthesize in an inactive form and undergoes dimerization and subsequent activation in response to dsRNA, a common replication intermediate during viral infection [187]. This activated PKR acts on downstream effectors to modulate both translation and transcription. The best-characterized substrate for PKR is the α subunit of the eukaryotic translational initiation factor 2 (eIF2α) which plays a critical role during the translation of cellular genes [188]. eIF2 comprise of three subunits: α, β and γ. During the translation, eIF2 promotes the guanine trisphosphate (GTP)-dependent delivery of Met-transfer RNA (tRNA) to the 40S ribosome, and then eIF2 is dissociated from the initiation complex and back to its active form via hydrolyze GTP [189]. However, PKR elicits phosphorylation of eIF2α, and prevents recycling of eIF2α. As a result, the initiation of both viral and cellular protein synthesize is inhibited. Additionally, PKR can modulate the activity of STATs in response to dsRNA. Studies indicate PKR functions in the phosphorylation of serine residues in STAT1 as well as STAT3 and

abrogation of this phosphorylation leads to disfunction of these STATs [190,191]. Moreover, PKR has also been reported to modulate the activity of NF-κB [192]. In summary, PKR has been involved in a number of antiviral mechanisms including induction of IFN response, cell apoptosis and cell-cycle arrest. Experiments in PKR-deficient mouse embryonic fibroblasts show that PKR is involved in protection against infection with several RNA viruses, including WNV, hepatitis D virus, HCV, Sindbis virus, HIV-1, encephalomyocarditis virus, and foot-and-mouth disease virus [193,194,195,196,197,198,199].

1.2.4.2 2'-5' OAS and RNaseL

Like PKR, OAS is synthesized in an inactive form and in response to the cofactor dsRNA [200]. The OAS proteins are distinguished by their capacity to synthesize 2', 5'-linked phosphodiester bonds to polymerize ATP into oligomers of adenosine. 2'-5' oligonucleotides of adenylate specially activate latent form of RNase L which functions to cleave single-stranded RNA at the 3' side of the UpAp or UpUp regions [201]. In this way, OAS in combination with RNaseL leads to decay of viral RNA. Moreover, OAS protein also functions as PRRs for the detection of viral dsRNA in the cytoplasm [202]. Single RNA cleaved by RNaseL can generate short dsRNA which can be senesed by the other cytoplasmic PRRs, such as RIG-I and MDA5, resulting in the induction of type I IFN gene expression. Corresponding with this, the RNaseL-deficient cells show deficiency in the IFN-β production because of the reduced signaling through these PRR [203].

To date, there are four OAS genes identified in humans, termed OAS1, OAS2 OAS3 and OASL (OAS-like). OAS1, OAS2 OAS3 have considerable homology to each other,

and contain one, two and three conserved 'OAS' domain respectively. OASL is distinctive from the first three ones for its unique cellular location. Each of OAS protein has unique biological function and is differentially expressed and induced after activation. The antiviral function of OAS proteins has been demonstrated by using RNaseL-deficient mice [204]. The mice without RNaseL is more susceptible to RNA viruses from Reoviridae, Picornaviridae, Paramyxoviridae, Togaviridae, Flaviviridae, Retroviridae, and Orthomyxoviridae families [205].

1.2.4.3 The Mx proteins

Mx proteins were another prominent antiviral protein induced by IFNs. It was observed that the sensitivity of many inbred mouse strains to orthomyxomavirus was solely due to mutations within the *Mx* locus on chromosome 16 in a mouse strain (A2G) [206]. To date, Mx proteins comprise MxA and MxB in humans and Mx1 and Mx2 in mice. The cellular localization of Mx proteins varies among the different isoforms and is also species-dependent. Since different viruses may replicate in varied cell compartments, the differential distribution of Mx proteins may allow each protein to target viruses in specific sites [207]. The two human Mx proteins are cytoplasmic, while only MxA has demonstrated antiviral activity which directly against both nuclear and cytoplasma viruses.

As a GTPase family member, the MX protein has a large N-terminal GTPase domain, a central interacting domain (CID), and a leucine zipper (LZ) motif [208]. It has been demonstrated that CID and LZ are responsible for recognizing viral nucleocapsid-like structures. Mx proteins are able to manage exocytic events and mediate vesicle trafficking to trap essential viral components, and result in inhibiting viral replication at

early time points [209] . Studies have shown that human MxA present antiviral activity for Coxsackie virus from the Picornaviridae family, HBV from the Hepadnaviridae family and all infectious genera of the Bunyaviridae family [210,211].

1.2.4.4 ISG15, ISG54 and ISG56

In addition to PKR, OAS and Mx, other ISGs have been demonstrated to play important roles in the antiviral response. ISG15 is a 17 kDa protein which is one of the most prominent ISGs induced in response to viral infection or type I IFN treatment. ISG15 shares several common properties with other ubiquitin-like molecules, but its activity is tightly regulated by type I signaling pathway. Recent studies suggest ISG15 regulated ubiquitin-like protein response (ISGylation) functions in many aspects of immunity, including intracellular signal transduction as well as acquire immune functions [212]. Unlike ubiquitylation, ISGylation does not trigger protein degradation, but rather inhibit ubiqutin's activating effects. Many important factors in IFN signaling are targeted by ISG15, and these substrates include JAK1, STAT1, RIG-1, and several antiviral proteins MxA, PKR and RNaseL [213]. The consequences of ISG15 conjugation include stabilizing proteins against degradation. The direct evidence for the antiviral activity of ISG15 comes from studies of knockout mice. Mice ablated for *Isg*15 become more susceptibile to viruses including herpes simplex virus 1 (HSV-1), the influenza A and B viruese, and Sindbis virus (SV). Supporting this, ablation of the deISGylation enzyme, USP18, in mice increased the sensitivity to IFN treatment and the resistance to virus infection, notably VSV and ECMV [214,215]. ISG15 appears to upregulate the efficiency of the IFN response globally. The in-vitro studies show that macrophages from Ubp18 deficient mice show an increase in the strength and duration

of the IFN-inducible transcription response and MEFs from these mice have prolonged IFN-signalling responses [216].

ISG54 and ISG56 are another two genes induced strongly but transiently in response to interferons, double-stranded RNA and a variety of viruses. These two proteins have similar function in binding to the translation initiation factor eIF3 and inhibiting translation. Human ISG56 interacts with the eIF3e subunit [217]and inhibits its ability to stabilize the eIF2–GTP–tRNAiMet complex [218]. On the other hand, the murine ISG56 bind to eIF3c and block the activity of eIF3. Thus, the formation of translational initiation complex, eIF2–GTP–tRNAiMet–mRNA–40S ribosomal subunit is inhibited [219].

1.2.5 Type I IFN induced antiviral status

In addition to induce some ISGs which directly inhibit viral replication, IFNs also profoundly modulate cellular status and immunity to clear viral infection.

1.2.5.1 IFN induced cell apoptosis and cell cycle arrest

The cell apoptosis induced by type I IFN is essential for hosts to remove infected cells and finally clear viral infection. As mentioned above, interferon stimulated genes like PKR and OAS affect apoptosis especially in circumstances where apoptosis depends on viral dsRNA. Besides these ISGs, studies indicate that procaspase genes are stimulated after IFN treatment. For instance, studies in cells of different lineages indicate that type I IFN activated STAT have a proapoptotic effect through transcription-dependent activation of caspases, Bcl-xL, and death receptors and ligands such as Fas and TRAIL [220,221]. Caspases-1 and caspase-11 activated by STAT1 further lead to the cleavage of the effector caspases-3 [222]. Moreover, activated STATs may also regulate apoptosis through non-transcriptional mechanism by interacting with p53 and TRADD or by inhibiting the anti-apoptotic protein NF-κB [223,224].

In addition to establishing an antiviral state by triggering cell apoptosis, IFNs also regulate genes relating to cell cycle. The best understood example is the G1/S CDK inhibitor p21 also known as pWAF, CIP. Upregulation of p21 or other CDK inhibitors by IFNs lead to cell growth arrest at the G1/S transition point [225]. The interferon-inducible p200 (IFI-200) family of proteins also function in regulate cell cycle progression. The p200 proteins have been implicated in modulate the activities of multiple transcriptional factors such as Rb, p53 and E2F which play essential roles in

cell cycle regulation and differentiation. For example, IFN induced p200 proteins are able to lead p53 activation and inhibit E2F-dependent gene expression of S-phase-specific genes. As a result, cells are arrested in G1 phases, and viral replication activities are also inhibited [226].

1.2.5.2 Immunomodulation

It has been established that IFN-α/β is important in promoting both innate and adaptive immune response [227]. For instance, type I IFNs can promote the maturation of NK cells which are further activated by IFN stimulated perforin and granzymes [227]. Another obvious example is the upregulation of major histocompatibility complex (MHC) I and components of the antigen-presenting machinery [228]. Moreover, the maturation of dendritic cells can be promoted after type I IFN treatment [229]. These mechanisms help the presentation of antigens and trigger the transition from innate to adaptive immune response. IFNs can trigger the production of IL-15 which is important for survival of memory cytotoxic T cells and NK-cells [230]. IFNs also induce B cell maturation and function in immunoglobulin class switching. Finally, type I IFNs mediate the expressions of chemokines and chemokine receptors allowing differential trafficking of immune effectors to sites of inflammation [231].

1.3 Viral evasion of type I IFN response

Despite the powerful antiviral activity of type I IFN, most viruses are still able to evade the IFN response and multiply extensively to establish a solid infection. There are mainly three ways by which viruses circumvent the IFN response by (i) minimizing the production of IFN; (ii) disruption of IFN-inducible signaling; (iii) interrupt the action of IFN stimulated genes with antiviral activity. For different viruses, they may cope with the IFN system by using different strategies. Furthermore, in order to achieve high replication and persistent infection, most viruses often use combinations of these strategies.

1.3.1 Viral inhibition of IFN production

Suppressing the cellular IFN production is essential for viral transmissions in different cells. Supposing that a virus blocks IFN signaling but fails to limit IFN production, the IFNs produced by infected cells can drove the neighbor cells into antiviral status. Consequently, this virus may not spread rapidly, and further replication may also be inhibited. To establish persistent infection, many viruses can interfere with the IFN production by mainly two ways: (i) Interfere with PRR activation; (ii) Inactivation of the IFN transcriptional factors.

1.3.1.1 Interfere with PRR activation

PRRs like TLR3, RIG-1 and MDA-5 are often interfered by viruses to inhibit the type I IFN production. As mentioned above, the amount of IFN induced by a virus is firstly dependent upon the recognition of viral factors by PRR. Thus, virus can interfere with this step by concealing the viral factor from PRR detection or by blocking PRR activation.

Viral dsRNA is a representative viral factor, and recognition of dsRNA by PRR is a key in IFN induction. Thus, any exposure of viral RNA is critical. In this respect, the viral proteins associated with viral RNA can be considered as important IFN antagonists which concealing RNA from recognition by host cells. Additionally, it also 'hides' the dsRNA from the dsRNA dependent antiviral gene products like PKR, and OAS, as well as minimizes dsRNA induced apoptosis. For example, West Nile virus (WNV) can induce IRF-3 activation and trigger type I IFN response and ISGs expression, but these activities are delayed until late points in the viral replication cycle [232]. The delay of IFN response is not due to the expression of a IFN antagonist

which blocking IRF3 activity [233], instead it is likely that WNV shields viral components from PRR detection and delays host response until its replication cycle has been completed. Also, the similar situation is observed in influenza virus. The viral protein NS1 is a multifunctional protein which regulates the virus replication, enhances viral mRNAs translation and inhibit 3' end processing of celluar pre-mRNAs. Moreover, as an IFN antagonist, NS1 can bind with dsRNA to disrupt the processes of PRR signaling and inhibit IRF-3 and NF-κB activation [234]. Recombinant viruses with NS1 mutants (R38 and K41) defective in dsRNA binding lead to increased IFN production and attenuated pathogenicity in mice [235]. A special mechanism used by Bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) is binding extracelluar dsRNA to block the activation of TLR3. BVDV secretes a structure glycoprotein with dsRNA binding and RNase activities. Both properties of the protein have been found to be involved in IFN induction by extracelluar dsRNA [236].

Another strategy to interfere with PRR activation is direct targeting the function of the pattern receptors or their adaptors. For example, the V protein of paramyxovirus genus, Sendai virus, and Simian virus 5, can binds MDA-5 which is function as a sensor for recognition viral dsRNA. As a consequence, the RNA induced IFN production is minimized [237,238]. Virus like herpes simplex virus type 1 (HSV-1) has evolved a mechanism to block IFN production by interfering autophagy specifically. As mentioned before, TLR3/7/9 are expressed on endomsomes of plasmacytoid dendritic cells (pDCs) and the viral recognitions by these receptors are partially required the autophagy pathway which producing cytosolic replication intermediates of viruses [239]. Studies demonstrate that HSV-encoded neurovirulence protein ICP34.5 inhibits

autophagy pathway and interfering PRR activation through binding to the mammalian autophagy protein Beclin1 [240]. Viral proteins targeting the adaptors of recognition receptors are exemplified by positive RNA viruses. HCV NS3/4A protease is an essential viral protein required for processing of the immature viral polyprotein. In order to inhibit IFN induction, NS3/4A is able to cleave the TLR3/4 adapter TRIF and disrupt its association with TRAF3 and/or TBK1 [241]. Also, NS3/4A can cleave the RIG-I adaptor IPS-1 and precludes its mitochondrial localization by removing the transmembrane anchor from IPS-1. By targeting multiple TIR proteins including TRIF, Vaccinia virus (VACV) proteins A52 and A46 can block activity of TLR3 and TLR4 and inhibit the induction of IFN [242].

1.3.1.2 Inactivation of the IFN transcriptional factors

The downstream signal cascades of PRRs including IRF kinases, IRFs themselves as well as NF-κB could also be the target of the virus. For example, IRF3 is the target of a variety of RNA and DNA viruses. The phosphoproteins (P) of Borna disease virus (BDV) and rabies virus (RABV) and the G1 protein of Hantavirus inhibit the activation of IRF3 and IRF7 by TBK1, as does the N1 protein of VACV. Rabies virus P protein interferes the phosphorylation of both IRF7 and IRF3, by TBK1 and by IKKi [243]. The IRF3 dimerization, nuclear import is depended on its phosphorylation status and as a result the IFN transcription activation by IRF3 is inhibited in the presence of P [244]. Moreover, the rabies virus P protein is also found active in inhibiting JAK/STAT signaling [245]. Besides interfering phosphorylation of IRF3, viral proteins can also target IRF3 by leading it into degradation. N protein of both Bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) blocks IFN induction by leading

proteasome-mediated degradation of IRF3, as does non-structural protein 1 of rotavirus which not only mediate IRF-3 but also IRF-5 and IRF-7 degradation [246,247,248]. In case of HSV, viral protein ICP0 can recruit activated IRF-3 and CBP/p300 and prevent them from interaction with host chromatin. This leads to the inactivation and accelerated degradation of IRF-3, resulting in reduced transcription of IFN-β.

IRF7 is also a target of virus proteins. An example is provided by Epstein-Barr virus (EBV). BZLF-1, an EBV encoded protein, can inhibit IRF7 activity by physically binding with cytoplasmic and nuclear IRF7 [249]. Herpes viruses have acquired additional weapons to interfere with the functions of cellular IRFs. For Human herpes virus 8 (HHV-8), the causative agent of Kaposi sarcoma, developed multiple methods to inhibit IFN production. The immediate-early 1 protein of HHV-6 inhibits IFN production by interfering IRF-3 functions, and that of HHV-8 inhibits IFN production by targeting IRF-7 for degradation. Moreover, HHV-8 encodes several IRF orthologues, so-called vIRFs, which has homology to the IRF family of transcription factors. vIRFs can function as dominant negative mutants to block endogenous IRF function or mimic host IRFs functioning to interfere IFN gene expression. To data, three vIRFs (vIRF-1, vIRF-2, and vIRF-3/LANA2) of HHV-8 have been characterized. vIRF1 prevents the function of the enhanceosome via associating with CBP/p300 and IRF3 [[250]. The vIRF3 (LANA3), interacts with IRF7, and then suppress the expression of IFN-βand IFN stimulated genes [251].

