INTERACTION OF TYPE I INTERFERONS AND MTOR SIGNALING UNDERLYING PRRSV INFECTION

by

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Abstract

Animal metabolic and immune systems integrate and inter-regulate to exert effective immune responses to distinct pathogens. The signaling pathway mediated by mechanistic target of rapamycin (mTOR) is critical in cellular metabolism and implicated in host antiviral responses. Recent studies highlight the significance of the mTOR signaling pathway in the interferon (IFN) response. Type I IFNs mediate host defense, particularly, against viral infections, and have myriad roles in antiviral innate and adaptive immunity. In addition to their well-known antiviral properties, type I IFNs also affect host metabolism. However, little is known about how animal type I IFN signaling coordinates immunometabolic reactions during antiviral defense. Therefore, understanding the interaction of mTOR signaling and the type I IFN system becomes increasingly important in potentiating antiviral immunity.

Tissue macrophages (MΦs) are a primary IFN producer during viral infection, and their polarization to different activation statuses is critical for regulation of immune and metabolic homeostasis. Using porcine reproductive and respiratory syndrome virus (PRRSV) as a model, we found that genes in the mTOR signaling pathway were regulated differently in PRRSV-infected porcine alveolar MΦs at different activation statuses. Therefore we hypothesize that: 1) the mTOR signaling pathway involves host anti-PRRSV regulation; 2) mTOR signaling interacts with IFN signaling to modulate the antiviral response; and 3) different type I IFN subtypes (such as IFN-α1 and IFN-β) regulate mTOR signaling differently. We show that modulation of mTOR signaling regulated PRRSV infection in MARC-145 cells and porcine primary cells, in part, through regulating production and signaling of type I IFNs. In addition, expression and phosphorylation of two key components in the mTOR signaling pathway, AKT and p70 S6 kinase, were regulated by type I IFNs and PRRSV infection.

Taken together, we determined that the mTOR signaling pathway, a key pathway in regulation of cell metabolism, also mediates the type I IFN response, a key immune response in PRRSV infection. Our findings reveal that the mTOR signaling pathway potentially has a bi-directional loop with the type I IFN system and implies that some components in the mTOR signaling pathway can serve as targets for augmentation of antiviral immunity and therapeutic designs.

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Chapter 1 - General introduction

In animals, the innate immune system acts as the first line in restriction of viral invasion or replication, and launches early antiviral response [1]. At the beginning of cell sensing viral infection, cell-associated receptors recognize viral components such as single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), and viral genomic DNA and proteins to mount immune responses [2-5]. Through a cascade of signaling transduction, viral infected cells or activated cells are elicited to increase production of immune effectors, primarily including interferons (IFNs), other proinflammatory cytokines/chemokines, and antimicrobial peptides [2, 3, 6-8]. For example, IFNs, in particular, type I IFNs as key antiviral cytokines, are induced via interferon regulatory factors (mainly IRF3 and IRF7), and NF-κB is responsible in modulating most proinflammatory cytokines and type I IFNs [6, 7, 9, 10]. Following engagement of viral infection and induction of these potential immune effectors, multiple cellular systems including both immune and metabolic systems are adapted to effectively control pathogenic invasion [1, 11-14]. In this context, IFN signaling is the key in antiviral immune regulation, and it has also been discovered to have multiple roles in mediating cellular growth and proliferation rates [10, 15-20]. Similarly, some key pathways in regulation of cell growth and metabolism, such as that mediated by mechanistic target of rapamycin (mTOR), have been found to be important in regulation of immune responses [6, 12, 13, 21]. However, currently there is a lack of platforms to dissect this inter-systemic interaction during viral infection. Here, type I IFN- and mTOR-mediated pathways were used to decipher the interaction between cell immune and metabolic systems during viral infection.

1.1 Type I IFNs

Over the past 50 years IFNs have been discovered and used clinically in human and animals [15, 16]. IFNs comprise an essential family of cytokines in animal immune systems and have a pivotal influence on biological actions, especially those relevant to antiviral defense and carcinogenesis [17, 20].

The interferon family is divided into three classes, type I, II, and III IFNs [16]. They are distinguished by their distinct receptor complexes, display different expression pattern and have numerous functions in innate and adaptive immune responses [22]. Type I IFNs are transduced by their heterodimeric receptor complex (IFNAR1/2), and form the largest subgroup, comprised of more than 10 subtypes such as IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω generally displayed in most mammalian species. Some species-specific subtypes include IFN-δ (pigs and horses), IFN-τ (cattle), IFN-ζ (mice) and IFN-αω (pigs, horses and cattle) [16, 23]. In addition, IFN-α, IFN-δ and IFN-ω are subtypes containing multiple genes. The human genome contains 13 IFN-α functional genes, and the swine genome contains 25, 11 and 8 functional genes of IFN-α, IFN-δ, and IFN-ω, respectively [23]. IFN-γ is the only type II IFN, functioning as a homodimer and signaling via its receptor complex (IFNGR1/2) [24, 25]. Type III IFNs, are composed of IFN-λ1, IFN-λ2, IFN-λ3 (also known as IL-29, IL-28A and IL-28B, respectively) and IFN-λ4, which interact with distinct heterodimeric receptors of IFRL1/IL-10R2 to transmit signals [26]. Type I IFNs are primarily thought to be antiviral mediators, are rapidly induced upon viral infection, and play an indispensable role in producing rapid innate immune responses and effective adaptive immune responses [10].

Sensing pathogen associated molecular patterns (PAMPs) of viral components by cellular pattern recognition receptors (PRRs) is the first step to initiate and regulate type I IFN response

[19]. The selective features of PRRs for viral molecules and different cell types potentially induce differential production of specific IFN subtypes. Cytoplasmic PRRs, such as RIG-I-like receptors (RLRs), are specialized to recognize nucleic acids from RNA viruses. After binding to viral RNA, RLRs undergo a conformational change and expose their N-terminal caspase activation and recruitment domains (CARDs) to interact with the adaptor molecule, mitochondrial antiviral signaling protein (MAVS) for further generating antiviral signaling [10]. Membrane-bound Toll-like receptors (TLRs) also are critical to detect viral glycoproteins and nucleic acids and are recruited to their respective adaptors like myeloid differentiation primary response gene 88 (MyD88) for TLR7 and TLR9, and tumor necrosis factor receptor associated factor (TRIF) for TLR3 when viral nucleic acid species are recognized [10, 26]. In addition to PRRs, a cytosolic PAMP sensor, named cyclic GMP-AMP (cGAMP) synthase (cGAS), is initiated by sensing foreign DNA and activates the stimulator of IFN genes (STING) [27]. Upon activation of MAVS, MyD88, TRIF and STING, TRAF family is recruited to trigger mitogen activated protein (MAP) kinase, IKK complex and IKK-related kinases TBK1/IKKε, resulting in activation and nuclear translocation of AP-1, IRF3, IRF7 and NF-κB to specifically bind to various promoters of type I IFN genes, increasing their production [18].

Once type I IFNs are induced by viral infection, they are secreted by infected cells and stimulate target cells to start type I IFN action signaling [9, 21]. Upon type I IFNs binding to their specific receptors in the surface of target cells, ligand-dependent rearrangement, dimerization and transphosphorylation of receptors propagate downstream signaling cascades [28]. Receptor aggregation allows autophosphorylation and activation of associated JAKs/TYKs, IFNAR1 interacting with tyrosine kinase 2 (TYK2), IFNAR2 with Janus activated kinase 1 (JAK1) [29]. Consequently phosphorylation and activation of STATs (signal transducers and

activators of transcription) are regulated to constitute functional homodimers or heterodimers which stimulate transcription of interferon-stimulated genes (ISGs) after nuclear translocation to bind their specific promoter sites [30]. Beyond classic JAKs/STATs signaling pathways, other important signaling cascades are also induced by type I IFNs for optimizing transcription of diverse ISGs, including MAPKKK (mitogen-activated protein kinase kinase kinase) pathway, PI3K (phosphoinositide 3-kinase)-AKT pathway, and mTOR (mechanistic target of rapamycin) signaling pathway [14, 24, 31]. Signaling cascades downstream are generated through actions of myriad ISGs including antiviral effectors (e.g., ISG15 and Mx1), negative regulators (e.g., USP18 and SOC) and positive regulators (e.g. STAT1/2 and IRF1, 3, 7, 9) [18, 21]. Following activation of these signaling pathways, a wide range of protective and destructive biological activities are evoked [32, 33]. Therefore, a balanced type I IFN response is critical to induce host immune responses for defense and survival (Figure 1.1).