NF-κB is another target for virus to countermeasure IFN production. As noted, NF-κB is required in the activation of the IFN-β promoter. Thus, several viruses have developed mechanism to suppress NF-kB function directly. For example, African

swine fever virus encodes an IκB homology that inhibits the activity of NF-κB [252]. The core protein of HCV inhibits NF-κB signaling by targeting the IKK complex [253], and so does the human papillomavirus (HPV) type 16 E7 protein [254]. However, the activated NF-κB plays an important role in cell proliferation and apoptosis [255]. For this reason, any virus that manipulates NF-κB activity specifically may make itself susceptible to enhanced induction of apoptosis.

1.3.2 Viral inhibition of IFN signaling

Suppressing IFN induction is important for virus transmission, and inhibition of IFN signaling is always required for virus to establish solid infection. There are several clear advantages for viruses to interfere IFN signaling. Firstly, type I, II and III IFNs share the signaling pathway (JAK/STATs) which induces cellular antiviral status. By inhibiting one cascade activity in IFN signaling pathway, the virus may suppress the production of antiviral ISGs and evade from the multiple IFNs stimulated antiviral effects. Secondly, type I IFN signaling activation is required for adaptive immune response including cytotoxic T lymphocytes (CTLs) effect and antibody production. Thus, viral inhibition of IFN signaling also delays or reduces the antiviral effects produced by adaptive immunity. Thirdly, the viral infected cells would be resistant to the IFNs produced from other cells or the IFNs treatment by blocking IFN signaling pathway. At last, IFN signaling activation is needed for 'second-wave' IFN induction. Thus, viral inhibition on IFN signaling not only blocks the production of antiviral proteins but also the expression of RIG-1, MDA5, IPS-1/MAVS, IRF-3 and IRF-7 which are responsible in a positive feedback loop of IFNs synthesis.

IFN signaling pathway included mainly six main components which are IFNAR1/R2, JAK1, Tyk2, STAT1, STAT2 and IRF9. Viral proteins are able to block all this cascade by down-regulating the protein levels or inhibiting their activities. Firstly, virus can block IFN signaling by neutralizing the secreted IFNs or the corresponding receptors. For example, IFN-α/β-binding proteins encoded by VACV and most other orthopoxviruses can directly interact with IFN-α/β and thus inhibit the interaction between IFN-α/β and receptors[256]. Some viruses like MYXV, ECTV, and CPXV

also encodes protein that target IFN-γ [257]. The virus that target IFN receptor is exemplified by HHV-8. The K3 and K5 proteins encoded by HHV-8 can reduce the IFNGR1 level by inducting protein degradation [258]. More recently, the viral infection induced unfolded protein response (UPR) is demonstrated to attenuate IFN response by phosphorylation-dependent degradation of IFNAR1. Upon viral infection, endoplasmic reticulum resident protein kinase named PKR-like ER kinase (PERK) is phosphorylated, and p-PERK can target IFNAR1 for ubiquitination and degradation [259].

The activities of the IFNAR-associated JAK kinases including JAK1 and Tyk2 are also interfered by several viruses. The NS5 protein encoded by Japanese encephalitis virus inhibits Tyk2 activation via activating protein tyrosine phosphatases [260]. The E6 protein of HPV18 directly interacts with TYK2 to impair its function while the Large T protein encoded by polyoma virus target another kinase JAK1 [261,262].

Comparing with JAK kinases, the downstream signaling cascades STATS are more common target for viruses. Viruses evolved multiple mechanisms to interfere the function of STATs in IFN signal pathway which is exemplified by paramyxoviruses. Most family members of paramyxoviruses inhibit STAT function via V and P proteins. The V protein of Rubulavirus genus blocks IFN signaling by targeting STATs for proteasomal degradation. For examples, Parainfluenza virus 5 and Parainfluenza virus type 2 target STAT1 and STAT2 respectively, while Mumps virus targets both STAT1 and STAT3 [263,264]. In contrast, the V proteins from other paramyxovirus genus including Nipah and Hendra viruses interfere with the function of STATs by sequester STAT1 and STAT2 in high molecular mass complexes [265]. Also, the P protein of

Rabies virus (RABV) sequesters STATs by forming an inactive complex. Unlike the V proteins of Paramyxoviruses, the RABV P protein only interacts with the STATs after tyrosine phosphorylation rather than inactive ones [266]. For the Sendai virus, P/V genes encoded another set of proteins named the C proteins which also block IFN signalling by sequestering STATs, increasing their turnover and altering the pattern of STAT1 phosphorylation [265]. Other positive RNA viruses or DNA viruses can also target STATs for interfering IFN response. The core protein of HCV is reported to interact with STAT1 to inhibit its phosphorylation and interaction with STAT2 [267]. The ORF6 proteins of SARS coronavirus inhibit STAT1 nuclear translocation but not affect the STAT phosphorylation [268]. The adenovirus E1A proteins can bind not only STAT1 but also IRF9 to interfere the formation of ISGF3 complex [269].

Additionally, virus can suppress IFN signaling by regulate members of the SOCS family. SOCSs proteins are potent inhibitors of JAK/STAT signaling. SOCS1 has been characterized to block IFN signaling by directly interacting with JAK1, whereas SOCS3 and CIS directly bind with the phosphorylated receptor to prevent the recruitment and phosphorylation of STATs [270]. Many viruses, like respiratory syncytial virus, HCV, HPV evolve to inhibit STATs activation by modulate SOCS1 or SOCS3 expression [270].

1.3.3 Antagonists encoded by members of picornavirus family

Picornavirus family contains varied members which develop different strategies to antagonize type I IFN response. Studies on these viruses are mainly focus on their ability to inhibit IFN induction. Both MDA5 and RIG-1 are reported as sensors in picornavirus infection. Different picornaviruses like poliovirus, rhinoviruses, echovirus, and encephalomyocarditis virus can suppresses production of IFN-α/β by interfering with MDA-5 and RIG-1 [271,272,273]. For instance, rhinovirus type 1a and encephalomyocarditis virus target MDA-5 to degradation in a proteasome- and caspase-dependent manner and the degradation correlates with the cleavage of poly ADP-ribose polymerase (PARP), which is the hallmark of apoptosis. Studies on the poliovirus demonstrated the virus can reduce the IFN-β secretion by repressing cellular secretary pathway [274]. Additionaly, viral proteinase 2A of poliovirus is essential for viral replication in type I interferon treated cells [275]. This suggests that 2A protein of poliovirus may play an inhibitory role in downstream interferon signaling or in the antiviral activities of ISGs. The leader proteinase of foot and mouth disease virus can inhibit type I IFN production by cleaving p65-RelA [276,277]. Also, the 3Cpro of hepatitis A virus inhibit type I IFN production by cleaving mitochondrial antiviral signaling proteins [278].

In the case of EV71, a more recent study on $3C^{pro}$ demonstrated this viral protease was associated with RIG-I. This association would disrupt the recruitment of an adaptor IPS-1 by RIG-I and result in repression of IFN-α/β [279]. To our knowledge, whether and how EV71 antagonist downstream type I IFN signaling pathway is still elusive. The study on murine infection model indicated that administration of type I IFN before

viral infection protected mice from EV71 challenge while little protective effect was achieved when administration of IFN post viral infection [108]. Recent work in our lab also demonstrated that the conventional IFN can control EV71 infection and replication only in high does concentration [110]. These observations suggested that the EV71 as many other virus may develop the mechanism to circumvent the IFN response.

1.3.4 Thesis hypothesis and objective

Hypothesis: it is predicted that EV71 has developed mechanism to antagonize type I IFN response. In addition, viral protein encoded by EV71 may play an essential role in this process.

Objectives:

I. To accurately describe the viral kinetics of EV71 in human RD cells

II. To investigate the effects of EV71 infection on different aspects of type I IFN signaling and explore the potential mechanism used by EV71 to antagonize IFN response.

Chapter 2 Materials and Methods

2.1 *Cells and Virus propagation*

RD (rhabdomyosarcoma), 293T and HeLa cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 U/ml penicillin and 100μg/ml streptomycin. EV71 stain SHZH98 (GenBank accession number AF302996.1) was obtained from Shenzhen Center for Disease Control and Prevention, Shenzhen, China. To prepare virus stocks, viruses were propagated on a 90% confluent monolayer cells in DMEM with 2% FBS. When >50% of the cells have detached from the plate, viral stock was prepared by lysis cells with three consecutive freeze-thaw cycles. After the third cycle, briefly centrifuge to pellet debris and store at -80°C. The viral titers were measured by the end-point dilution assays and expressed as plaque-forming unit (PFU) per milliliter according to the Spearman-Karber method.

2.2 *Viral infection and cytopathic effects assay*

One day after plated, the cultured cells were infected with nil or EV71 at different MOI (multiplicity of infection) as indicated. Briefly, plated cells were washed twice with PBS and infected with EV71. Time was set as zero after adsorption for 1 hour. The inocula were removed and cells were washed twice with PBS to remove unattached virus before adding culture medium to each well. The cells were cultured at 37°C in 5% CO2. The cell morphology was monitored and recorded by using a phase-contrast microscope associated with a CCD camera and computer at different time points. The infected cells and culture supernatants were harvested for isolating RNA and proteins.

2.3 *MTT assay*

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed to determine the cell viability upon EV71 infection. Briefly, RD cells were set in 96-well plates at $1x10^4$ cells per well 24 hours before infected with EV71 at MOI 1 or MOI 10. The medium was then replaced with 0.5 mg/ml MTT medium at different time points and incubated for another 4 hours. The MTT solution was removed from the wells and the formazan crystals were dissolved in DMSO. The absorbances of the formazan products were measured at 550 nm with the reference wavelength at 690 nm.

2.4 *Plasmids*

All EV71 protein-encoding plasmids were generated in the *pcDNA4/HisMax B* (Invitrogen, USA) background. Primer sequences used for the generation of these constructs are listed in table 2.4. Each cDNA fragment was directionally cloned into the *NotI* and *XbarI* sites of the plasmid. Human *IFNAR1* (Accession NM_000629.2) was amplified by RT-PCR from RNA isolated from 293T cells, and IFNAR1 expression plasmid was constructed by inserting the cDNA of *IFNAR1* into *pcDNA4/HisMax B* vector. Mutation of Cys^{110} $2A^{pro}$ to Ala^{110} of EV71 and Ser^{535} to Ala^{535} of IFNAR1 was carried out by site-directed mutagenesis with one step muatagenesis kit (GeneTailor Site-Directed Mutagenesis System, Invitrogen, USA). To express 2A and mutant 2A in *E. coli*, 2A and 2A mutant fragments were amplified and cloned into *PET-28a* (Novagen, USA) vector by using primer PET-2AF/R which containing *EcoRI* and *XhoI* recognition sites respectively.

Gene	Sequences of primers	
2A	F	5'- GGGCGGCCGCATGGGGAAATTTGGGCAACAG -3'
	R	5'- GCTCTAGACTGCTCCATAGCTTCTTC -3'
2B	F	5'- GGGCGGCCGCATGGGCGTGTCCGATTACATC-3'
	R	5'- GCTCTAGATTGCTTATGAGCGATGGGGATCC -3'
2C	F	5'- GGGCGGCCGCATGAGCGATTCCTGGCTAAAGAAG -3'
	R	5'- GCTCTAGATTGGAATAGAGCCTCGATTGTGTTG -3'
3A	F	5'- GGGCGGCCGCATGGGCCCACCCAAGTTCA -3'
	R	5'- GCTCTAGATTGAAACCCGGCAAAGAGC -3'
3B	F	5'- GGGCGGCCGCATGGGTGCGTATTCTGGTGCT-3'
	R	5'- GCTCTAGATTGCACTGTGGCTGTGC -3'
3C	F	5'- GGGCGGCCGCATGGGGTCCGACCTTGATTTTGCT -3'
	R	5'- GCTCTAGATTGCCTACTAGCAAAGTAACTCC -3'
3D	F	5'- GGGCGGCCGCATGGGAGAGATCCAGTGGGT -3'
	R	5'- GCTCTAGAAAATAACTCGAGCCAATTGCGTC -3'
VP1	F	5'- GGGCGGCCGCATGGGAGATAGGGTGGCAGAT -3'
	R	5'- GCTCTAGAGAGAGTGGTGATCGCTG -3'
VP2	F	5'- GGGCGGCCGCATGTCCCCATCCGCTGAG -3'
	R	5'- GCTCTAGACTGGGTAACTGCTTGCCTAAG -3'
Vp3	F	5'-GGGCGGCCGCATGGGTTTTCCCACTGAATTG-3'
	R	5'- GCTCTAGACTGGATGGTGCCCGTCT -3'
VP4	F	5'- GGGCGGCCGCATGGGTTCACAGGTGTCCA -3'

	R	5'- GCTCTAGACTTCAGTGGCGCTGCCATTT -3'
IFNAR1	F	5'- CGGGATCCAATGATGGTCGTCCTCCTGG -3'
	R	5'- TGCTCTAGATCATACAAAGTCCTGCTGTAGTTC -3'
2A (for PET vector)	F	5'- CCGAATTCATGGGGAAATTTGGGCAACAG -3'
	R	5'- GCACTCGAGCTGCTCCATAGCTTCTTC -3'
2A mu331	F	5'-TCACTCAGAACCTGGTGAT**GCC**GGCGGTATCC -3'
	R	5'- ATCACCAGGTTCTGAGTGACCTTGTGCGAG -3'
IFNRmu535	F	5'- GTTCCCAAACTAGCCAAGATGCCGGAAATTATTC -3'
	R	5'- ATCTTGGCTAGTTTGGGAACTGTATTTTTTATG -3'

Table 2.4 Primers used for cloning. The recognition sites for related restriction enzymes are underlined, and mutant site sequences in 2A were bolded. F, forward; R, reverse.

2.5 *Transfection and cell treatment*

HEK293 or HeLa cells were plated in 12-well clusters at 1 x 10^5 cells per well and allowed to adhere for 1 day prior to transfection. The next day, transfections were performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's recommendations. For interferon stimulation, IFN-α2b (PBL, USA) was added at indicated concentration at 24h after transfection and cells were harvested at indicated time after stimulation. Proteins and RNA were extracted and applied for Western Blot and Quantitative reverse transcription-PCR analysis.

2.6 *RNA extraction and cDNA synthesis*

Celluar RNA was extracted by using TRIzol reagent (Invitrogen, USA). Briefly, about 2 x10^5 cells (for 12 well-plate assay) were homogenized in 0.5mL Trizol. Completely lysis was performed by incubated cells at room temperature for 2-3 mins, and then 0.1mL chloroform was added followed by vigorously shaken for 15 sec. Next, mixture was centrifuged at 12,000g for 15min at 4°C, and the colorless upper aqueous phase (about 0.2mL) was transferred to the new centrifuge tube, precipitated by adding 0.2mL isopropyl alcohol. The RNA precipitate was achieved by centrifuging at 12,000g for 10min at 4°C and washing twice with 75% ethanol. Finally, purified RNA was dissolved with RNA-free water. The quality and quantity of total RNA were deterimined by measuring the absorbance at 260/280nm by NanoDrop ND-1000 (NanoDrop Technology, Wilmington, DE, USA). 1 μg of isolated RNA was reverse-transcribed into cDNA with random primer by using Reverse Transcription System (Promega, USA).

For intracellular viral RNA quantification, the total cellular RNA was isolated from EV71 infected cells. For quantification of the extracellular virions, the virions were first isolated from the culture media of infected cells. The media were harvested and briefly centrifuged to remove cell debris. Viral core particles were then precipitated with 10% polyethylene glycol 8000 containing 0.5M NaCl at 4°C overnight. After centrifugation for 30 min at 16,000 g, viral particles were pelleted and treated with 100 μg/ml of RNaseA (Sigma, USA). To isolate the intracellular virions, EV71 infected cells were lysed with lysis buffer (1% Triton 100 and 1 x Roche protease inhibitor cocktail in PBS). Then the cell lysates were used to isolated viral particles as described above. The intracellular viral RNA and RNA of extracellular virions as well as intracellular virions was isolated as described above and used for one-step real-time quantitative PCR. To set up the standard curve of infectious viruses, the viral titers were first determined by CPE assay. Then the viral RNA was extracted from those infectious EV71 viruses. RNA was diluted at tenfold serial and used to reflect the calculated PFU from 10 to 1×10^7 live virions.