1.2 mTOR signaling pathway

mTOR, an evolutionarily conserved serine/threonine kinase, acts as an overriding node for maintenance of homeostasis in animal cells [34-36]. mTOR kinase forms two functionally distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC1), to exert multiple functions through regulation of various elements downstream [36]. mTORC1 consists of five components: mTOR, the catalytic subunits of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein (mLST8); proline-riche AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) [36]. Except mTOR, mLST8 and Deptor, mTORC2 includes rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), and protein observed with Rictor-1 (Protor-1) [36]. Genetic deletion

of Raptor and Rictor, fundamental subunits for mTORC1 and mTORC2 respectively, has provided significant insight into functions of mTORC1 and mTORC2 [37]. Over many years, diverse mTOR inhibitors have been developed, such as rapamycin, PP242, Everolimus, ARQ 092 and CC214-2, and some of them including rapamycin are approved for clinical therapy [38, 39]. The two mTOR complexes display different sensitivity to some of these inhibitors. Rapamycin and its analogs are the first generation mTOR inhibitors, which associate with 12 kDa FK506-binding protein (FKBP12) to form a complex interacting with FRB (FKBP12-rapamycin binding) domain in mTOR kinase to disrupt formation of mTORC1 [40]. Only mTORC1 activity is inhibited by rapamycin in a short time period, but prolonged rapamycin treatment also affects mTORC2 activity [41]. PP242 is a non-selective inhibitor, targets the adenosine triphosphate (ATP)–binding site of mTOR, and suppresses both mTORC1 and mTORC2 activities [42].

The mTOR signaling network comprised of mTORC1- and mTORC2-signaling pathways, senses and integrates both intracellular and extracellular signals to regulate various cellular processes (e.g. metabolism, growth, proliferation and survival) [36]. In recent studies, it has been increasingly recognized that the mTOR signaling pathway is also a vital regulator of innate and adaptive immune responses [12-14]. For example, Keating et al. demonstrated that the mTOR signaling pathway engages host antibody response to produce cross protection against lethal influenza infection [43]; mTORC1- and mTORC2-signaling pathways involves IFN production and action signaling [25, 31, 44-52]; mTOR-mediated regulation of interleukin production and signaling has been shown in human monocytic cells [53-55]; polarized macrophages (MΦs) are modulated by disrupting mTOR signaling pathway [56, 57]. Thus the mTOR signaling pathway provides a key link between immune responses and metabolism to

modulate functions of immune cell populations, such as monocytes, MΦs, T cells, B cells, dendritic cells (DCs) and neutral killer (NK) cells [13].

Upon stimulation of external signals such as cytokines and growth factors, the mTOR signaling pathway is initiated via upstream PI3K-AKT (Figure 1.1) [14, 34, 46, 51]. PI3K and mTORC2 activate AKT that mediates inhibitory phosphorylation of tuberous sclerosis protein 2 (TSC2), which is associated with TSC1 to repress the phosphotransferase activity of mTOR through inhibiting RAS-related small GTPase RAS homologue enriched in brain (RHEB) [58, 59]. Previous studies found that PI3K can be activated by several type I IFNs (IFN-α, IFN-β and IFN-ω) via inducing tyrosine phosphorylation insulin receptor substrate 1 (IRS1) or IRS2 [24, 60, 61]. After IFNs evoking the PI3K signaling pathway downstream of JAKs, STAT1 is phosphorylated at Ser727 to induce STAT1-mediated gene transcription via ISREs or GAS (IFNγ activation site) elements [24]. In addition to JAKs/STATs signaling pathway, IFN signaling also is mediated by the PI3K-AKT-mTOR-ULK1-p38 mitogen-activated protein kinase (MAPK) pathway, revealing a critical role for the PI3K-AKT-mTOR signaling pathway in IFN-driven gene transcription [31, 62, 63].

1.3 Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of most economically important swine disease that causes losses of over $800 million annually in United States, is a small, enveloped, single-stranded, positive-sense RNA (+ssRNA) virus [64, 65]. It belongs to genus Arterivirus, family Arteriviridae, order Nidovirales, and was first identified in early 1990s in Europe (Lelystad virus, LV) and the United States (VR2332) [66, 67]. The PRRSV genome has approximately 15kb and comprises 11 open reading frames (ORFs)

encoding 16 nonstructural proteins: NSP1α, NSPβ, NSP2-6, NSP2TF, NSP2N, NSP7a, NSP7b and NSP8-12 and eight structural proteins: glycoprotein (GP) 2-5, GP5a, nucleocapsid (N), non-glycosylated membrane (M) and envelope (E) proteins [68-70]. PRRSV is monocytotropic and primarily targets porcine alveolar MΦs and monocyte-derived DCs (mDCs) to cause infection [71-73]. Except for MΦs and mDCs, a cell line generated from kidney of African green monkey (MARC-145) is fully permissive to the virus and routinely used for supporting PRRSV replication *in vitro* [74]. PRRSV has evolved diverse mechanisms to evade and dysregulate effective host immune responses. Induction and signaling of type I IFNs, a group of critical antiviral cytokines, are suppressed by PRRSV infection as repeatedly observed in cells or pigs [69, 70, 75]. For example, PRRSV NSP1α, NSP1β, NSP2, NSP4, NSP11 and N protein regulate type I IFN production by adjusting phosphorylation and nuclear translocation of IRFs or/and activation of NF-κB signaling [68, 76-84]. Induction and processing of key antiviral ISGs, such as Mx1, ISG15 and ISG56, also are modulated by PRRSV infection [68]. Moreover, previous studies showed that: activation of PI3K-AKT signaling pathway is regulated by PRRSV infection in a time-dependent manner [85, 86]; PRRSV infection activates mTORC1-signaling and its inhibitor, rapamycin, regulates PRRSV [87]; PI3K inhibition, AKT1 overexpression or mTORC1 inhibitor (rapamycin) treatment modulates viral gene transcription and protein synthesis [88]; PI3K-AKT signaling pathway is involved in regulation of PRRSV-mediated apoptosis and virus entry [89, 90].

1.4 Potential interaction of mTOR and type I IFN signaling in antiviral regulation

Current studies reveal that the mTOR signaling pathway displays increasing importance in host innate and adaptive immunity [12-14]. However, functions of the mTOR signaling

pathway in antiviral immunity, in particular of regulation of IFN response, remain poorly defined. In this study, we focus on its function in type I IFN production and signaling in antiviral response.

It has been shown that both mTORC1- and mTORC2-signaling pathways are critical for type I IFN production and signaling [31, 49, 91]. Cao et al. report that PI3K-mTOR-p70 S6 kinase pathway is required for TLR-induced type I IFN production in plasmacytoid DCs [50]. Type I IFNs coordinate mTOR signaling to selectively regulate transcription and translation of hundreds of ISGs [25, 44, 46, 49]. A recent study further demonstrates that ULK1 acts as a link between the type I IFN response and the mTOR signaling pathway and that the mTOR-ULK1 pathway plays an indispensable role for gene transcription mediated by ISREs and GAS elements in type I IFN signaling [62]. In addition, the mTOR signaling pathway also can be activated/regulated by type I IFN response [25, 49, 51]. The function of the mTOR signaling pathway in anti-PRRSV innate immune response remains unknown, particularly in anti-PRRSV type I IFN response, albeit a few studies have shown that PI3K-AKT-mTOR signaling pathway involves regulation of PRRSV replication [85-90]. Furthermore, mammalian type I IFNs consists of multiple subtypes with different antiviral activity [15, 16]. Antiviral activities of human and porcine type I IFNs have been well analyzed, and porcine type I IFNs show different anti-PRRSV activity that also is cell type-dependent [23, 92]. However, it has not been characterized how distinct type I IFNs regulate the mTOR signaling pathway. Therefore, PRRSV was chosen as a model to decipher the potential interaction of mTOR and IFN signaling in antiviral responses. The aims of this study were to: 1) identify functions of mTOR signaling in anti-PRRSV regulation; 2) investigate regulation of anti-PRRSV type I IFN response by the mTOR

signaling pathway; and 3) explore modulation of the mTOR signaling pathway by diverse type I IFNs (Figure 1.1).

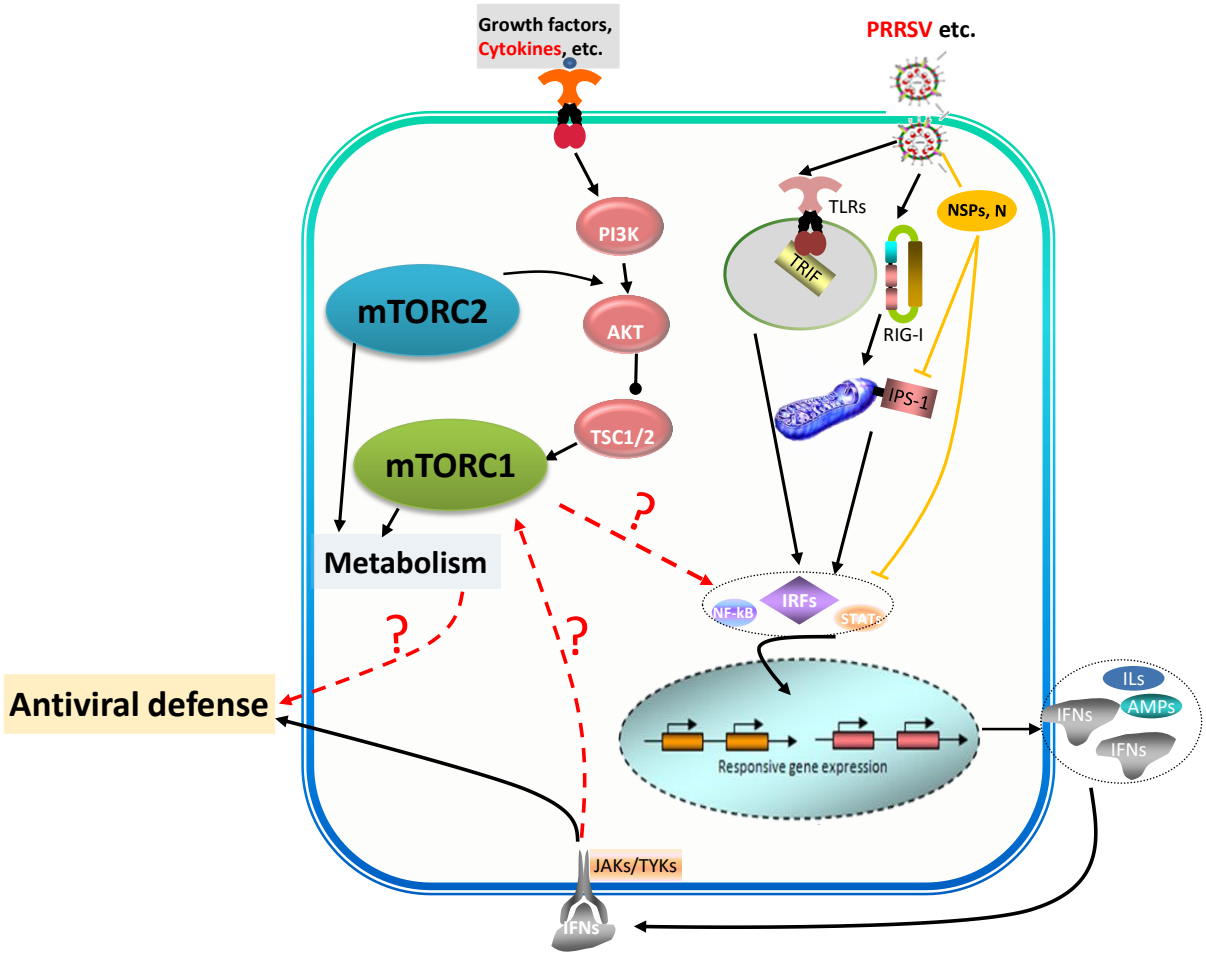


Figure 1.1 The mTOR signaling pathway interacts with the type I IFN system to regulate the antiviral response.

Viral infection activates IFN signaling to produce type I IFNs and other proteins, exerting antiviral defense mainly via the JAKs/TYKs pathway. The mTOR signaling network, consisting of mTORC1- and mTORC2-signaling pathways, senses external signals primarily to modulate metabolism, cell growth and differentiation through the PI3K-AKT signal transduction pathway. AKT indirectly regulates mTORC1 activity via actions of TSC1/2. In our study, PRRSV was used as a model to decipher potential interaction of the mTOR signaling pathway and the type I IFN system in antiviral immunity. We hypothesize that 1) mTOR signaling pathway is involved in anti-PRRSV defense; 2) mTOR signaling interacts with type I IFN signaling to regulate antiviral responses; and 3) type I IFNs modulate mTOR signaling pathway differently.

Chapter 2 - Materials and Methods

2.1 Cells and viruses

Experiments involved in animals and viruses were approved by the Kansas State University Institutional Animal Care and Use and Biosafety Committees. Animal procedures and isolation of porcine alveolar MΦs and peripheral blood mononuclear cells (PBMCs) were previously described [93, 94]. In brief, 5-week-old clinically healthy pigs from a herd without viral infection history were used for collection of primary cells. PBMCs were isolated from blood collected by jugular venipuncture from anesthetized pigs, using Histopaque-1077 (Sigma, Saint Louis, MO). Immediately after euthanasia, MΦs were obtained by lavaging lungs with 1× PBS (pH7.4, Sigma), then washing cells three times with RPMI (Roswell Park Memorial Institute)-1640 medium (Gibco, Carlsbad, CA). Isolated primary cells were used immediately or cryopreserved in Recovery™ Cell Culture Freezing Medium (Gibco). African green monkey kidney (MARC-145, ATCC) cells were grown in Modified Eagle's medium (MEM, Gibco) containing 8% fetal bovine serum (FBS, Gibco) and 1× Antibiotic-Antimycotic (Gibco), 293FT cells (Invitrogen, Carlsbad, CA) in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1× MEM Non-Essential Amino Acids Solution (NEAA, GIBCO). MΦs and PBMCs were maintained in RPMI-1640 medium containing 10% FBS and 1× Antibiotic-Antimycotic. mDCs were generated from PBMCs stimulated with IL-4 (2ng/ml) and GM-CSF (5ng/ml) (R&D Systems, Minneapolis, MN) and maintained in RPIM-1640 medium containing 10% FBS and 1× Antibiotic-Antimycotic [95]. P129-GFP and DsRed-labelled PRRSV were used in this study [93, 94, 96].

2.2 Cell polarization, viral infection and transcriptomic shotgun sequencing

MΦs have long been considered as one of most important immune effector and regulator cells, are distributed widely throughout the body, display multiple roles in both innate and adaptive immune responses, and also serve an indispensable role in inflammation and its resolution [97-99]. To maintain its various functions, MΦs undergo phenotypical polarization in response to diverse environmental stimulants [100]. Typical activation statues characterized in MΦ polarization include classical (M1) and alternative (M2) states [93, 94, 100, 101]. M1 status is induced in response to IFN-γ and bacterial products, such as lipopolysaccharides (LPS) [93]. M2 status is further categorized into three subclasses: M2a, induced by type II cytokines IL-4 or IL-13; M2b, obtained by triggering Fcγ receptors plus a TLR stimulus; and M2c, activated by glucocorticoid (GC), IL-10 and/or TGF-β [93].