2.7 Protein expression and purification

The expressing plasmids (pET-28a 2A-his/ pET-28a m2A-his) were constructed and $2A^{pro}$/ $m2A^{pro}$ were expressed as fusion products in *E.coli* strain BL21 (DE3). Cells were grown in LB media until the optical density reached 0.6, and then IPTG was added to final concentration of 0.5 mM. Cells were grown at 30°C for an additional 4 hours before harvested by centrifugation, and then resuspended in buffer A (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 20% glycerol, 1 mM DTT). Cells were lysized by sonication Bacterial cell lysates were centrifuged for 30 min at 10,000g and proteins

were precipitated from the soluble fraction. The pellets which contained the majority of 2A-his were solubilized with 8M urea in buffer A, and then purified under denaturing condition by nitriloacetic acid-nickel chromatography. These proteins were renatured for 2 days with gradual dilution of 6, 4, 2 and 0 M urea containing buffer A. The $2A^{pro}$ and $m2A^{pro}$ protein concentration were measured using the method of Bradford (Bio-Rad, USA).

2.8 Direct cleavage assays of $2A^{pro}$

293T cells were transfected with IFNAR1 expression plasmid, and cell lysis was collected at 24 hours after transfection. To test the protease activity of $2A^{pro}$ on IFNAR1 in vitro, 30μg cell lysis was incubated with 500ng purified $2A^{pro}$ and $m2A^{pro}$ respectively. Reaction buffer contained 100mM NaCl, 1mM $MgCl_2$, 0.4mM 2-mercaptoethanol, and 5% glycerol. After 4 hours incubation at 37°C, protein samples were boiled for further Western blotting analysis.

2.9 Real-Time Quantitative PCR

The RNA viral loads were quantified by using one-step quantitative real-time PCR (qRT-PCR) with QuantiTect SYBR Green RT-PCR Kit (Qiagen) and specific forward EV71-VP1F and reverse EV71-VP1R primers targeting a conserved region of the VP1 gene [110]. PCR assay was carried out in 20 μl volume consisting of 10 μl of 2 × Quantitect SYBR green RT-PCR Master Mix, containing HotstarTaq DNA polymerase, 1 μl of 10 μM of each oligonucleotide primer, 0.2 μl of 100 × QuantiTect RT Mix (containing Omniscript and Sensiscript reverse transcriptases) and 2 μl of RNA extracted from samples or from ten-folds serial diluted virus RNA standard (from 10^7 to

10 copies). The target fragment amplification was carried out as follows: reverse transcription at 50 °C for 30 min; initial activation of HotStar Taq DNA Polymerase at 95 °C for 15 min; 45 cycles in four steps: 94 °C for 10 s, 56 °C for 30 s, 72 °C for 30 s. At the end of amplification cycles, melting temperature analysis was carried out by a slow increase in temperature (0.1 °C /s) up to 95 °C.

To quantify transcription level of other target genes, the indicated cDNA was used and qRT-PCR was carried out with Power SYBR Green Master Mix (Applied Biosystems, USA). The PCR reaction was set up under the following thermal cycling conditions: 95°C, 10 min; 45 cycles of 95°C, 15 s and 60°C, 1 min. Fluorescence signals were collected by the machine during the extension phase of each PCR cycle. The CT value was normalized to that of GAPDH. Primers used in qRT-PCR were listed in table 2.9. All samples were run in triplicate and the experiment was repeated three times. The relative mRNA level of each target gene was expressed as a fold change to corresponding controls (set as 1).

Gene	Genbank Accession No.	Sequences of primers
VP1	AAG23181	F 5'-GCAGCCCAAAAGAACTTCAC-3'
		R 5'-ATTTCAGCAGCTTGGAGTGC-3'
IFN-β	NM_002176	F 5'-GACCAACAAGTGTCTCCTCCAAA-3'
		R 5'-GAACTGCTGCAGCTGCTTAATC-3'
ISG15	NM_005101	F 5'-ATGGGCTGGGACCTGACG-3'
		R 5'-GCCAATCTTCTGGGTGATCTG-3'
ISG56	NM_001548	F 5'- TCCCCTAAGGCAGGCTGTC-3'
		R 5'-GACATGTTGGCTAGAGCTTCTTC-3'
MXA	NM_002462	F 5'-GCTTGCTTTCACAGATGTTTCG-3'
		R 5'-AAGGGATGTGGCTGGAGATG-3'
OAS1	NM_016816	F 5'-TCCACCTGCTTCACAGAACTACA-3'
		R 5'-TGGGCTGTGTTGAAATGTGTTT-3'
GAPDH	NM_002046	F 5'-GATTCCACCCATGGCAAATTCCA-3'
		R 5'-TGGTGATGGGATTTCCATTGATGA-3'

Table 2.9 Primers for Quantitative Real-time PCR Analysis.

2.10 Western Blotting analysis

Total cellular protein extracts were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 x Roche protease inhibitor cocktail) with occasional vortexing. Lysates were then collected by centrifugation at 14,000 rpm for 10 min at 4°C and protein concentrations were determined by the Bradford method (Bio-Rad, USA). The same amount of total protein for each sample was loaded and separated by 8% to 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked by 5% skim milk in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) followed by an incubation with specific antibodies. The unphosphorylated forms of STAT1 and STAT2 were detected using anti-STAT1 and anti-STAT2 (sc-346 and sc-476, Santa Cruz Biotechnology, USA). Antibodies (9171 and 4441, Cell Signaling Technology, USA) were used to detect phosphorylated forms of STAT1 and STAT2. The phosphorylated forms of Jak1 and Tyk2 were detected by using anti- Phospho-Jak1 and anti- Phospho-Tyk2 (3331 and 9321, Cell Signaling Technology, USA). The IFNAR1 and ubiquitin were detected with anti-IFNAR1 (sc-7391, Santa Cruz Biotechnology) and anti-ubiquitin (P4D1, Cell Signaling Technology, USA) antibodies respectively. Target proteins were finally visualized with corresponding secondary antibodies (Santa Cruz Biotechnology, USA) in a chemiluminescence detection system (Amersham Biosciences, USA). Each immunoblot was done at least thrice to confirm the results. The signal intensity of the relative band was quantified by using Quantity One software (BioRad, USA).

2.11 Immunoprecipitation

Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1 mM NaF, 1 mM sodium orthovandate and a protease inhibitor cocktail (Roche, Mannheim, Germany). For immunoprecipitation of IFNAR1, Dynabeads Protein G (Invitrogen, USA) was first incubated with IFNAR1 antibody (sc-7391, Santa Cruz Biotechnology, USA) according the manufacture's recommendations. 500 μg of total protein in 500 μL lysis buffer was incubated with the dynabeads which has bounded with primary antibody overnight at 4°C. After washing, the beads were boiled in Laemmli buffer and proteins were applied for Western Blot analysis.

2.12 Immunofluorescence

Inactivated virions were done by UV irradiation for 10 min with a 350-nm wavelength source at a distance of 10 cm. The irradiation can break viral DNA, and therefore inhibit the expression of viral genes. Cells cultured in 24-well plates were infected with EV71 at MOI of 10 or UV-inactivated virus. The mock-infected cells were set as control. 9h p.i., cells were fixed with cold methanol at -10°C for 5 min. After three washes with PBS, cells were blocked with 10% FBS in PBS for 40min. Samples were next incubated with anti-IFN-α/βRα (sc-7391, Santa Cruz Biotechnology, USA) and anti-VP1 (PAB7631-D01P, Abnova, USA) for 1 h. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG; Invitrogen, USA) and rhodamine-conjugated goat anti-rabbit IgG (Invitrogen, USA) at a dilution of 1:200 was used to visualize

IFNAR1 and VP1 proteins. The slides were with a Nikon fluorescence microscope and photographed with a CCD camera.

2.13 Statistical Analysis

Results are expressed as mean ± standard deviation (SD). All statistical analyses were carried out with SPSS 14.0 software (SPSS Inc.). Two-tailed Student's t test was applied for two-group comparison. A p value < 0.05 was considered statistically significant.

Chapter 3 Results

This chapter was divided into two parts. In the first part, viral kinetics of EV71 in RD cells was characterized; and in the second part, mechanism of EV71 to antagonize type I IFN response to escape the antiviral effects of the host was delinated.

3.1 Viral Kinetics

It was known that viral kinetics provided basic information for the study of viral pathogenesis. Thus, the EV71 kinetics was firstly investigated. RD cells were infected with EV71 at MOI 1 and MOI 10, and then the cell growth, viral protein expression, viral replication, package and secretion were measured at different times post infection.

3.1.1 Cytopathic effects and the kinetic of cell viability

The morphological changes were shown as early as 6 hours post infection (p.i.) when RD cells were infected with EV71 at either MOI 1 or MOI 10 (Figure 3.1.1A, panel f and j). Initially, the cells rounded up and became more refractile. As the culture progressed, some infected cells detached from the culture plate and floated into the medium (Figure 3.1.1A, panel g, h, k and l). Compared with the cells infected at MOI 1, more cells were unhealthy at 6 hours p.i. when infected at MOI 10 (Figure 3.1.1A, panel j vs f). Later on, the cells infected with EV71 both at MOI 1 and MOI 10 underwent significant cell death and detached from the surface of culture dishes (Figure 1A, panels g and k). At 24 hours p.i, most of cells were detached from the surface of the plate in both of these two different infected groups (Figure 3.1.1A, panels h and l). The MTT assay showed that the viability of the cells infected with EV71 at MOI 1 did not significantly decrease at 12 hours p.i. while slightly reduced for the cells were infected at MOI 10 (Figure 3.1.1B). At 18 and 24 hours p.i., the viability of the cells infected with EV71 either at MOI 1 or MOI 10 significantly decreased.

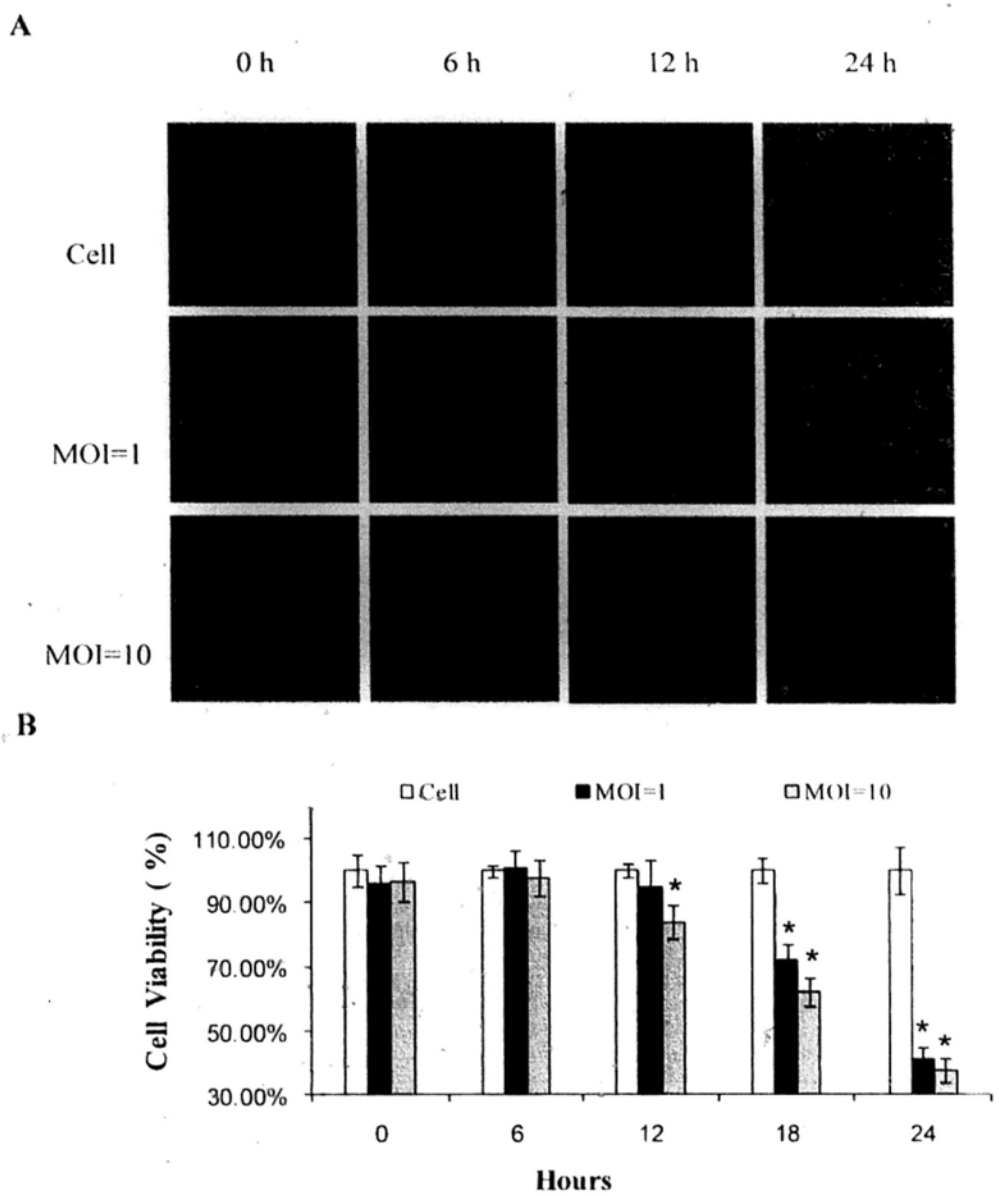


Figure 3.1.1 Cytopathic effects and kinetics of cell viability upon EV71 infection. RD cells were infected with EV71 at MOI 1 or MOI 10. A. The cytopathic effects were shown by cell morphological changes (original magnification, ×100); B. Cell viability was measured by MTT assays at different time points after EV71 infection. Data are shown as mean ± standard deviation (SD) of three independent experiments, each done in triplicate. *, $p < 0.05$.

3.1.2 The kinetics of viral replication

To examine the kinetics of viral replication, the levels of intracellular viral RNA were measured by quantitative RT-PCR at each time point. As shown in figure 3.1.2, the intracellular viral RNA began to increase at as early as 3 hours p.i. and the exponential phase was from 3 to 6 hours p.i in both infected groups. In the case of infection at MOI 1, the intracellular viral RNA continually increased from 6 to 12 hours p.i., and then gradually decreased until 24 hours p.i.. In case of MOI 10, the intracellular viral RNA reached a peak between 6 and 9 hours p.i., and then began to decrease.

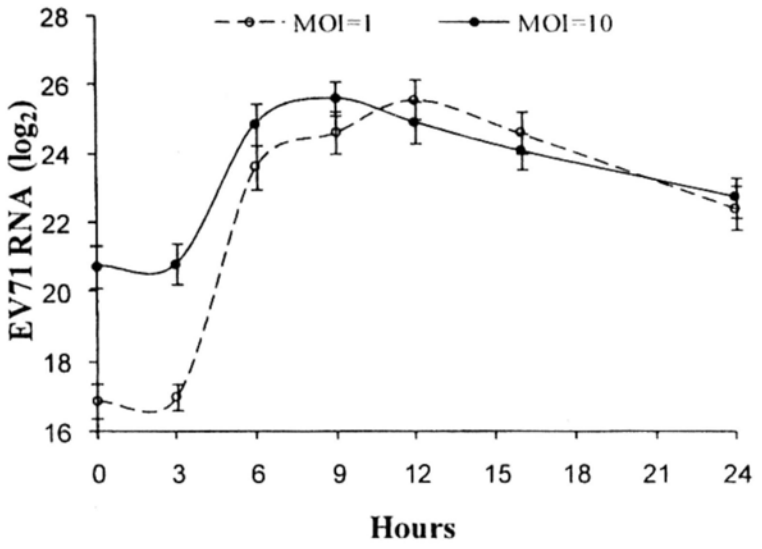


Figure 3.1.2 The kinetics of EV71 Replication. RD cells were infected with EV71 virus at MOI=1 or MOI=10. At the indicated time points, the levels of total intracelluar viral RNA were measured by qRT- PCR. Data are shown as mean ± SD of three independent experiments; each was done in triplicate.

3.1.3 The kinetics of viral protein synthesis

The intracellular viral protein VP1 could not be detected until 6 hours p.i. in the cells infected at either MOI 1 or MOI 10 (Figure 3.1.3). The VP1 protein level reached the peak at 9 hours p.i. in the cells infected with EV71 at both MOI 1 and MOI 10. Obviously, the VP1 levels were much higher in the cells infected at MOI 10 than that of MOI 1. In the case of infection at MOI 1, the VP1 level was maintained until 12 hours p.i.; whereas the VP1 protein level fast decreased after 9 hours p.i. when the cells were infected with EV71 at MOI 10.

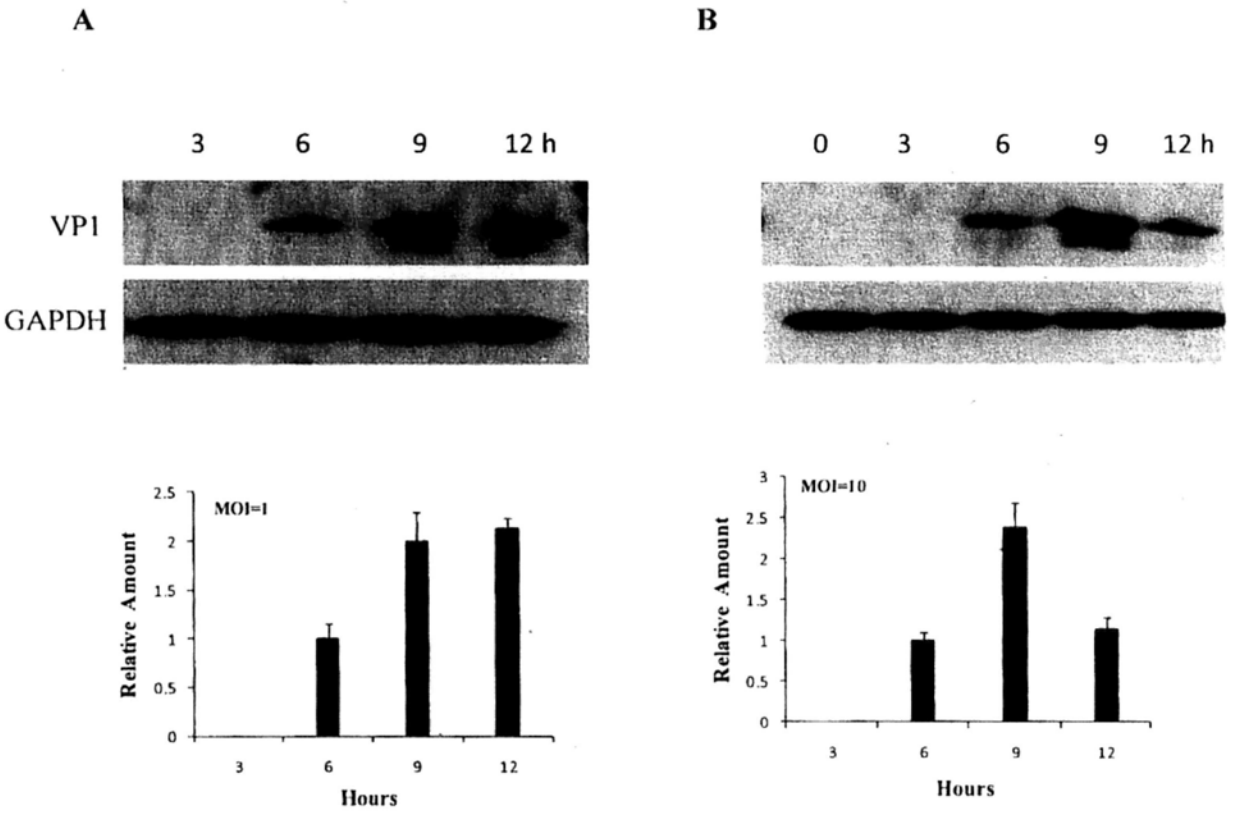


Figure 3.1.3 The kinetics of virus VP1 protein synthesis. RD cells were infected with EV71 virus at MOI=1 (A) and MOI=10 (B). The intracellular viral protein VP1 was measured by Western blotting. The relative VP1 levels (the density of VP1/GAPDH) were calculated and shown as solid bars.

3.1.4 The kinetics of viral package

To determine the kinetics of virus package, the intracellular EV71 virions were quantified at different time points post infection. At 3 hours p.i, the intracellular virions obviously decreased. Thereafter, the intracellular virions began to increase and enter into the exponential phase until 12 hours p.i. when the amount of intracellular virions reached to the peak. The viral package kinetics was comparable for both MOI 1 and MOI 10 infected groups (Figure 3.1.4). The total amounts of intracellular virions were then decreased from 12 to 24 hours p.i.

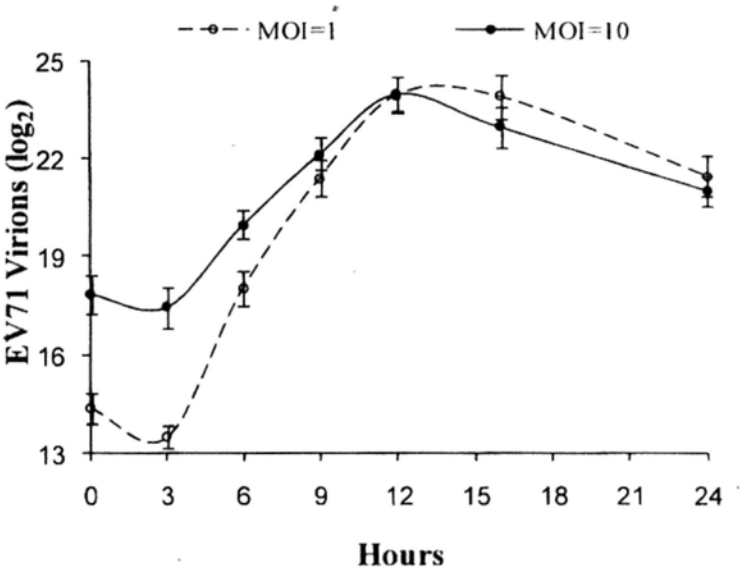


Figure 3.1.4 The kinetics of EV71 virus package. RD cells were infected with EV71 virus at MOI=1 or MOI=10. The intracellular virus particles were isolated to measure the virion RNA by qRT-PCR. Data are shown as mean ± SD of three independent experiments; each was done in triplicate.

3.1.5 The kinetics of viral secretion

To determine the kinetics of virus secretion, the extracellular EV71 virions were quantitated. The EV71 virions began to be released from the cells infected either at MOI 1 or MOI 10 three hours p.i. (Figure 3.1.5). The amounts of extracellular EV71 virions in the cultures of the two groups were constitutively increased. From 3 to 6 hours p.i., the virions were slowly secreted into the culture media, and the virus secretion entered into the exponential phase from 6 to 12 hours p.i.. After 12 hours p.i., the increasing rate declined and the total amount of extracellular virions reached maximal at 24 hours p.i.. For cells infected at MOI 1 or MOI 10, the virions in the culture media were almost the same at 24 hours p.i..

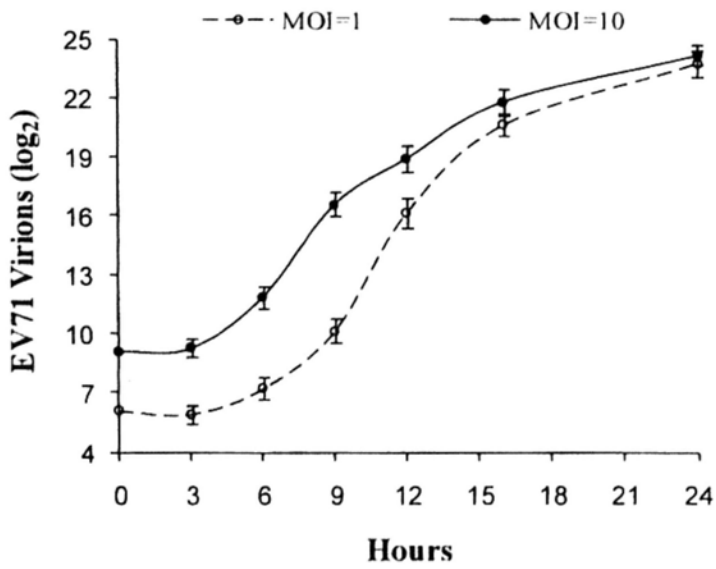


Figure 3.1.5 The kinetics of EV71 virus secretion. RD cells were infected with EV71 virus at MOI=1 or MOI=10. Extracellular EV71 virions in the culture media were measured by qRT-PCR at different time points post infection. Data are shown as mean ± SD of three independent experiments; each was done in triplicate.

3.2 *EV71 antagonize type I IFN response*

3.2.1 EV71 infection activated Type I IFN production but inhibited ISGs activation

Most types of cells can sense the viral infection and activated IFN-β production at the first of time. Through IFN signaling pathway, the secreted IFN-β next stimulate mounts of ISGs which play detrimental roles in anti viral infection. Therefore, the levels of IFN-β and related ISGs are regarded as hallmarks in these two steps. To determine effect of EV 71 infection on type I IFN response, the mRNA level of IFN-β and a subset of ISGs were investigated at different time points post virus infection. The human rhabdomyosarcoma (RD) cells were infected with EV71 (MOI1 or MOI10) or mock treated as described in Materials and Methods. Cellular RNA was isolated at 3, 6, 9, 12, 24 hours post infection and mRNAs encoding IFN-β, ISG15, ISG56, MXA and OAS1 were quantified by quantitative RT-PCR. When infected with EV71 at MOI of 10, little cells could be collected at 24h p.i. because of the CPE. To our surprise, IFN-β level was highly increased following the infection (Fig 3.2.1 A, B). The induction of IFN-β was activated at 6h p.i., and the mRNA level of IFN-β is higher in cells infected at relatively higher MOI. For cells infected at MOI of 1, mRNA level of IFN-β was continuously increased following virus infection and reach 25 folds higher than in mock infected cells at 24h p.i. For cells infected at MOI 10, the induction of IFN-β was faster, and mRNA level of IFN-β led to peak at 9 p.i. These results demonstrated that the transcription of IFN-β was actually activated in the process of EV71 replication.

In contrast to the activation of IFN-β, the mRNA levels of a group of ISGs were unchanged and even little decreased following the EV71 infection as compared with mock infected cells (Fig. 3.2.1 C, D). These data suggested that host cells sensed the EV71 infection and activated IFN-β production. However, the IFN-β stimulated by EV71 infection did not take effects as the downstram ISGs, which function as antiviral effectors, were inhibited by the EV71.

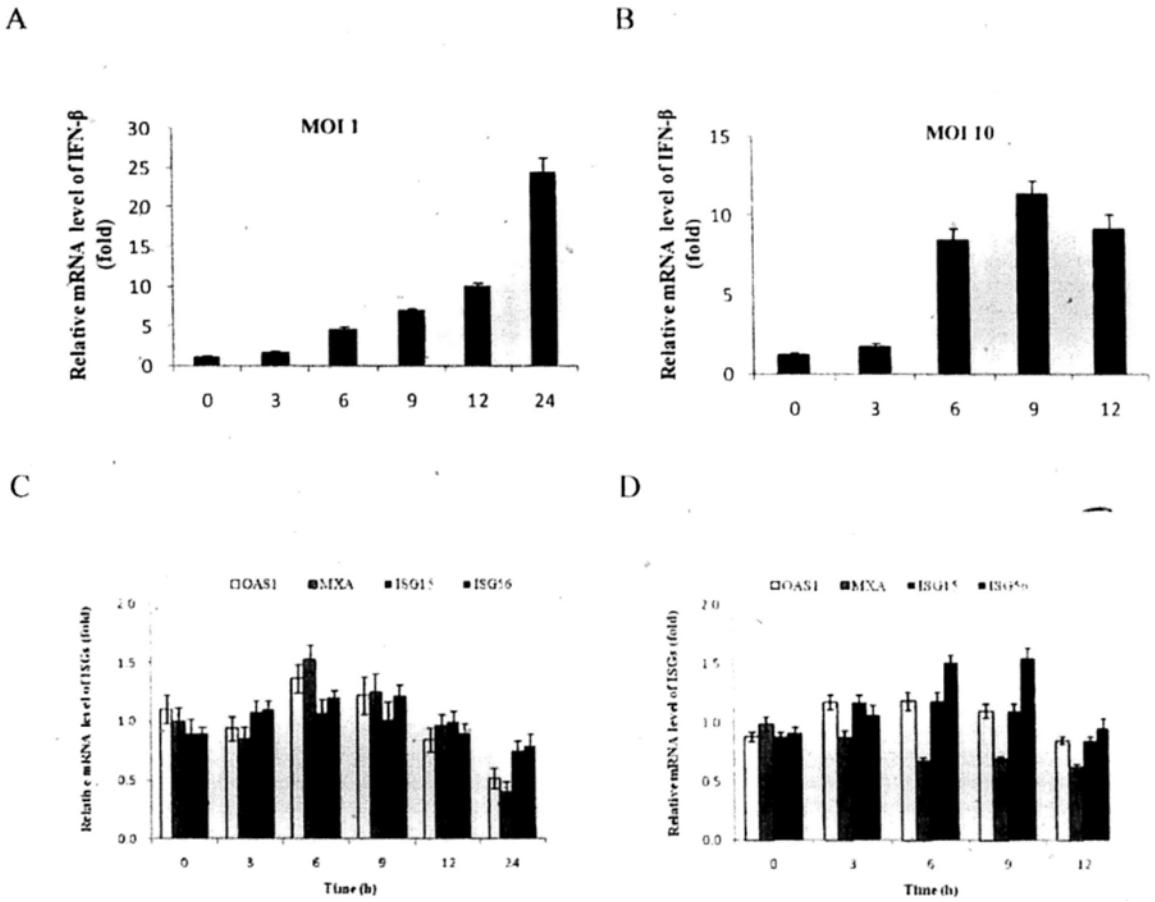


Figure 3.2.1 EV71 infection activated Type I IFN production but inhibited ISGs activation. RD cells were mock-infected or infected with EV71 at MOI 1 (A, C) or MOI 10 (B, D). Cells were collected at 0, 3, 6, 9, 12, 24 h after infection. The expression of IFN-β (A, B) and ISGs (C, D) were measured by quantitative RT-PCR. Data are shown as mean ± SD of three independent experiments; each was done in triplicate.

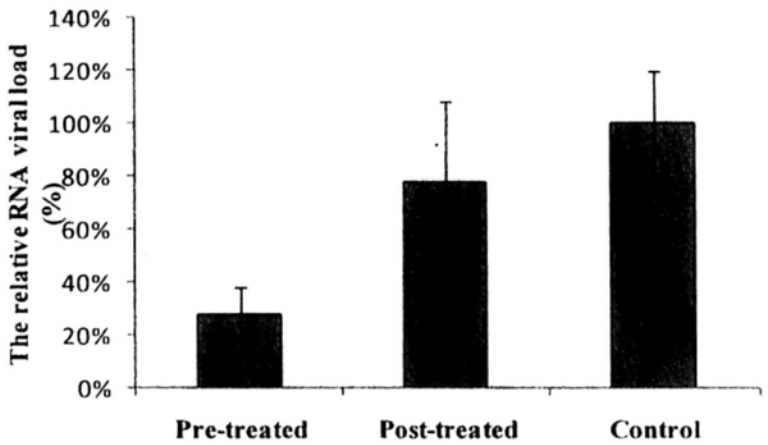
3.2.2 EV71 antagonized the antiviral effect of IFN-α treatment

Promoted by previous results, I further tested the ability of EV71 to subvert exogenous IFN-α mediated antiviral effect. RD cells were infected with EV71 at MOI of 1, and IFN-α treatment (1000U/ml) was performed at 6h before or post viral infection. Mock treated cells were set as a control. 24h after infection, cells were collected and RNA levels of EV71 were determined by quantitative RT-PCR. As shown in figure3.2.2A, the treatment of IFN-α before viral infection effectively reduced RNA viral loads in infected cells. In IFN treated cells, the EV71 RNA levels were 30% of virus control group. In contrast to preinfection treatment, no significant protection was observed when IFN was added after EV71 infection.