Procedures of MΦ polarization were performed as previously described [93, 94]. Briefly, porcine alveolar MΦs were stimulated with LPS, IFN-α1, IFN-γ, IL-4 and IL-10 at 20 ng/ml for 30 h (R&D Systems), followed by infection of P129-GFP at a multiplicity of infection (MOI) of 0.1 for 5 h. After polarization and infection, cells were washed twice with fresh culture medium, and then total RNA was extracted from 3×10^7 cells of each treatment using a RNA/DNA/protein purification kit (Norgen Biotek, Ontario, Canada). To qualify for constructing RNA-Seq libraries, RNA concentration and quality were evaluated with a Nano-Drop 8000 spectrometer (NanoDrop, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to insure RNA samples with A260/A280>1.8 and RNA integrity number (RIN)>7.0. All transcriptomic shotgun sequencing was conducted following the procedures of Illumina Pipeline (BGI Americas, Cambridge, MA). Genome-wide transcriptomic analysis was performed using 25-30 M clean reads per sample. Data analyses were conducted as described [93, 94].

2.3 Antiviral analysis

MARC-145 cells were treated with mTOR inhibitors, rapamycin and PP242, or mTOR activator, MHY1485 at different concentration for 12, 24, 36 or 48 h (Sigma), and then infected with DsRed-labelled PRRSV at MOI of 1.0 for 36-42 h. All images were collected using a Nikon fluorescent microscopy (Shinjuku, Tokyo, Japan) at a magnification of 20×, and viral infection was quantified with SpectraMax i3 (Molecular Devices, Sunnyvale, CA). Porcine primary cells were infected with DsRed-labelled PRRSV at MOI of 0.5 along with mTOR inhibitors or activator for 14-18 h, visualized with fluorescent microscopy and quantified using SpectraMax i3. All chemicals used were dissolved in dimethyl sulfoxide (DMSO, cell culture grade, Sigma).

2.4 Bioassays and ELISA

MARC-145 cells were treated with mTOR inhibitors or activator for 24 h, and infected with DsRed-labelled PRRSV at MOI of 1.0 for another 24 h. Supernatants from each cell culture sample were inactivated with UV light for 1 h, and used to measure IFNs with a bioassay in MARC-145 cells stably transformed with IRF3-, IRF7- or Mx1-promoter driven luciferase reporter system, or an ELISA kit (R&D system) for detecting IFN-α subtypes [102]. In brief, MARC-145 (IRF3, IRF7, or Mx1) cells were treated with inactivated supernatants for 24 h, lysed with Glo lysis buffer and quantified by Steady-Glo® Luciferase Assay System (Promega, Madison, WI).

2.5 Real-Time PCR and western blotting assay

Porcine alveolar MΦs were infected by DsRed-labelled PRRSV at MOI of 0.5 along with mTOR inhibitors or activator treatment for 12 h, and total RNA was extracted from 2×10^5 cells of each treatment using RNA/DNA/protein purification kit. Real-Time PCR (RT-PCR) assay

was performed using GoTaq® 2-Step RT-PCR System (Promega). Total cDNA was reversely transcribed from RNA pools (2 μg RNA in a 20-μl reaction mixture) using random primers. RT-PCR analysis was conducted using a StepOnePlus™ RT-PCR system (Applied Biosystems, Grand Island, NY). Reactions were run with 1 μl cDNA in a 20-μl reaction mixture, and set at 95°C for 2 min followed by 40 amplification cycle of 95°C for 15s, 60°C for 1 min with a melting curve, 95°C for 15s, 60°C for 1 min, a ramp from 60°C to 95°C at an 1% rate, and 95°C for 15s. Critical threshold (Ct) values and melt curves were monitored and collected with enclosed software. Relative gene expression data were first normalized against Ct values of the housekeeping gene (β-Actin), and the relative expression index ($2^{-\Delta\Delta Ct}$) was determined compared with expression levels of control sample for stimulated regulation.

MARC-145 cells were treated with porcine IFN-α1 and IFN-β (R&D system), or infected with DsRed-labelled PRRSV for 24 h, then lysed using 100 μl CelLytic™ M (Sigma) for 1×10^6 cells supplemented with protease inhibitors (Sigma) at 4°C for 30 min. Soluble proteins were collected by centrifugation at 12000× g, 4°C for 15 min. After combining with 4× LDS sample buffer, proteins were resolved on a 4–12% gradient Bis-Tris gel and transferred to a PVDF membrane using NuPAGE® electrophoresis system (NuPAGE Novex, Invitrogen). After blocking with 5% non-fat dry milk (Bio-Rad, Hercules, CA), filters were probed with primary antibodies against AKT, Phospho-AKT (Ser473), p70 S6 kinase, Phospho-p70 S6 kinase (Thr389) or β-Actin at 1:1000 dilution, followed by blotting with a 1:2000 fold dilution of HRP-linked secondary antibody (Cell Signaling Technology, Danvers, MA). Blotted filters were imaged with a Kodak Image Station 4000 (Rochester, NY).

2.6 Gene Silencing based on CRISPR/Cas9 system

The clustered regularly interspaced short palindromic repeat (CRISPR), a short and repetitive segment of prokaryotic DNA, was first identified in microbially adaptive immune system to confer resistance to foreign viruses/phages and plasmids [103]. The CRISPR/Cas9 (CRISPR-associated nuclease 9) system has been widely utilized for sequence-specific regulation of gene expression [104]. Over the past several years, this RNA-guided genomic editing technique has been developed extensively, involved in transcription repression and activation, specific sequence replacement or insertion, and gene mutation or knockout [105, 106]. The target genes in mammalian cells can be quickly, easily and efficiently down-regulated by using Cas9 with one or few single guide RNAs (sgRNAs) [107]. In this study, we established a stably Cas9-expressing MARC-145 cell line and down-regulated Rictor and Raptor expression by combining 3 or 5 sgRNAs, using a lentivirus-based platform [107-109].

pHR-Cas9-2A-puro and phU6/BB-GFP plasmids were constructed on the basis of pHR-SFFV-dCas9-BFP (Addgene, #46910), pSpCas9(BB)-2A-GFP (Addgene, #48138) and pgRNA-humanized (Addgene, #44248) [107-109]. The fragment of Cas9-2A was amplified from pSpCas9(BB)-2A-GFP and inserted into pHR-SFFV-dCas9-BFP using *Mlu*I and *BamH*I, followed by puromycin fragment amplified from pgRNA-humanized with *BamH*I and *Sbf*I. phU6/BB-GFP was achieved by replacing murine U6 promoter in pgRNA-humanized with human U6 promoter from pSpCas9(BB)-2A-GFP using *Xba*I and *Not*I, and all *Bbs*I sites were mutated in pgRNA-humanized vector backbone. All sgRNA expression constructs were obtained with *Bbs*I by inserting an annealed oligo pair encoding 20-nt guide sequences [107]. All restrictive enzymes, Quick Ligation™ Kit, Quick-Load® Taq 2× Master Mix and Phusion® High-Fidelity PCR Kit were purchased from New England Biolabs (Ipswich, MA).

Lentiviral constructs for efficient transfection and expression of Cas9 and sgRNA in mammalian cells were produced using a 2nd generation lentiviral system with pMD2.G (Addgene, #12259) and psPAX2 (Addgene, #12260). 293FT cells were transfected with envelop plasmid (pMD2.G), packaging plasmid (psPAX2) and transfer plasmid (pHR-Cas9-2A-puro or phU6/BB-xx-GFP) at a ratio of 0.9:1.5:2.1 or 0.9:1.5:1.5 (μg). Briefly, 293FT cells were grown in a 24 well plate overnight to reach 70% confluence and transfected with totally 1 μg DNA using XtremeGene9 transfection reagent (Roche, Indianapolis, IN) at a transfection reagent:DNA ratio of 2.5:1. After incubation for 8 h, growth medium was refreshed, and lentivirus-containing supernatants were collected for transducing target cells at 48 h post-transfection. The MARC-145 cell line that stably expresses Cas9-2A-puro was established by lentiviral transduction, and selected with a complete culture medium including 7 μg/ml puromycin (InvivoGen, San Diego, CA). The pure cell line was acquired by serial dilution. After attaining MARC-145-Cas9 cells, they were transduced with sgRNA expressing lentiviruses to down-regulate target genes.

2.7 Tables

Table 2.1 RT-PCR primer sequences for porcine type I IFNs and their receptors [102].