To further confirm the inhibition role of EV71 on the antiviral effect of exogenous IFN-α treatment, the mRNA levels of IFN induced antiviral effectors were measured in infected cells. RD cells were either mock or EV71 infected at MOI of 10. At 9h p.i., cells were stimulated with IFN-α2b (100U/ml) for another 1 hour. After treatment, the quantitative RT-PCR was applied to detect relative mRNA level of ISGs. As shown in figure3.2.2B, the IFN stimulation induced robust expression of all checked ISGs in mock infected cells as expected while not in EV71 infected cells. Comparing to mock infected cells, ISGs activation in EV71 infected cells was dramatically suppressed after IFN-α treatment.

Taken together, these results revealed that EV71 developed a defense mechanism to eliminate the innate interferon immune response against viral infection and benefit its survival and replication.

A



B

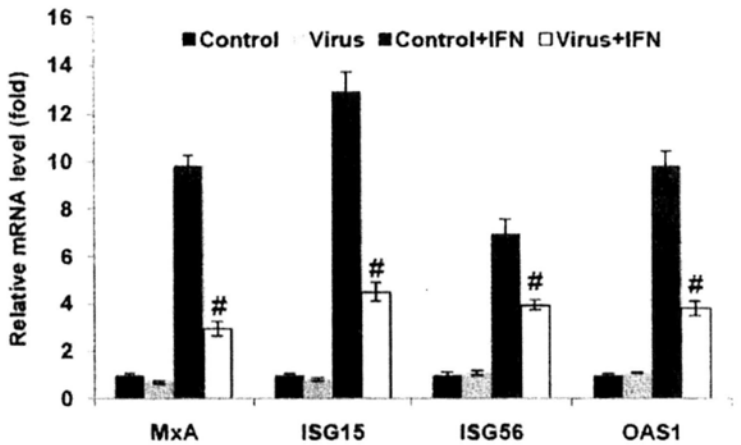
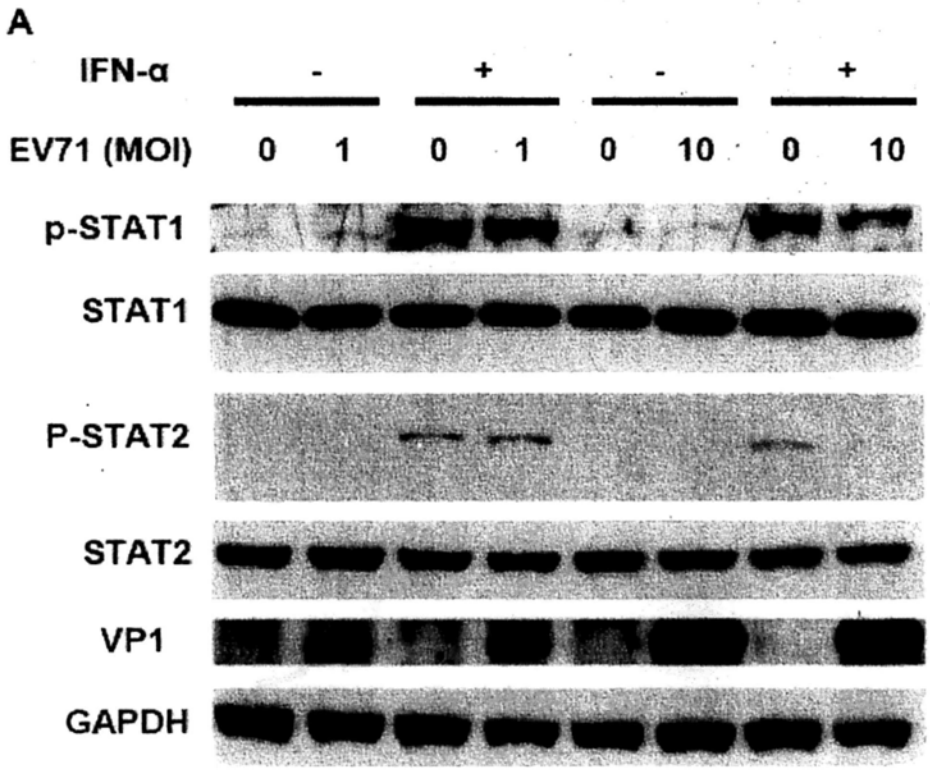


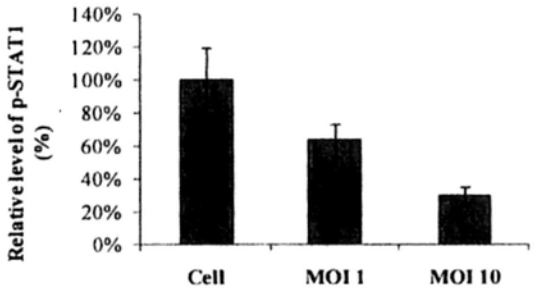
Figure 3.2.2 EV71 antagonized the antiviral effect of IFN-α treatment. (A) RD cells were infected with EV71 at MOI of 1, and IFN-α treatment (1000U/ml) was performed at 6 h before or post viral infection. Mock treated cells were set as a control. Cellular

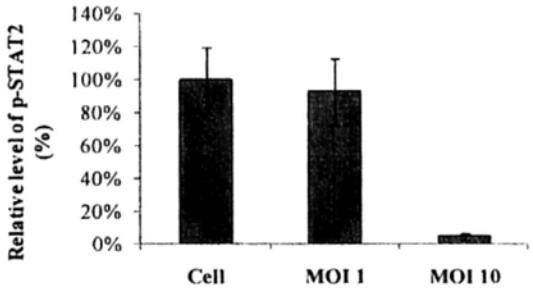
viral loads were quantified at 24h p.i. (B) RD cells were infected with EV71 at MOI 10. Nine hours after infection, cell were stimulated with IFN-α2b (100 U/mL) for another 2 hours. Celluar RNA was extracted and expression levels of relative ISGs were measured by real-time PCR. The expression level of each gene was calculated relative to GAPDH gene expression and normalized to mock treated cells. Data are shown as mean ± SD of three independent experiments; each was done in triplicate.

3.2.3 Phosphorylation of STAT1 and STAT2 was inhibited by EV71

Antagonizing IFN signaling is a possible mechanism to explain the observed defect in ISG activation above. The STATs proteins (STAT1 and STAT2) play a pivotal role in type I IFN signal transduction pathway and thus lots of viruses evolve to inhibit type I IFN signaling by interfering with proteins levels or the activities of the STATs [264,280,281,282]. Therefore, the statuses of STATs in EV71 infected cells were next analyzed by examining the expression level and phosphorylation patterns of STATs. RD or HeLa cells were either mock or EV71 infected at indicated ratio (MOI 1 or 10). At 9h p.i., cells were subsequently stimulated with IFN-α2b at 100 U/mL for another 30mins. The levels of total STATs and phosphorylated STATs were assessed in Western blots by using specific antibodies respectively. As indicated in figure 3.2.3A, the total proteins levels of STAT1 and STAT2 were not significantly altered by EV71 infection in RD cells. However, IFN-α2b treatment induced a rapid and easily discernable STAT1 and STAT2 phosphorylation in mock infected cells. In contrast, there was a notable reduction in IFN-inducible STATs phosphorylation in EV71 infected cells and the suppressive effect was much more significant when cells were infected at higher MOI (Fig 3.2.3A). The similar results were also obtained in HeLa cells (Fig 3.2.3B).


A
IFN-α
-
+
-
+
EV71 (MOI)
0
1
0
1
0
10
0
10
p-STAT1
STAT1
P-STAT2
STAT2
VP1
GAPDH


Relative level of p-STAT1 (%)
140%
120%
100%
80%
60%
40%
20%
0%
Cell
MOI 1
MOI 10


Relative level of p-STAT2 (%)
140%
120%
100%
80%
60%
40%
20%
0%
Cell
MOI 1
MOI 10

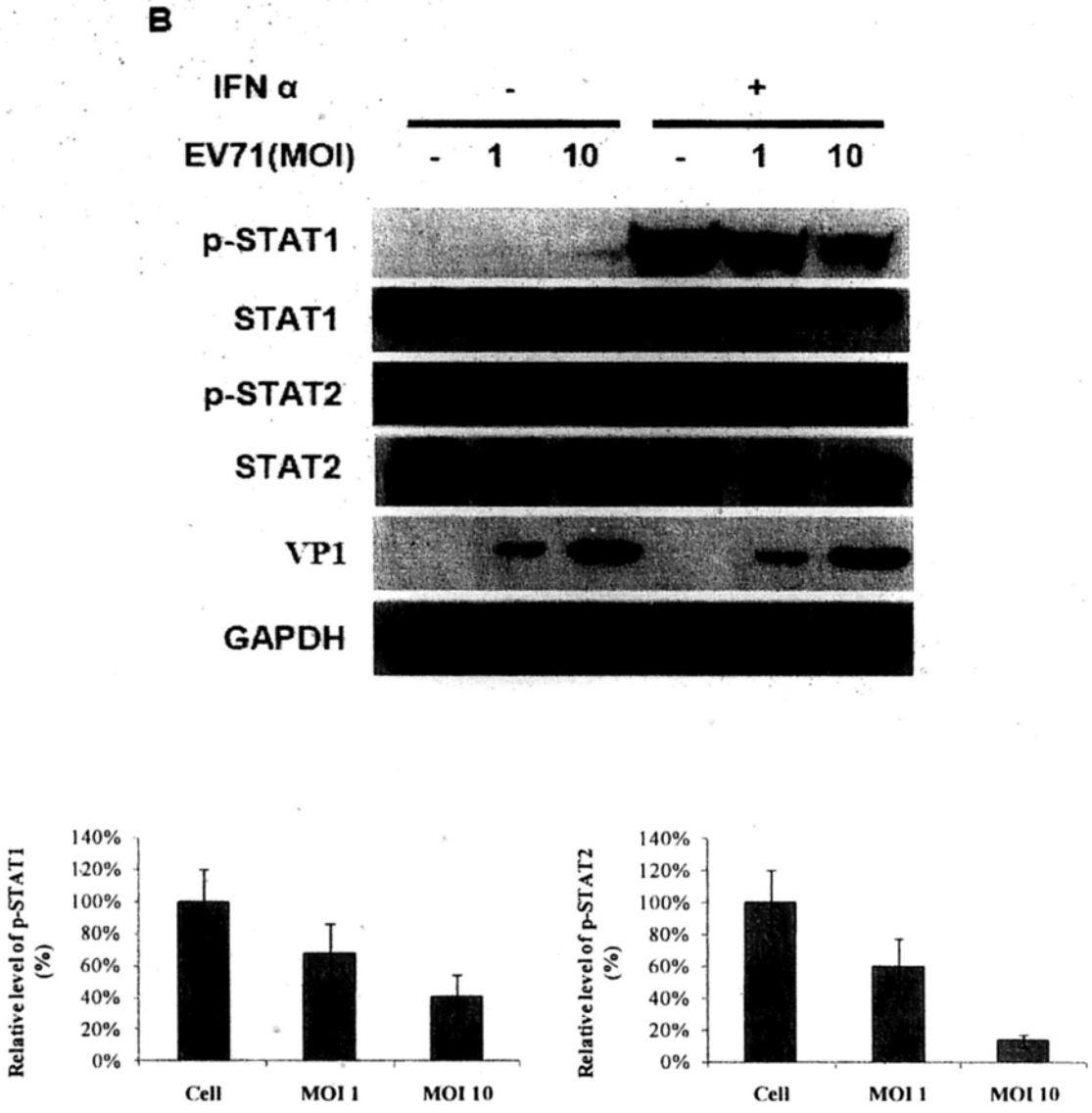


Figure 3.2.3 EV 71 infection inhibited IFN induced STAT phosphorylation. RD cells (A) or HeLa cells (B) were infected with EV71 at MOI 1 or MOI 10 for 9 h and cells were left untreated (-) or treated (+) with IFN-α2b (100 U/mL) for another 30 mins. Western blots was performed by detecting STAT1, STAT2, phosphorylated STAT1, phosphorylated STAT2 and viral structure protein VP1 with relative antibody. GAPDH was also detected as loading controls. Relative level of phosphorylated STAT1 and STAT2 after IFN treatment in virus infected cells was present as a comparison with the

signal intensity in mock-infected control. Data are shown as mean ± SD of three independent experiments; each was done in triplicate.

3.2.4 EV71 Blocked IFN-mediated Jak/STAT signaling by reducing IFNAR1 protein level

Given the effects of EV71 on inhibition of the IFN-induced phosphorylation of STATs, we next examined the influence of viral infection on upstream molecules involved in IFN signaling. The phosphorylation of the tyrosine kinase JAK1 and Tyk2 is regarded as the first step in activation of the IFN signal transduction. To reveal the effect of EV71 infection on the activation of JAK1 and Tyk2, RD cells were infected with EV71 at MOI of 10. 9 hours post infection, cells were treated with IFN-α2b (100U/mL) for 10min and the phosphorylation of JAK1 and Tyk2 was analyzed by using specific antibodies. As shown in figure 3.2.4 A, both kinases were phosphorylated in control cells treated with IFN-α while only background levels of phosphorylation kinases were detected in the infected cells.

Because the phosphorylation of JAK1 and Tyk2 is the first event in the IFN-signaling cascade, the above results suggested that the EV71 may play an inhibition role on the IFN-α/β receptor. To prove this hypothesis, we examined the protein levels of interferon-α receptor I (IFNAR1) with mock or EV71 infection at MOI of 10. Then the RD cells were treated with IFN-α2b for another 30minutes at different time points. The total cells were collected to determine the phosphorylated STAT1 and expression level of IFNAR1 by Western Blotting. As shown in figure 3.2.4B, the inhibition of IFNAR1 started at 6h p.i. when the viral protein like VP1 was expressed at a relative high level. More significant reduction of IFNAR1 was observed at 9h p.i.. Compared with the mock infected cells, the p-STAT1 level in EV71 infected cells was also significantly reduced at this time point, which was consistent with the inhibition of ISGs activation (Fig 3.2.1).

It seemed that the reduction of IFNAR1 occurred at 6h p.i. while the level of the phosphorylated STAT1 began to decline thereafter accompanying with large accumulation of intracellular viral proteins. These results suggested that viral protein(s) accumulated during viral reproduction may play an essential role in repressing IFNAR1 level.