Primer name	Sequence (5' to 3')		Product size (bp)
IFN-α1	Sense Antisense	GGC TCT GGT GCA TGA GAT GT GCC TTC TTC CTG AAT CTG TCT TA	337
IFN-α5/6	Sense Antisense	GCA CAA ATG AGG AGA ATA TCT CCT CCT GAG TCT GTC TTG	437
IFN-α7/11	Sense Antisense	GGG ACT TTG GAT CCC CTC AT GTG GAG GAA GAG AAG GAT G	369
IFN-α9	Sense Antisense	GTG CTG CTC AGC TGC AAG AGT CCT CCT CCA GCA GGG GC	384
IFN-α12	Sense Antisense	CCT CAG CCT TCC TCA CGG T CTC ATG ACT TCT GCC CTG AT	509
IFN-αω	Sense Antisense	AGA TCT TCC GCC TCT TCA GCA CAA TTC TGG TTT CCA CCC TGA CAA CCT	261
IFN-β	Sense Antisense	ATG TCA GAA GCT CCT GGG ACA GTT AGG TCA TCC ATC TGC CCA TCA AGT	246
IFN-δ1	Sense Antisense	TAT AAG CTT CTG GCA GGA GT AGC CTT GAG TCA TCT TGT	205

IFN-δ3/4/5	Sense Antisense	AGA ACT TGT CTG CTG TCC ATT TTT GGA GAA GAC ACC GGA	209
IFN-δ6/7	Sense Antisense	CAA TGG CCC ACA TCC ATT TGC T AGA TGT GTC ACA AGT GTG CCT	214
IFN-δ8/9	Sense Antisense	ATG CTC TGC TCC ACT CCT GC GTG CCT TGA GTC ATC TGG ATT GG	194
IFN-ε	Sense Antisense	TTG GTA CTG CTG GCT TCT TCC ACT AAC TGC CCT GAA GAG GCT GAA GAT	255
IFN-κ	Sense Antisense	GCA GAA TGA GCC ATT CGT TTC CCA TCC TCT TCC TCC TGC AAG CAT TGT	259
IFN-ω1	Sense Antisense	TGG TGC TTC TGC GTC AGA TG CTC ACC TGC ACC AAG CAG GAC	265
IFN-ω2	Sense Antisense	TTC GTG CTC TCT CTA CCG ATG CAG AGA TGG CCT GGA CCT	225
IFN-ω4	Sense Antisense	TCT GCA TCA GAT GAG GAG AC CAA ATG TCT GCT CTT CCA TCT	278
IFN-ω5	Sense Antisense	TCA TGC TCT CTC TAC TGA CAG C TGG AGC TTG TCC AGG AGG A	300
IFNAR1	Sense Antisense	ACC ACA GTG AAA CAT CAC CTG CCT TGT TGA TGA CGG GAG GAA ACA GGA	349

IFNAR2	Sense Antisense	TCA ACG GGA ATC AGA GTC GTC AGA TCA GGA AAT ACC CAG GCG GAC AAT	180
β-Actin	Sense Antisense	TCG CCG ACA GGA TGC AGA AGG A AGG TGG ACA GCG AGG CCA GGA T	129

Table 2.2 sgRNA primer sequences

Primer name	Sequence (5’ to 3’)	
sgRictor-1	Sense	CAC CGC CGA TCG CCG CCA TAT TGA
	Antisense	AAA CTC AAT ATG GCG GCG ATC GGC
sgRictor-2	Sense	CAC CGA TCT GAC CCG AGG TAA CGC G
	Antisense	AAA CCG CGT TAC CTC GGG TCA GAT C
sgRictor-3	Sense	CAC CGA CAA GAC CTC CAG TTC CAG A
	Antisense	AAA CTC TGG AAC TGG AGG TCT TGT C
sgRictor-4	Sense	CAC CGT AGC AGT GAT CCA AAA GGA
	Antisense	AAA CTC CTT TTG GAT CAC TGC TAC
sgRictor-5	Sense	CAC CGT CTT TCA GGT TTC ATC CCA G
	Antisense	AAA CCT GGG ATG AAA CCT GAA AGA C
sgRaptor-1	Sense	CAC CGG TCC TGG CCT TCA GCC CCG
	Antisense	AAA CCG GGG CTG AAG GCC AGG ACC
sgRaptor-2	Sense	CAC CGC ATT TCG GAC TCC ATC AGT G
	Antisense	AAA CCA CTG ATG GAG TCC GAA ATG C
sgRaptor-3	Sense	CAC CGG GAA ACT ACC AAG TTC AAG
	Antisense	AAA CCT TGA ACT TGG TAG TTT CCC

Chapter 3 - Results

3.1 Genes in mTOR signaling pathway were differentially expressed in PRRSV-infected MΦs at different activation statuses

Genome-wide analysis of gene regulation was conducted in PRRSV-infected porcine alveolar MΦs at different activation states as described [93, 94]. The differentially expressed genes (DEGs) involved in mTOR signaling pathway including mTOR complexes, their upstream regulators and downstream effectors, were extracted for further analysis (Figure 3.1A). Some of them displayed significantly differential expression and were selected as candidate targets to analyze antiviral regulation of MΦs by mTOR signaling (Figures 3.1B and 3.1C). mTOR kinase, the pivotal component in both mTORC1 and mTORC2, was up-regulated in IFN-γ (M1) and IL-4 (M2a) stimulations and down-regulated in LPS (M1) and IL-10 (M2c) treatments; however, mTOR kinase was less regulated by antiviral IFN-α1, indicating that mTOR is more relative to MΦ activation status, or antiviral regulation through cell polarization [110]. Two downstream effectors of mTORC1 and mTORC2, RPS6KB2 (also called p70 S6 kinase) and AKT3, were differentially regulated by MΦ polarization. The key subunit of mTORC2, Rictor, was greatly up-regulated by IFN-α1 [47, 111]. ULK1, acting as a crosslink for IFN-mTOR, was down-regulated by IFN-γ, LPS and IFN-α1, and up-regulated by IL-4 and IL-10 [62]. Genes related to the mTOR pathway included a large group of DEGs in PRRSV-infection MΦs at different activation statuses, which suggests that the mTOR signaling pathway is closely involved in anti-PRRSV regulation.

3.2 Regulation of PRRSV infection by the mTOR signaling pathway

The involvement of mTOR signaling in regulation of anti-PRRSV response was first investigated using a pharmaceutical approach. Two mTOR inhibitors, rapamycin and PP242, and an mTOR activator, MHY1485, were selected to modulate the mTOR signaling pathway [42, 112, 113]. Compared with rapamycin, which shows selective suppression primarily on mTORC1, PP242 is a non-selective inhibitor acting on both mTORC1- and mTORC2-signaling pathways. PRRSV infection was substantially repressed by mTOR inhibitors; especially, the non-selective inhibitor, PP242, which showed a much better effect than rapamycin in MARC-145 cells (Figures. 3.2A, 3.2B and 3.3A). Cells treated by both rapamycin and PP242 resulted in an additive effect on viral repression. In contrast, the activator, MHY1485 reversed the inhibition of mTOR activity by PP242. The addition of MHY1485 at physiological doses to PRRSV-infected cells, which were pre-treated with PP242, displayed a reverse effect on PP242-suppression of PRRSV replication (Figures 3.2C and 3.3B) [114]. However, MHY1485 at a high concentration of 8 μM inhibited PRRSV infection moderately, which could be relevant to its inhibitory effect on cell autophagy (Figures 3.2B and 3.3A) [113, 115]. All three tested inhibitors and the activator affect viral infection in a dose- and time-dependent manner (Figure 3.3A). In particular, prolonging rapamycin treatment significantly reduced PRRSV infection in MARC-145 cells. Although primarily targeting mTORC1, it was shown that long-term exposure of cells to rapamycin also led to suppression of mTORC2 activity [116].

In addition, we used porcine primary cells to confirm the results from MARC-145 cells, an African kidney cell line frequently used for culture of PRRSV *in vitro*. PP242 displayed an inhibitory effect on PRRSV infection in both MΦs and mDCs with dose-dependence as shown in MARC-145 cells (Figures 3.4A and 3.4B). Rapamycin showed inhibition on PRRSV infection

only in mDCs. The activator, MHY1485, conferred undetected effect on PRRSV infection in both MΦs and mDCs. No cytotoxic effect was shown in MARC-145 cells and porcine primary cells treated with mTOR inhibitors or activator. Results suggest that inhibition of mTOR signaling provides a potential route to regulate anti-PRRSV response and host antiviral immunity is significantly modulated by mTOR signaling pathway.

3.3 mTOR signaling modulates the type I IFN response

IFNs serve as critical mediators in regulation of overall antiviral response. Through interaction with their specific receptors, type I IFNs induce expression of a myriad of ISGs to exert antiviral and other biological functions [1, 18, 19]. It is implied that mTOR activity is closely related to type I IFN production and signal augmentation, but its involvement in PRRSV infection has not been studied [14, 31, 45-47, 49, 91, 117]. To test if mTOR signaling is involved in IFN production upon PRRSV infection, we family-wide analyzed porcine type I IFN gene expression in virus-infected porcine cells with pharmaceutical modulation of mTOR signaling. Porcine alveolar MΦs were infected with PRRSV along with mTOR inhibitors or activator, and the expression of type I IFNs and their receptors was examined using validated primers [102]. We observed that PRRSV infection repressed expression of most type I IFN subtypes, including IFN-α1/α5/6/α9/12, IFN-δ1/3/4/5 and IFN-ω1/2/5, which are effective anti-PRRSV IFNs (Figure 3.5A) [92, 102]. PP242 significantly reversed suppression of all analyzed type I IFN subtypes except for IFN-αω and IFN-β (Figure 3.5B). In comparison to PP242, rapamycin showed much less effect on PRRSV-induced inhibition of type I IFNs, and the activator MHY1485 only enhanced production of IFN-α9, IFN-δ6/7/8/9 and IFN-ω1/2 significantly. In addition, PP242 treatment conferred high expression levels of type I IFN receptors. However, mTOR activator MHY1485 suppressed expression of receptor genes of IFNAR1/IFNAR2. We measured IFN-α

proteins secreted in culture supernatants collected from MARC-145 cells treated with mTOR inhibitors/activator. We showed that mTOR catalytic inhibitor PP242 was able to heighten IFN-α production, the most effective IFN subtype that acts against PRRSV (Figure 3.6A) [92].

ISGs are collection of hundreds of genes up-regulated in response to IFN production, and play multiple roles in antiviral regulation. Typical ISGs, including PRRs, IRFs, and other signal transducing proteins, work in magnifying IFN signaling or directly inactivating viruses [18]. IRFs, particularly IRF3 and IRF7, are critical transcription factors in mediation of both IFN production and action. Using the promoter-reporter system constructed with central promoter elements of human IRF3, IRF7 and Mx1 genes [102], we showed that PRRSV infection suppressed promoter activity of IRF3, IRF7 and Mx1, and the suppression was successfully reversed with treatment of mTOR inhibitors, rapamycin and PP242 at physiological concentration (Figures 3.6B, 3.6C and 3.6D). In contrast, mTOR activator, MHY1485, showed little effect on PRRSV-suppression on promoter activity of IRF3, IRF7 and Mx1 genes. These findings indicate that PRRSV replication and antiviral response in cells are significantly regulated through modulation of mTOR signaling, which in turn affects both cell metabolic and immune statuses. Furthermore, through detection of type I IFN production at mRNA and protein levels as well as using bio-assays to monitor ISG-stimulating activity, we showed that regulation of mTOR signaling in antiviral response functions, at least in part, through changing production or action of type I IFNs.

3.4 Gene silencing mTOR signaling regulates PRRSV infection

Using a pharmaceutical approach, we have demonstrated that mTOR signaling regulates cell antiviral responses partly through modulation of the type I IFN system [25, 44, 45, 47, 51]. Here, using gene silencing, we further confirmed this observation. Raptor and Rictor are two key

subunits of mTORC1 and mTORC2, respectively. Studies showed that knockout of Raptor and Rictor completely blocked mTORC1- or mTORC2-pathway activity, respectively [111, 118, 119]. Use of a newly developed genome editing system, CRISRP/Cas9, we suppressed transcription of either Rictor or Raptor near 60% in MARC-145 cells (Figures 3.7A and 3.7B). Consistent with our observation using mTOR inhibitors, genetic suppression of Rictor, the essential factor for mTORC2, significantly suppressed PRRSV infection in MARC-145 cells (Figures 3.7C and 3.7D). In contrast, PRRSV infection was only slightly inhibited by silencing Raptor expression. Our tests repeatedly showed that inhibition of mTORC2 activity by inhibitors (such as PP242 or prolonging treatment with rapamycin) or gene silencing of Rictor provided better protection against PRRSV infection. Bioassays of culture supernatants in stimulation of ISG promoter activity verified that silencing the Rictor gene enhanced the cellular capacity to up-regulate promoter activity of ISG genes including IRF3, IRF7, and Mx1, plausibly through increasing type I IFN production in Rictor-silent cells (Figures 3.7E, 3.7F and 3.7G).

3.5 Type I IFNs modulate mTOR signaling

Previous studies have revealed that the mTOR signaling pathway interacts and affects IFN signaling transduction [25, 44, 45, 47, 51, 52, 117, 120]. For example, activation of AKT-mTOR pathway promoted mRNA translation of IFN-stimulated genes [44]. However the regulation of mTOR cascade by different type I IFN subtypes and viruses has not been well investigated. In this study, we found that phosphorylation and expression of p70 S6 kinase and AKT, the key downstream effectors of mTORC1 and mTORC2 respectively, were remarkably regulated by porcine IFN-α1 and IFN-β, and PRRSV infection in MARC-145 cells (Figure 3.8). IFN-α1 and PRRSV infection stimulated phosphorylation of p70 S6 kinase at T389, which phosphorylates ribosomal protein S6 and translation initiation factor, eIF4B, and acts

downstream of mTOR signaling; however, only IFN-α1 elevated p70 S6 kinase expression [121, 122]. In addition, IFN treatments and PRRSV infection increased expression of AKT and decreased its phosphorylation at S473, indicating that IFN-signaling may interact with mTOR-signaling through regulation of AKT at different levels. In summary, type I IFNs could interact with the mTOR-signaling cascade at several pathway components. Modulation of the mTOR signaling pathway provides novel targets to regulate virus-host interaction and to potentiate antiviral responses.