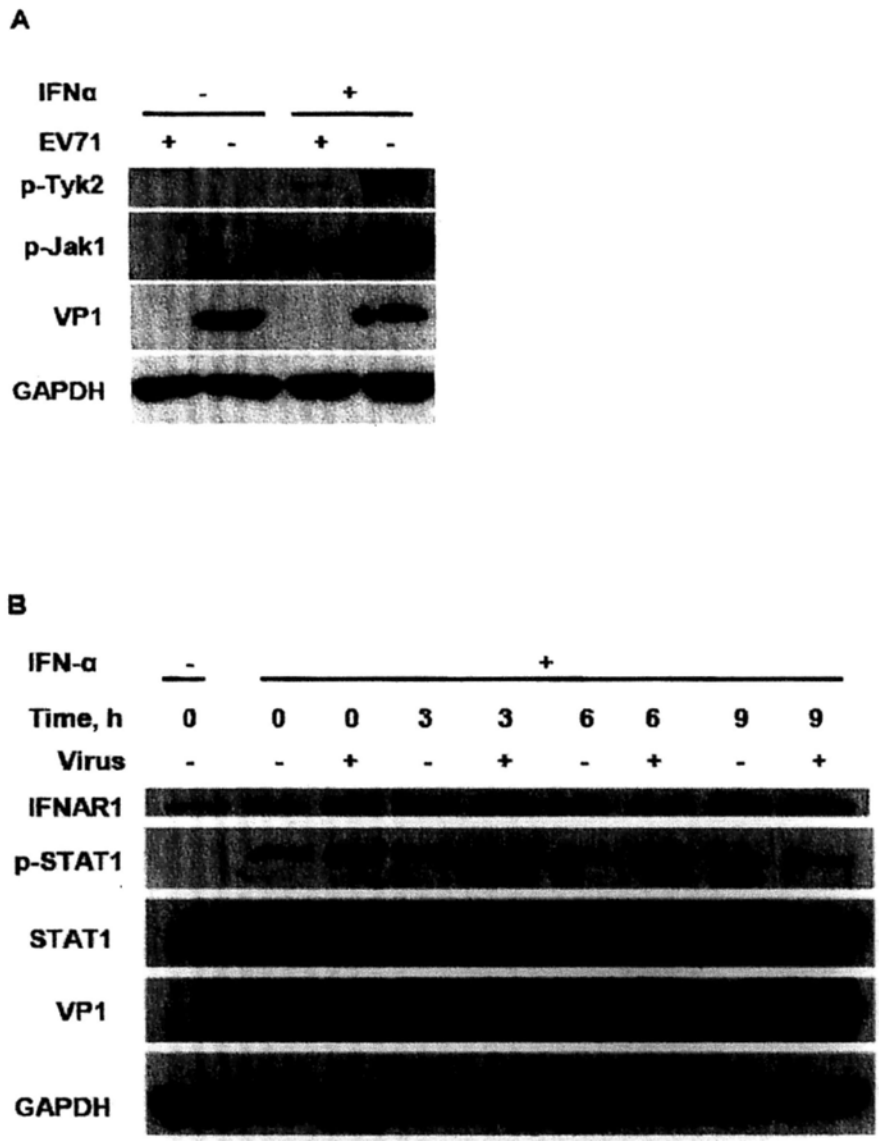


Figure 3.2.4 EV71 inhibited type I IFN-mediated Jak/STAT signaling by reducing IFNAR1 protein level. (A) RD cells were infected with EV71 at MOI of 10 for 9h and cells were left untreated (-) or treated (+) with IFN-α2b for another 10minutes. phosphorylated Jak1 (p-Jak1), phosphorylated Tyk2 (p-Tyk2) and VP1 was detected with specific antibodies. GAPDH was also detected as loading controls. (B) RD cells

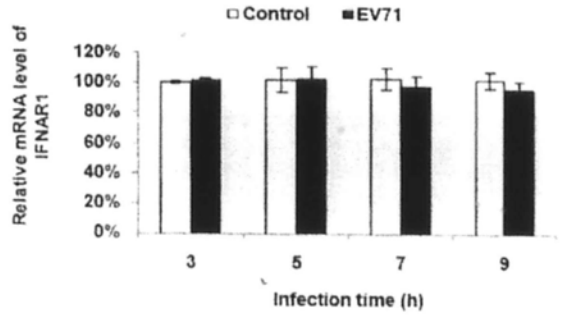
were mock-infected or infected with EV71 at MOI 10. Cells were treated with IFN-α2b for another 30 minutes at 0, 3, 6, 9 h after infection. The mock infected cells without IFN treatment was set as a negative control. The expression of IFNAR1, STAT1, VP1, and p-STAT1 were measured with related antibodies.

3.2.5 EV71 promote internalization of IFNAR1

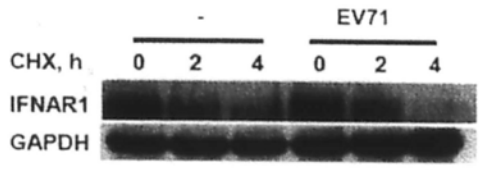
The downregulation of IFNAR1 could occur through different mechanisms including a decrease in protein synthesis and/or increase in protein degradation. The protein synthesis can be regulated at transcriptional or translational level. To investigate whether the transcription of IFNAR1 is affected by EV71, the mRNA level of IFNAR1 expression was analyzed at each time point post EV71 infection. The results showed that no alteration on the mRNA level of IFNAR1 was observed during the whole process of EV71 infection (Fig 3.2.5A), suggesting that the reduction of IFNAR1 should occur at post-transcriptional level. One possibility is that EV71 may reduce IFNAR1 level by suppressing the translation of this receptor. Previous studies have shown that EV71 infection led to the cleavage of eIF4G and then repressed the general mRNA translation. In this study, the EV71 infection only specifically reduced IFNAR1 level while other host proteins such as STATs and GAPDH kept unchanged (Fig 3.2.3). This suggested that the suppression of general translation may not be the major cause. Another possibility for the decreased IFNAR1 level may be caused by EV71-induced degradation. To investigate role of EV71 infection on IFNAR1 degradation, we used a standard approach to block protein synthesis by treating cells with cycloheximide (CHX). Obviously, the EV71 infection markedly accelerated the rate of degradation of exogenously expressed IFNAR1 (Fig 3.2.5B). To further prove our findings, we checked the ubiquitination status of IFNAR1. After co-immunoprecipitation, and blotted with an ubiquitin specific antibody, we observed that IFNAR1 was significantly ubiquitinated in EV71 infected cells when compared with the mock infected cells (Fig 3.2.5C).

As a transmembrane protein, IFNAR1 is firstly internalized and then degraded via the lysosomal pathway [175,283]. To uncover the potential mechanism used by EV71 to regulate IFNAR1 degradation, the subcellular distribution of the endogenous IFNAR1 protein was investigated by immunofluorescence assay. RD cells were infected with UV-inactivated virus or EV71 at MOI of 10. 9h p.i., cells were fixed and processed for immunofluorescence assays. The mock infected cells were set as control. As shown in Figure 3.2.5D, the internalization of IFNAR1 was observed in almost all the VP1 positive cells. In contrast, the internalization were not observed in control cells and cells infected with inactivated virus. These results suggested that EV71 infection could promote the internalization and degradation of IFNAR1 and the newly expressed viral protein(s) was required in this process.

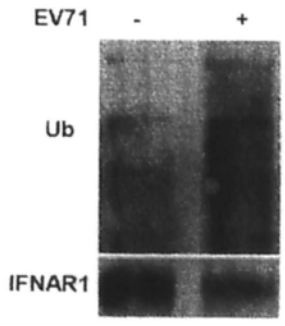
A


Control
EV71
Relative mRNA level of IFNAR1
120%
100%
80%
60%
40%
20%
0%
3
5
7
9
Infection time (h)

B


-
EV71
CHX, h
0
2
4
0
2
4
IFNAR1
GAPDH

C


EV71
-
+
Ub
IFNAR1

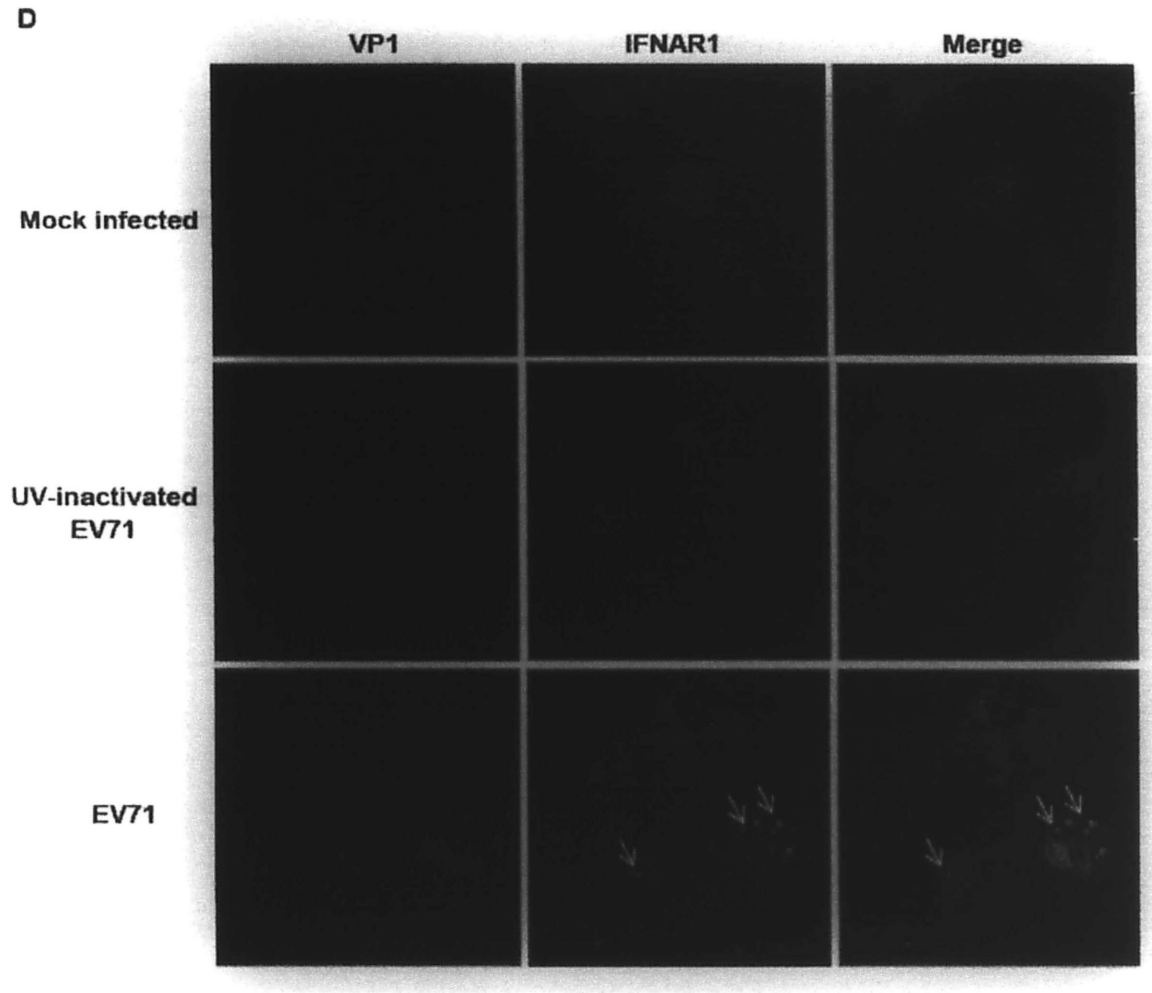


Figure 3.2.5 EV71 promoted the internalization and degradation of IFNAR1. (A) RD cells were mock-infected or infected with EV71 at MOI of 10. The mRNA levels of IFNAR1 in EV71 infected cells were measured by quantitative RT-PCR and calculated the relative level to GAPDH at different times post infection. The mock-infected cells were set as a control (100%). Data are shown as mean ± SD of three independent experiments; each was done in triplicate. (B) HeLa cells expressing His-tagged IFNAR1 proteins were mock infected or infected with EV71 at MOI of 10 for 6 hours. Thereafter, cells were treated with cycloheximide (CHX) for indicated times. Total level of IFNAR1 was analyzed with anti-His antibody. Relative level of IFNAR1 was normalized with

GAPDH. (C) Cell lysis from RD cells with or without EV71 infection were applied for co-immunoprecipitation assays. IFNAR1 was immunoprecipitated and blotted with the ubiquitin or IFNAR1 specific antibody. (D) RD cells infected with EV71 (MOI 10) or UV-inactivated virus were fixed and processed for immunofluorescence at 9h p.i.. The mock infected cells were set as control. The cells were fixed and incubated with VP1, IFNAR1 antibodies and then with rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG. The VP1 and IFNAR1 proteins were shown in Red and Green, respectively. The white arrows indicate the internalized IFNAR1 in EV71 infected cells.

3.2.6 Identification 2A as an antagonist of IFN signaling

In previous sections, it was demonstrated that EV71 infection inhibited IFN-induced STAT1 and STAT2 phosphorylation and then repressed the activation of ISGs (Figure 3.2.1 & 3.2.3). Studies from other viruses suggested viral protein especially non-structure protein may plays roles in this process (see chapter 1.3.2). To explore which viral protein is able to function as an antagonist to IFN signal pathway, plasmids for expressing 11 structural and nonstructural EV71 viral proteins were constructed. HEK 293 cells were transiently transfected with plasmids expressing each viral protein or control vector. 24 hours after transfecion, cells were stimulated with IFN-α2b (100U/ml) for another 2 hours. mRNA of MxA was selected as an indicator and quantified by qRT-PCR. The cells transfected with null vector were set as control. As shown in figure 3.2.6, IFN treatment promoted the transcription of MxA genes in control cells, and expression of 2B, 2C, 3A, 3B, 3C, 3D, VP1, VP2, VP3 and VP4 have no or little effect on inhibiting IFN-induced MXA transcription. While in $2A^{pro}$ expressing cells, the IFN-induced activation of MxA was significantly inhibited. These results suggested that the $2A^{pro}$ of EV71 may function as an antagonist of IFN signaling to suppress ISGs activation.

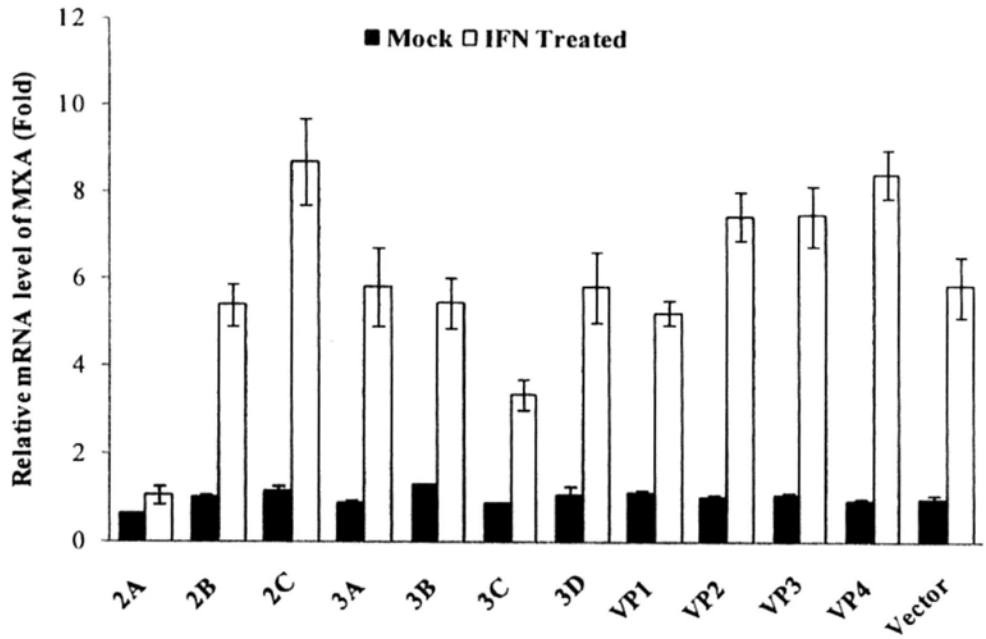


Figure 3.2.6 Inhibition of IFN-induced MxA activation by EV71 proteins. 293T cells were transfected with plasmids expressing individual EV71 proteins, and the null vector was used as control. At 24 h after transfection, cells were stimulated with IFN-α2b (100 U/mL) for another 2 hours. Then RNA was extracted and mRNA of MXA was quantified by real-time PCR. The expression level of MXA gene was calculated relative to GAPDH gene expression and normalized to control. Data are shown as mean ± SD of three independent experiments; each was done in triplicate.

3.2.7 2A Blocks Type I IFN-mediated phosphorylation of STAT proteins and Janus kinases by reducing IFNAR1

To assess the inhibitory role of the $2A^{pro}$ in IFNAR1, 293T cells were transfected with an empty vector or $2A^{pro}$ expressing plasmid, and treated with IFN-α2b (100U/mL) 24h after transfection. The IFNAR1 level and phosphorylation status of STATs were analyzed 30 minutes after IFN stimulation. As described in others studies, the expression of viral $2A^{pro}$ was hard to be directly detected due to its inhibition on its own translation [76,284], while the cleavage of eIF4G represented the functional $2A^{pro}$ was expressed in transfected cells (Fig 3.2.7A). Obviously, $2A^{pro}$ significantly decreased IFNAR1 level in 293T cells (Fig 3.2.7A). Repeated experiments showed that the endogenous IFNAR1 was reduced by $2A^{pro}$ by more than 90% percent after image quantification in both cases with or without IFN treatment. Consistent with the observations in EV71 infected cells (Figure3 & Figure4), $2A^{pro}$ also potently inhibited IFN-induced Janus kinases and STATs phosphorylation without notable change the total protein amount of STAT1 or STAT2 proteins (Fig 3.2.7A).