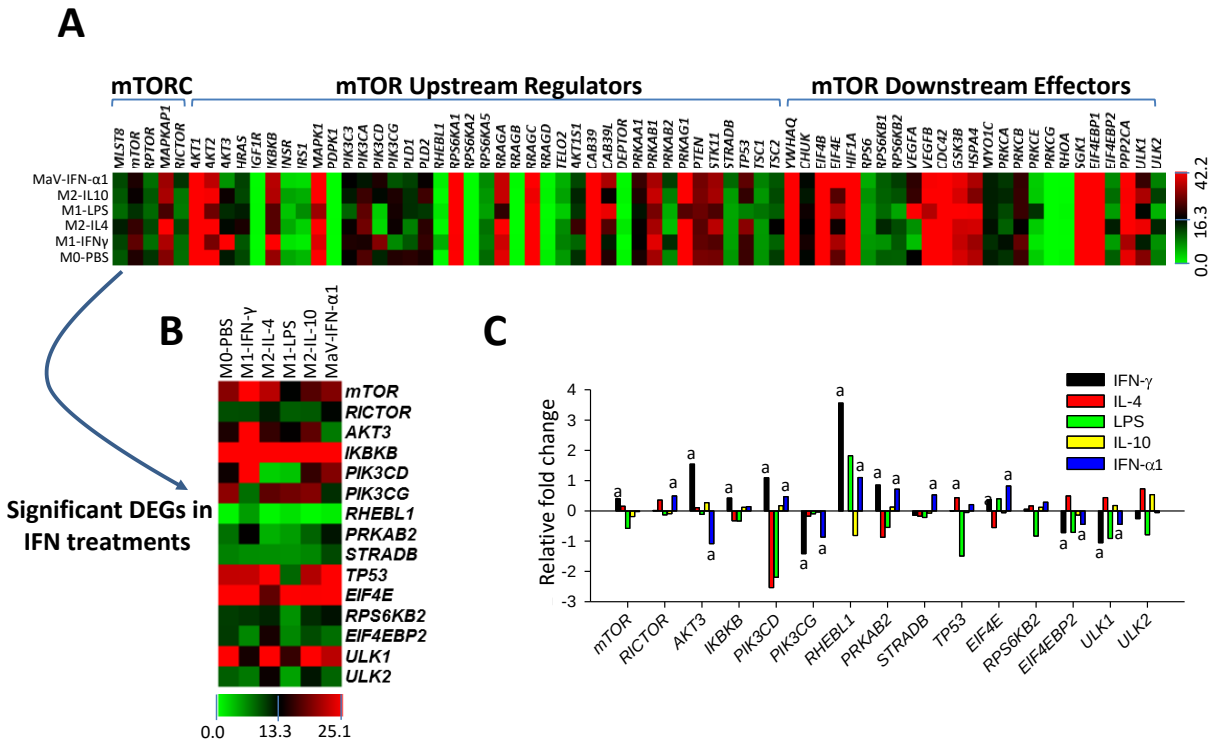


Figure 3.1 RNA-Seq analysis of DEGs in PRRSV-infected porcine alveolar MΦs at different activation statuses.

Subsets of genes involved in mTOR signaling pathway were extracted and displayed using a heatmap to show relative gene expression normalized as reads per kilobase of transcript per million reads mapped (RPKM) (A). Some DEGs, which were significantly regulated in cytokine treatments compared with the control, served as candidate genes for regulation of antiviral response, and were further emphasized using a heatmap (RPKM) (B) and a bar chart (log of fold changes to the control) at the bottom (C). a=*p* (FDR, false discovery rate)<0.001 to the control of PBS. All gene symbols are from NCBI Gene database (http://www.ncbi.nlm.nih.gov/gene/).

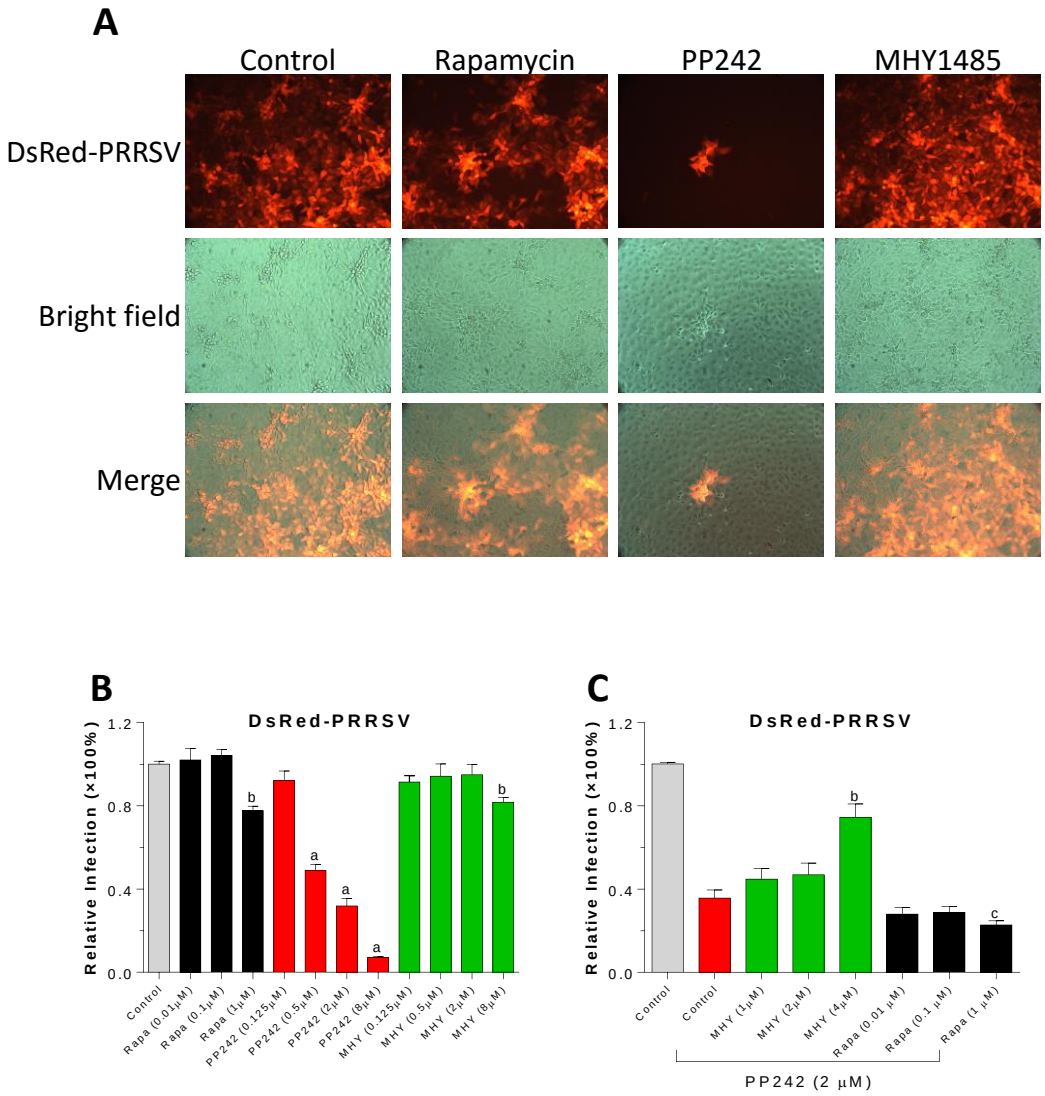


Figure 3.2 Regulation of PRRSV infection by mTOR inhibitors and activator in MARC-145 cells.

Cells were treated with 1 μM rapamycin, 2 μM PP242 or 2 μM MHY1485 for 24 h, then infected with DsRed-labelled PRRSV (MOI of 1), visualized and imaged with fluorescent microscopy (A) at 36 h post infection (hpi); or treated with serial dilution of mTOR inhibitors, rapamycin and PP242, and activator MHY1485, and quantified using SpectraMax i3 at 36 hpi (B); or pre-treated with 2 μM PP242 for 24h, then infected with DsRed-PRRSV along with MHY1485 or rapamycin at different concentration, and quantified with SpectraMax i3 at 36 hpi (C). n=3, a=p<0.001, b=p<0.01, c=p<0.05 to the control. Rapa: rapamycin, MHY: MHY1485.

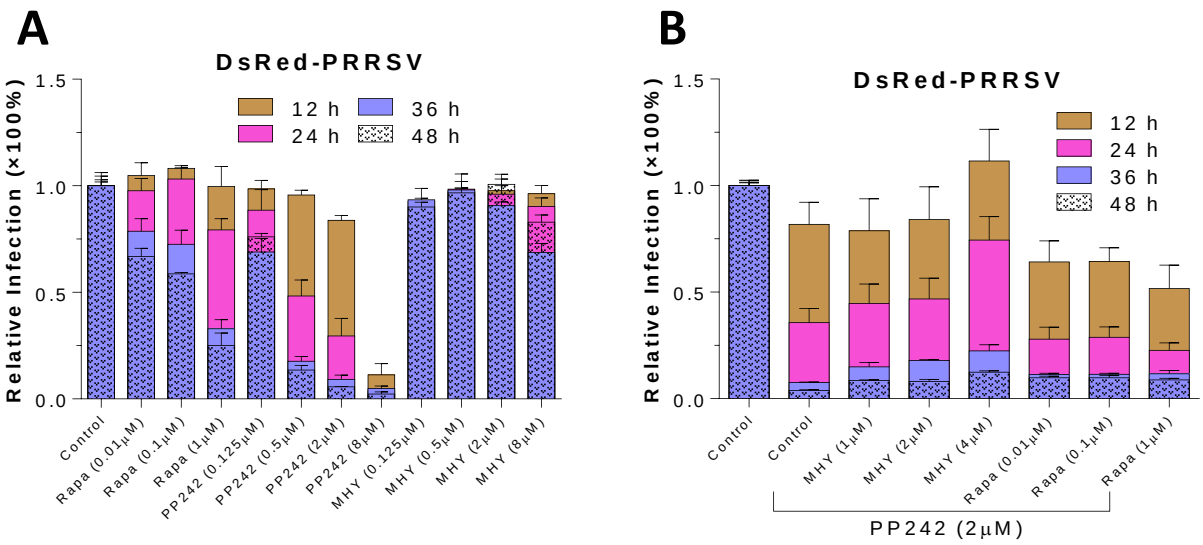


Figure 3.3 mTOR inhibitors and activator regulate PRRSV infection in a dose- and time-dependent manner in MARC-145 cells.