It has been shown that the phosphorylation of Ser535 residue is important for ubiquitination and degradation of IFNAR1, and mutation of Ser535 rendered $IFNAR1^{S535A}$ resistant to down-regulation in response to ubiquitination [285]. To assess the effect of $2A^{pro}$ on IFNAR1 degradation, $2A^{pro}$ and a His-tagged IFNAR1 or $IFNAR1^{S535A}$ were forced to co-express in 293T cells. As shown in Figure 3.2.7B, the wild type IFNAR1 was significantly reduced more than 90% but the mutated $IFNAR1^{S535A}$ was only reduced about less than 50%. These results suggested that $2A^{pro}$ promotes IFNAR1 degradation and the process is phosphorylation dependent.

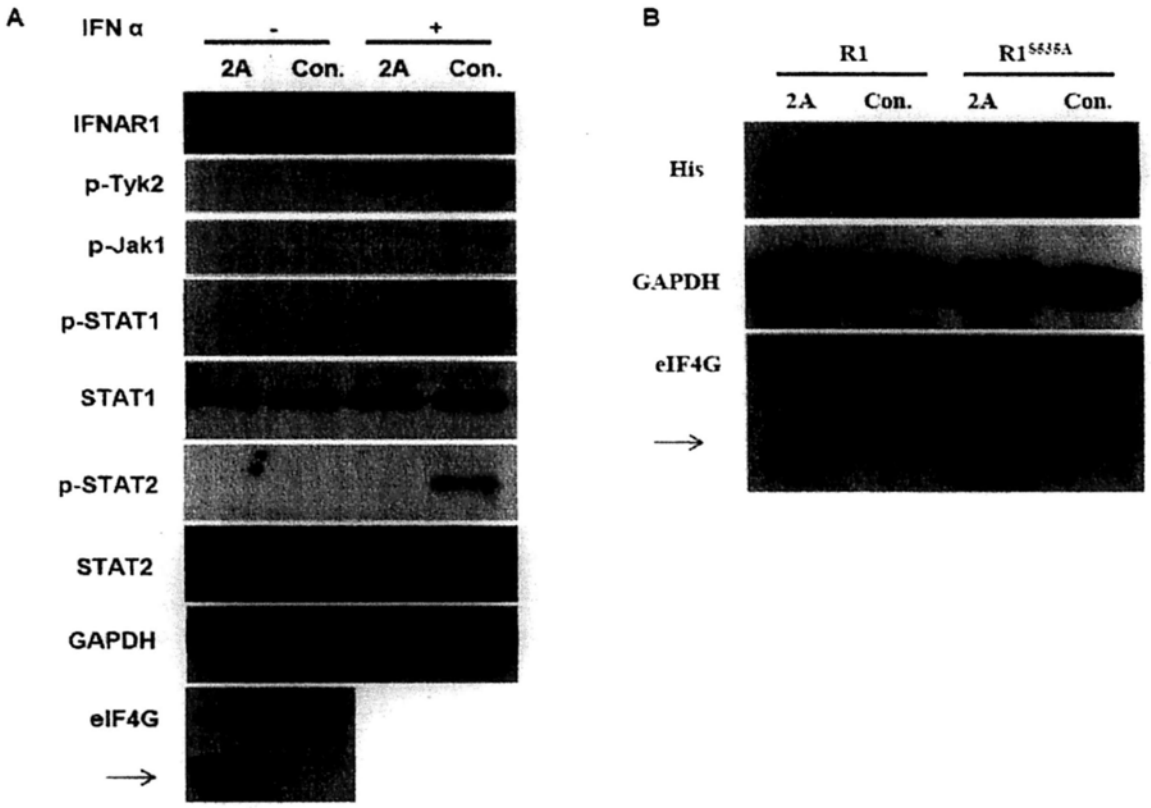


Figure 3.2.7 $2A^{pro}$ inhibited Jak/STAT signaling by promoting IFNAR1 degradation. (A) HEK 293T cells were transfected with $2A^{pro}$ expressing plasmid or null vector. 24 h after transfection, cells were treated with or without IFN-α2b. Western blots were performed by detecting IFNAR1, p-JAK1, p-Tyk2, STAT1, STAT2, p-STAT1, p-STAT2 and eIF4G with related antibodies. The arrow indicated the cleaved eIF4G in 293T cells. GAPDH was detected as loading controls. (B) The His-tagged IFNAR1 and $IFNAR1^{S535A}$ expression plasmids were transfected into HEK293T cells for 12 hours and followed by transfecting with $2A^{pro}$ expression plasmid or null vector control. IFNAR1 and $IFNAR1^{S535A}$ protein levels were analyzed at 36 h after tranfection. GAPDH was detected as loading controls.

3.2.8 Inhibition of IFN signaling is depend on the protease activity of $2A^{pro}$

EV71 $2A^{pro}$ is known as a viral protease. It catalyzes an essential cleavage in the polyprotein in virus replication (see chapter 1.1.3). On the other hand, $2A^{pro}$ also cleaves several host cellular proteins to facilitate viral replication, leading to viral pathogenesis [286,287,288]. It has been demonstrated that the Cys in amino acid 110 of $2A^{pro}$ is essential for its protease activity [289,290]. To test whether the inhibition effect of $2A^{pro}$ on IFN was depend on its protease activity, the plasmid expressing 2A protease with mutation in amino acid 110 from Cys to Ala was constructed. Thereafter, the plasmids expressing $2A^{pro}$ and mutant $2A^{pro}$ ($2A^{C110A}$) were tranfected into 293T cells, and cells tranfected with null plasmid were set as control. As shown in figure 3.2.8, the mutation on 110Cys abrogated the protease activity of $2A^{pro}$, and eIF4G was not cleaved in cells expressing mutated $2A^{pro}$. After IFN-α2b stimulation, cells expressing wild type $2A^{pro}$ significantly suppressed IFN-induced STATS phosphorylation while the mutant $2A^{pro}$ did not exhibit any inhibition activity on type I IFN signaling. No differences on IFN-α induced phosphrylation of STATs between cells expressing mutant $2A^{pro}$ and control vector was observed. Therefore, it was concluded that the protease activity of $2A^{pro}$ is essential for inhibiting type I IFN signaling.

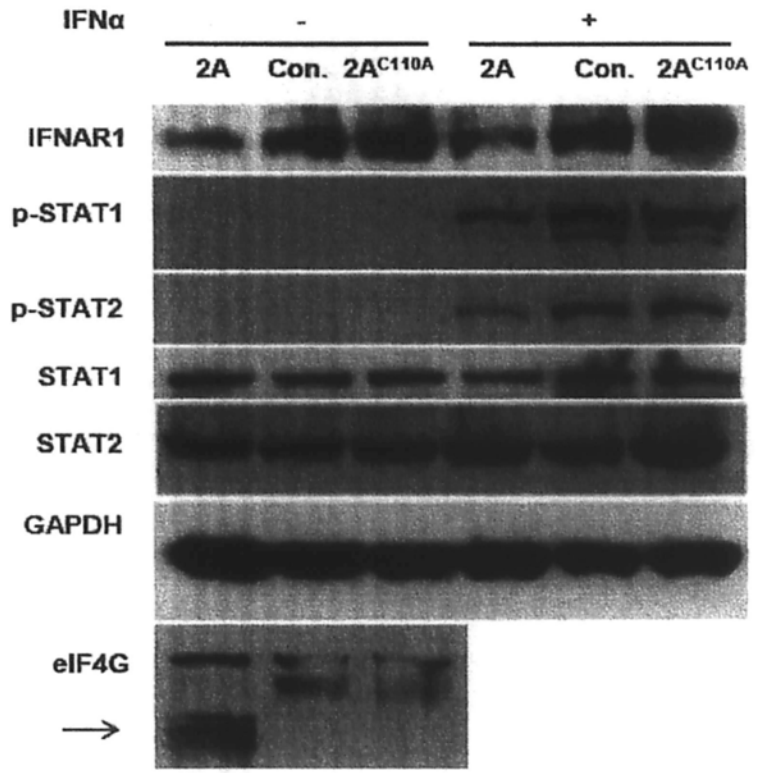


Figure 3.2.8 Protease activity is required for 2A inhibiting IFN signaling. 293T cells were transfected with 2A or mutant 2A ($2A^{C110A}$) expression plasmids or null vector as the control. 24 h after transfection, cells were treated without or with IFN-α2b (100 U/mL) for 30 mins. Western blots was performed by detecting IFNAR1, STAT1, STAT2, phosphorylated STAT1, phosphorylated STAT2 and eIF4G with related antibody. The arrow indicated the cleaved eIF4G. GAPDH was detected as loading controls.

3.2.9 $2A^{pro}$ can not directly cleave IFNAR

$2A^{pro}$ and mutant $2A^{pro}$ were successfully expressed in *E-coli* system (Fig 3.2.9A) and further renatured. 293T cells transfected with IFNAR1 expression plasmid were collected at 24hours after transfection, and cell lysis was incubated with purified $2A^{pro}$ and $m2A^{pro}$ for 4 hours as described in materials and methods. The protein level of IFNAR1 and cleavage of eIF4G were analyzed by Western blotting. As expected, the cleavage of eIF4G was observed in cell lysis incubated with $2A^{pro}$ but not $m2A^{pro}$ (Fig 3.2.9B). However, IFNAR1 protein level was not changed between cell lysis incubated with $2A^{pro}$ and $m2A^{pro}$.

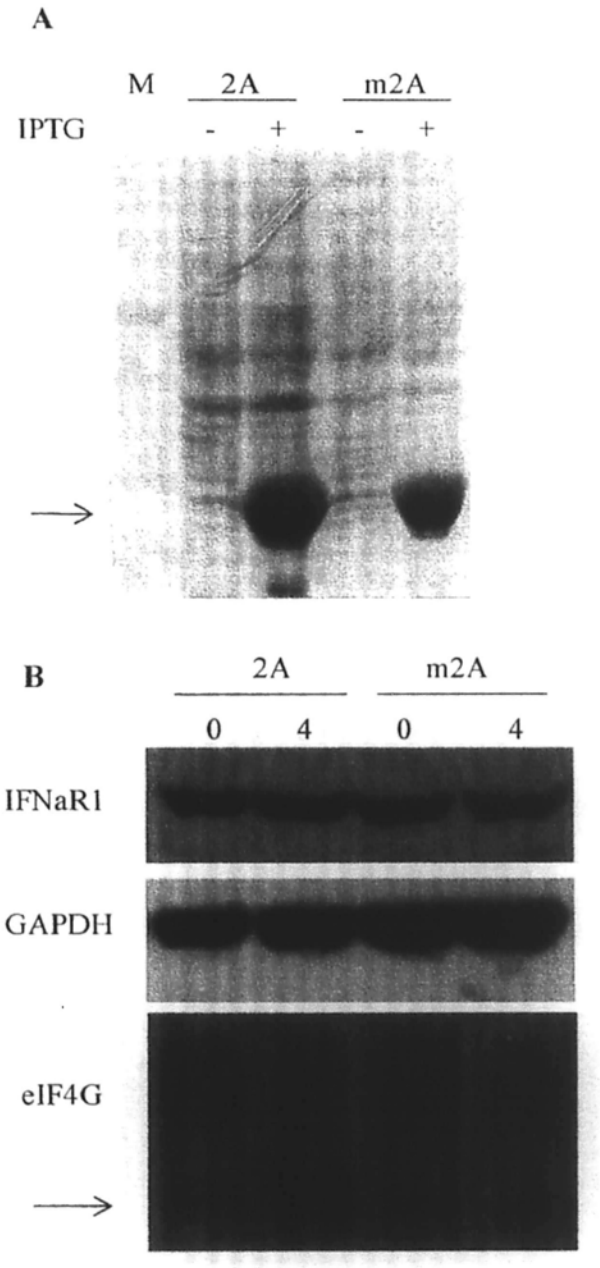


Figure 3.2.9 $2A^{pro}$ can not directly cleave IFNAR. (A) 12% SDS-PAGE gels stained with Coomassie brilliant blue showed the expression of $2A^{pro}$ in BL21 bacteria. *E.coli* strain BL21 transformed with 2A and m2A expression plasmid were collected before and after IPTG incubation. Target proteins were detected with anti-his antibody as arrow

indicated. M, Marker. (B) 293T cells transfected with IFNAR1 expression plasmid for 24 hours were lysed and incubated with the purified 2A and mutant 2A protein as indicated in chapter 2.8. Proteins were collected before and after incubation, and western blotting was applied to analyzed IFNAR1 and eIF4G. The cleavage of eIF4G was indicated with the arrow.

Chapter 4 Discussion

4.1 EV71 viral kinetics in RD cells

Viral kinetics is an important parameter for demonstrating viral activities in the host cells and provides basic information to understand the viral-host interactions and pathogenesis. Kinetics of some picornaviruses such as Swine Vesicular Disease Virus and Foot-and-Mouth Disease Virus have been described in several studies [49,50]. However, little information could be obtained on EV71. Some studies provided brief descriptions on EV71 RNA replication and growth kinetics of EV71infected cells while the infection ratios used in these studies were too low (MOI≤0.01) to guarantee the synchronicity of infection [291,292]. In this situation, some cells were undergoing cell death whereas others were to be infected by new EV71 viruses secreted from the first round infected cells. Therefore, the viral life cycle could not be accurately examined. In addition, except RNA synthesis, no information was provided about viral protein expression, virus package and secretion. In this thesis, the detailed viral kinetics of EV71 in human RD cells was comprehensively described. As RD cells infected by EV71 would develop cellular pathogenesis (CPE), these cells have been extensively used to investigate the viral activities of EV71 and host responses to EV71 infection [293,294,295,296]. To obtain a synchronized infection, RD cells were pulse infected at high MOIs (MOI 1 and 10) to ensure that the majority of the cells were primarily infected in our study. Following the infection, the unattached viruses were removed by washing the cells twice with PBS. This would minimize the interference of non-infectious virions. In this thesis, the kinetics of viral replication, gene expression, package and secretion as well as the effects of viral activities on host cells were carefully examined at different time points.

The intracellular virions of EV71 obviously decreased over 90% at 3 hours p.i. (Fig 3.1.4) while the total intracellular RNA copies almost kept at the same levels (Fig 3.1.2). This result suggested the virions immediately uncoated after entry and virus replication was inactive within the first three hours upon infection. During this phase, the viral RNA could be translated to generate viral proteins essential for viral replication. From 3 to 6 hours p.i., the virus underwent fast replication and the total intracellular viral RNA was accumulated rapidly (Fig 3.1.2). Similar results were also reported in other poliovirus infection [297]. The total intracellular viral RNA increased by over 64 folds within the period. In the meantime, the viral gene expression also initiated along with viral replication, as viral VP1 proteins could be clearly detected in the host cells at 6 hours (Fig 3.1.3). The viral package also started (Fig 3.1.4) but very few virions were secreted (Fig 3.1.5). At 6 hours p.i., about 1% (MOI 1) to 3% (MOI 10) of viral RNA was packaged into virions (Fig 3.1.2 and 3.1.4). Although the virus was rapidly replicating, the host cells were generally healthy in this period. From 6 to 9 hours p.i., some cells became unhealthy (Fig 3.1.1), the viral replication entered static stage as the total intracellular viral RNA only increased about 2 folds either in MOI 1 or MOI 10 group. In the case of MOI 1, the total intracellular viral RNA increased another 2 folds from 9 to12 hours p.i. while began to decrease 9 hours p.i. in the cells infected at MOI 10. This suggested that viral RNA in cells infected with higher MOI reached the maximal levels earlier. The viral gene expression and package also actively processed in this period. The viral protein VP1 levels reached the peak at 9 hours p.i. and fast decreased at 12 hours p.i. in MOI 10 group. In the case of MOI 1, the VP1 levels also reached maximal at 9 hours p.i. and maintained the same levels at 12 hours post infection. Accomplished with viral protein synthesis, the intracellular virions also rapidly increased over 16 folds

(MOI 10) or 64 folds (MOI 1). For both cases, the intracellular virions went up to a peak and about 30% of viral RNA was packaged into the virions at 12 hours p.i. (Figure 3.1.4 vs Figure 3.1.2). In the case of MOI 1, the extracellularly accumulated virions increased 8 and 64 folds from 6 to 9, and 9 to 12 hours, respectively; while the extracellularly accumulated virions increased 30 and 5 folds in the same periods in MOI 10 group. From 12 to 24 hours p.i., as more and more infected cells continuously became unhealthy and died, the intracellular viral RNA levels obviously decreased and the viral replication became less active. These findings suggested that the cells may no longer sustain further viral replication and died [73,77,80]. This was further supported by the data of intracellular and extracellular virion levels. The intracellular virions still kept high levels at 16 hours post infection although more and more virions were continuously secreted into the culture media. Since then, the ratio of packaged viral RNA to total viral RNA constantly kept about 50% in both MOI 1 and MOI 10 cases.

In summary, the viral kinetics model of EV71 in human RD cells was established. Upon infection, the virus uncoated within the first three hours and started to synthesize the essential viral proteins for replication. From 3 to 6 hours p.i., the virus rapidly replicated its genome RNA and initiate viral package. The fast viral package displayed from 3 to 12 hours p.i. and virion secretion constantly performed since 6 hours p.i. until the death of host cells. The host cells started to become unhealthy as early as 6 hours p.i. but still supported viral replication, package and secretion until death.

4.2 *EV71 antagonizes type I IFN response*

The type I IFN response functions as the first line defense against viral infection. By activating varies ISGs, type I IFNs generate an intracellular environment that restricts viral replication and promoting the presentation of viral antigens to the adaptive immune response [298,299,300]. Moreover, through type I IFN signaling pathway, IFN-α/β quickly launches signaling cascade and amplify the production of type I IFN. Although type I IFN response is exceedingly potent and rapid, most if not all viruses have evolved mechanisms to evade from the surveillance by either interfering the synthesis of IFNs or circumventing downstream antiviral signaling pathway.

As many other picornaviridae virus, dsRNA is produced during infectious cycle of EV71 and would be sensed by PRR to activate IFN-β production [155,156]. Previous studies on other picornaviruses mainly explored viral strategies on inhibiting type I IFN induction. These studies showed that different picornaviruses like poliovirus, rhinoviruses, echovirus, and encephalomyocarditis virus could suppresses the production of IFN-α/β by interfering with MDA-5 and RIG-I [271,272,273]. The poliovirus was also demonstrated to reduce the IFN-β secretion by repressing cellular secretory pathway [274]; The leader proteinase of foot and mouth disease virus inhibits type I IFN production by cleaving p65-RelA [276,277]; while the $3C^{pro}$ of hepatitis A virus inhibit type I IFN production by cleaving mitochondrial antiviral signaling protein (MAVS) [278]. EV 71 has also evolved to target the type I IFN induction. The more recent studies reveal that 3C protease of EV71 associates with RIG-I via the caspase recruitment domain. This interaction precludes the recruitment of the adaptor IPS-1 and

subsequent nuclear translocation of IRF3. As a result, the IFN-β transcription is inhibited during viral infection [279].

Herein a novel mechanism by which the EV71 alters type I IFN response was identified. EV71 inhibited type I IFN-induced ISGs activation by interfering IFN signal transduction. In EV71 infected cells, the IFN-β transcription activation was not totally inhibited and the mRNA level of IFN-β was increased about 25 folds at 24h following EV71 infection at MOI of 1 (Fig 3.2.1A&B). On the other side, the activation of ISGs which is the downstream of type I IFN signaling was not observed during the infection (Fig 3.2.1A&B). The effect of EV71 on repressing IFN-induced ISGs activation was further confirmed on RD cells treated with exogenous IFN-α2b. It is well demonstrated that phosphorylation of STAT1 and STAT2 is critical for type I IFN induced antiviral responses, and cells null mutations in these two transcriptional proteins are unresponsive to IFN-α/β and are highly susceptible to virus infection [301]. For this reason, the effect of EV71 infection on protein levels and phosporylated statuses of STATs were further investigated. The IFN inducible STATs phosphorylation was greatly inhibited in cells infected with EV71 at 9h p.i. , menwhile, the cellular viral loads reached to the peak (Fig 3.1.2 and Fig 3.2.3). This suggested that EV71 was able to abolish type I IFN signaling at early stage of infection and facilitate its replication and survival. These results partially explain why the viral load of EV71 can reach at high level soon after viral infection in murine model or culture cells [108,302].

Most of viruses circumvent the IFN response by specific viral protein: human cytomegalovirus (CMV) inhibits IFN-dependent STAT signaling by IE1-72KD viral protein [303]; influenza virus inhibit the JAK/STAT signaling by the non-structural

protein 1(NS1) though up-regulation of SOCS1 and SOCS3 [304]; HCV core protein not only suppresses STAT1 gene expression but also interacts with STAT1protein to interfere its normal function [267]. To further explore the mechanisms used by EV71 to countermeasure type I IFN signaling, 11 viral proteins of EV71were tested. 2A protease of EV71 was identified as an antagonist to type I IFN. Coincidence with the results observed in virus infected cells, expression EV71 $2A^{pro}$ alone in mammalian cells inhibited IFN-α induced STAT1 and STAT2 phosphorylation while had no obvious effect on total protein level of STATs. The upstream effectors of STAT proteins were next evaluated under $2A^{pro}$ expression. Similarly, IFN induced phosphorylation of Jak1 and Tyk2 was also inhibited by $2A^{pro}$. Finally, the protein level of IFNAR1, the first component in type I IFN signaling pathway was checked. The results showed that EV71 $2A^{pro}$ was able to selectively inhibit IFNAR1 protein level (Fig 3.2.7). IFNAR1 protein level was reduced in EV71 infected RD cells at 7 hours post infection, and IFN induced STAT1 phosphorylation was also repressed at following time points (Fig 3.2.8). These results inferred that the $2A^{pro}$-dependent reduction in IFNAR1 likely results in a decrease in IFN-inducible STATs phosphorylation and ISGs activation.

Previous studies have well demonstrated the substantial roles of IFNAR1 in protecting cells from viral infections. Mice with null mutations in IFNAR1 have indicated that IFNAR1 is essential for responses to IFN-α/β and is required for survival against most viral infections, myelopoiesis, as well as B and T cell mediated immune responses [305,306]. Other studies with cardiac fibroblasts and myocytes showed that the cardiac fibroblasts with higher basal levels of IFNAR1 and Jak-STAT components have correlated a stronger IFN response compared with the myocytes [307]. The clinical

investigations on HCV patients also showed that the expression of IFNAR1 and IFNAR2 genes in the liver is a useful index for predicting the long-term efficacy of interferon therapy in patients with chronic genotype 2a or 2b HCV infection [308]. Polymorphisms in promoters and genes encoding type I IFN receptors have been closely linked with susceptibility to a number of diseases including cerebral malaria [309], multiple sclerosis [310], trypanosomaiasis [311], HIV [312], and hepatitis B and C virus [313,314]. Viewed altogether, the reduction effect of EV71 $2A^{pro}$ on IFNAR1 protein level is an effective mechanism to render target cells less sensitive to IFN.

During EV71 life cycle, $2A^{pro}$ accompany with $3C^{pro}$ function as cysteine protease which make different cleavage in the viral polyprotein [315]. There is always a question why the virus has evolved two cysteine proteinases with similar catalytic mechanisms but different specificity. The most possible explanation is that the viruses could benefit from the presence of the additional proteinase. Previous studies shown $2A^{pro}$ is a multifunctional protein which is essential for viral replication. $2A^{pro}$ can cleave a variety of host proteins, including the translation proteins eIF4GI [286], eIF4GII [287], and poly (A)-binding protein [288], thereby enhance the viral protein expression which managed by its own IRES and cause the cap-dependented cellular translation shut-off. In addition, $2A^{pro}$ of poliovirus is reported to be involved in the process of viral RNA replication as well as in the viral RNA stability [316]. This study revealed that 2A functioned as an antagonist of type I IFNs by downregulation of IFNAR1. The *in vitro* experiments indicated that the reduction of IFNAR1 was not due to direct cleavage by $2A^{pro}$ (Fig 3.2.9). Currently, it was still unclear the exact mechanism used by $2A^{pro}$ to repress IFNAR1 level. However, it was noted that 2A inhibited protein but not mRNA level of

IFNAR1, and the inhibition was dependent on its protease activity since the single C110A substitution in the catalytic site significantly impaired its activity (Fig 3.2.8). These results also well explain the recent observation on poliovirus and enterovirus 70 that $2A^{pro}$ is essential for viral replication in type I interferon treated cells [275].

In conclusion, this study revealed a new mechanism to antagonize host type I IFN response by EV71 and provided an explanation for the previously observed resistance of this virus to IFN treatment in murine model and cultured cells [108]. 2A protease of EV71 was identified to counteract the type I IFN response by reducing IFNAR1 level. $2A^{pro}$ may represent a viral tool to antagnoize JAK/STAT signal transduction pathways in other enteroviruses and rhinoviruses. Moreover, this work may pave a way for new antiviral intervention strategies that target viral $2A^{pro}$ and/or cellular IFN signaling pathways.

4.3 Future studies and perspective:

In this study, EV71 was demonstrated to inhibit type I IFN signaling by reducing IFNAR1 level. Viral protease 2A may play an essential role in this process. However, the detailed mechanism of EV71 or $2A^{pro}$ on degradation of IFNAR1 is still elusive will be clarified in future studies.

Ubiquitination of plasma membrane receptors lead to internalization of these proteins and then the degradation via the lysosomal pathway [175,285,317]. This mechanism plays a pivotal role in down-regulation of the Type I IFN receptor and then the IFN signaling [318]. In this study, the EV71 infection was observed to promote IFNAR1 ubiqitination, internalization and degradation which result in the reduction of IFNAR1 level and inhibition of IFN signaling (Fig 3.2.5). Further studies will dedicate to the detailed mechanism used by EV71 to affect IFNAR1 stability. More recently, the hypothetical model for regulation of IFNAR1 degradation was proposed and mainly three ways were involved in the regulation process [319]. The first is the ligand-inducible pathway in which the activated Janus kinase Tyk2 promotes the binding of PDK2 to IFNAR1 and therefore leads to the phosophorylation and degerdation of IFNAR1 (Fig 4.3). Host cells use this way to limits the extent of IFN-α/β signaling when expose to these cytokines [318]. The second is UPR pathway which functions through PERK-dependent phosphorylation of IFNAR1. The PERK leads to the activation of p38 kinase which phosphorylated IFNAR1 at Ser532 by activating an unkown kinase. The phosophorylation at Ser532 priming phosphorylation of IFNAR1 at Ser535 by CK1α kinase and finally accerelate the IFNAR1 degradation. Viruses like VSV and HCV can regulate levels of IFNAR1 and evade from Type I IFN surveillance via this way [259].

The third way is the PRR (pathogen recognition receptors) pathway which also induces priming phosphorylation and degradation of IFNAR1 by activating p38 kinase. This pathway, which can be activated during HSV infection, negatively affects IFNAR1 stability and signaling [319]. As indicated by our data, the ligand-dependent pathway would be excluded since the Tyk2 activation (phosphorylation of Tyk2) was not detected during EV71 infection. Also, PRR pathway may be not the main reason although IFN-β transcription was activated in EV71 infection. The UV-inactivated virus which also contains viral RNA was not able to promote the internalization of IFNAR1 and inhibit IFN signaling (Fig 3.2.5D). Moreover, the downregulation of IFNAR1 was occurred at the later but not the early phase of EV71 infection which suggested viral protein expression may be required (Fig 3.2.4B).

As discussed above, it is plausible that EV71 promote IFNAR1 degradation via PERK pathway or the other way which is unkown yet. To dissect the potential mechanism, following experiments will be performed in future studies. In order to find out whether EV71-induced IFNAR1 degradation is depend on PERK or not, PERK will be knocked down by specific siRNA in EV71 infected cells and level of IFNAR1 will be analyzed when compared with control cells infected EV71 while treated with siRNA control. EV71 would use a unindenfied mechanism to downregulate IFNAR1 level if PERK pathway is excluded. As shown in figure 4.2, the activation of p38 plays a pivotal role in ligand-independent downregulation of IFNAR1. To define the role of p38 kinase in EV71-induced IFNAR1 degradation, the p38 kinase inhibitor (SB203580) will be used in the next experiment. If the p38 is required, EV71 may activate p38 through the third way rather than PERK and PRR pathway and promote phosphorylation as well as

degradation of IFNAR1. If the p38 is not required, EV71 may target the IFNAR1 via a new pathway which is not denpend on p38 activation.

Because 2A protease of EV71 antagnizes IFN signaling through the similar way as whole virus infection (Fig 3.2.7), this provide us another approach to clarify the mechanism of IFNAR1 degradation in EV71 infected cells. Previous experiments find that single amino acid mutation on 2A protease can abrogate the inhibition role of 2A on IFNAR1 (Fig 3.2.8) and 2A protease is not able to cleave IFNAR1 directly. In the future study, it will be clarified that whether the mutant 2A can compete with wild type 2A and inhibit its activity on downregulate IFNAR1 level. If the answer is yes, this suggests 2A may target an unknown protein and cleavage of this protein can nextly result in the reduction of IFNAR1. Mutant 2A protein which competes with wildtype 2A to bind with this target protein can inhibit the 2A effect on IFNAR1. Assays like pull-down and yeast two-hybrid have been frequently used for discovering and analyzing protein-protein interactions. In future studies, these assays would be useful to find out the 2A interacted protein and clarify the potential mechanism of EV71 reducing IFNAR1 level.

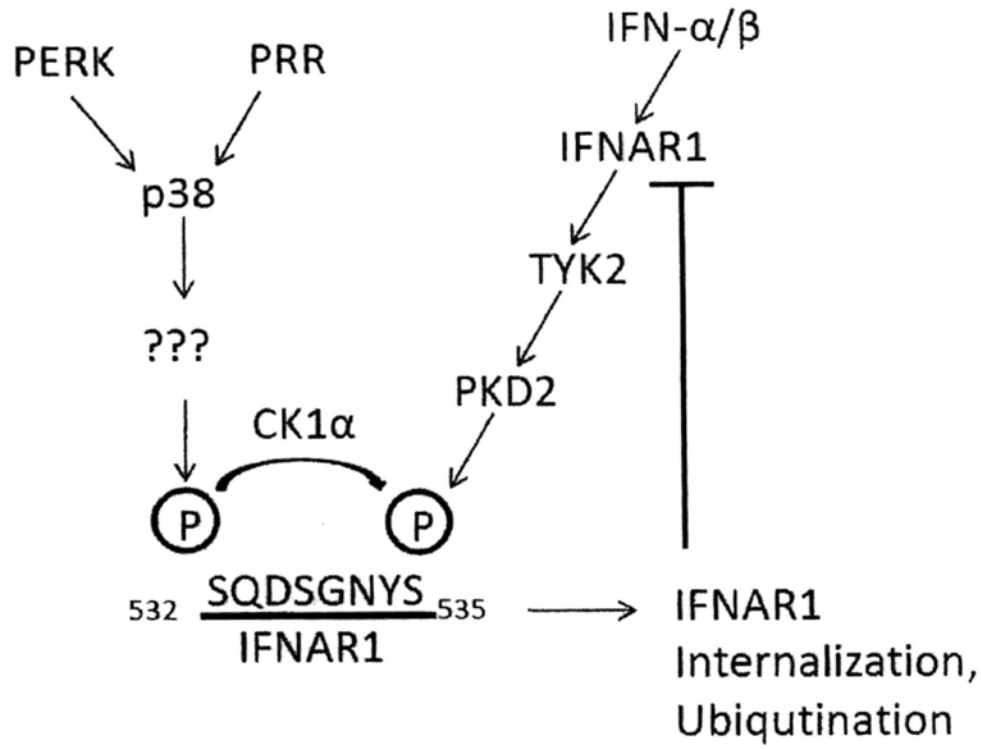


Figure 4.2 Hypothetical model for regulation of IFNAR1 degradation.

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