Cells were pre-treated with serial dilution of mTOR inhibitors, rapamycin and PP242, or activator MHY1485 for 12, 24, 36 or 48 h, then infected with DsRed-labelled PRRSV (MOI of 1), and quantified with a SpectraMax i3 at 36 hpi (A); or pre-treated with 2 μM PP242 for 12, 24, 36 or 48 h, then infected with DsRed-PRRSV along with MHY1485 or rapamycin at different concentration, and quantified with SpectraMax i3 at 36 hpi (B). Rapa: rapamycin, MHY: MHY1485.

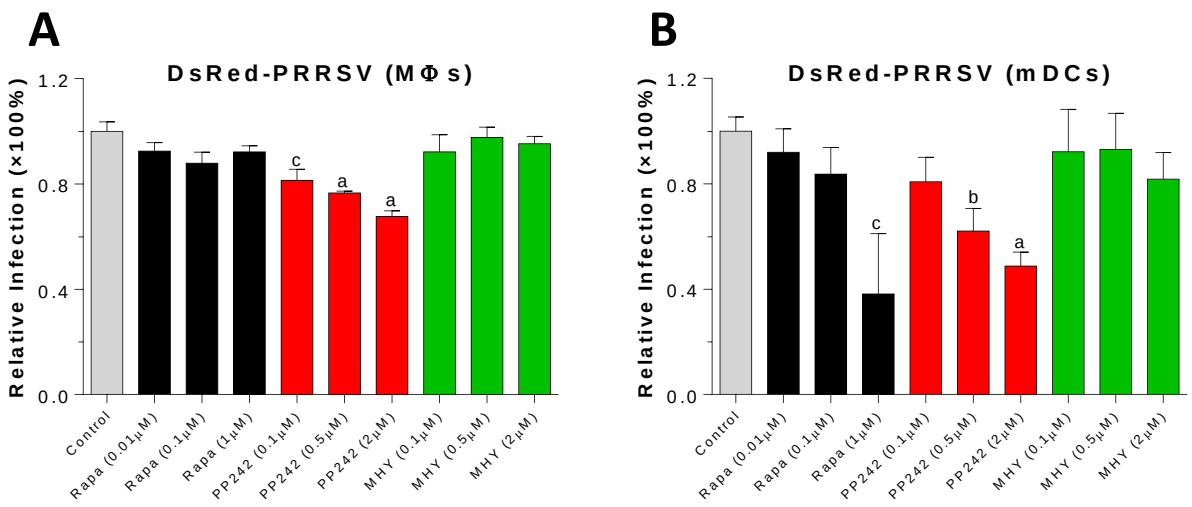


Figure 3.4 Repression of PRRSV infection by mTOR inhibitors in porcine alveolar MΦs and mDCs.

Cells were incubated with DsRed-labelled PRRSV (MOI of 0.5) for 1 h, and then treated with mTOR inhibitors (rapamycin or PP242) or activator (MHY1485) at indicated concentrations for another 20 h. Infected MΦs (A) and mDCs (B) were quantified using a SpectraMax i3 at 20 hpi. n=4, a=p<0.001, b=p<0.01, c=p<0.05 to the control. Rapa: rapamycin, MHY: MHY1485.

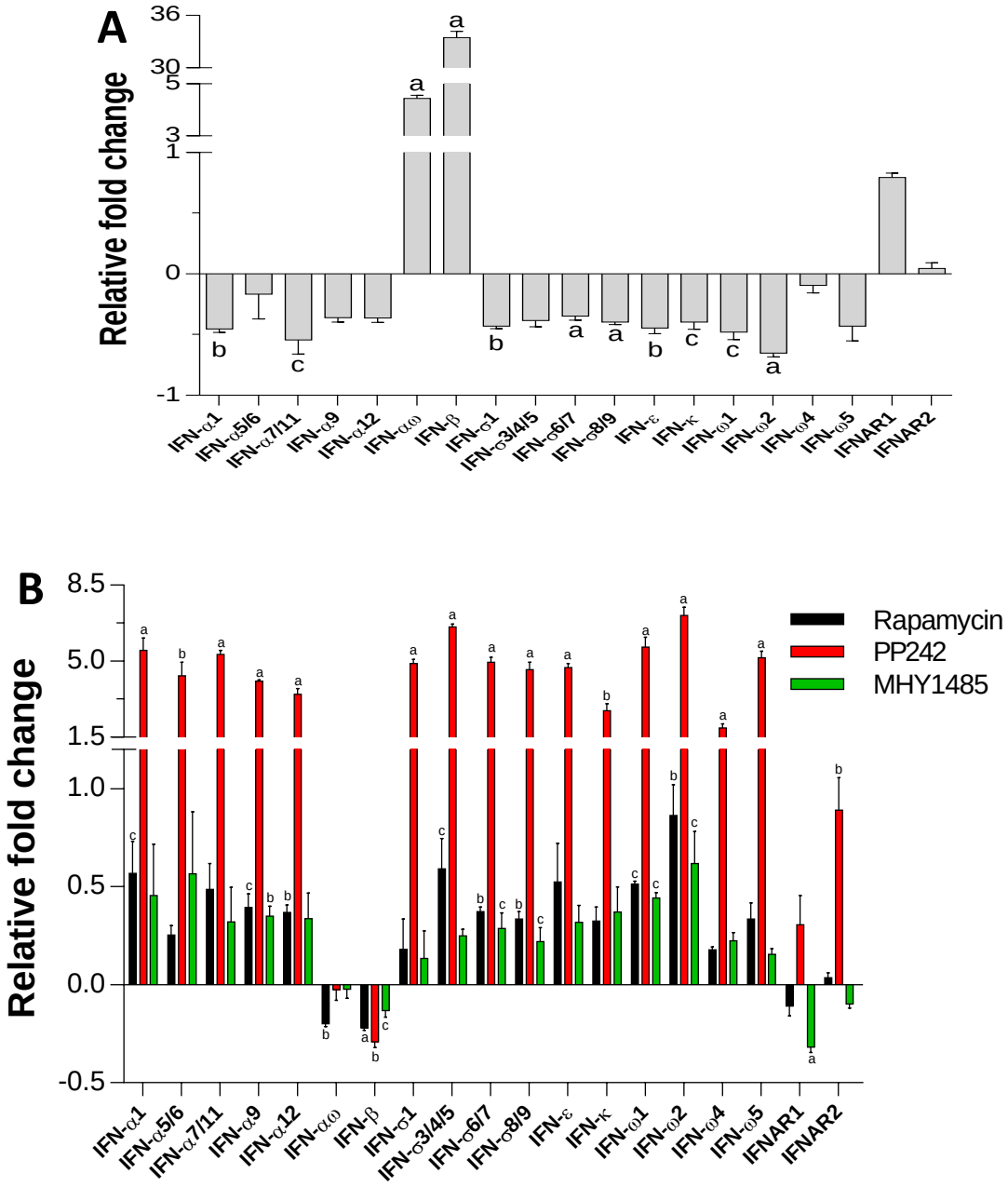


Figure 3.5 mTOR inhibitors or an mTOR activator modulate expression of type I IFNs and their receptors in PRRSV-infected porcine alveolar MΦs.

Cells were incubated with DsRed-labelled PRRSV (MOI of 0.5) for 1 h, and then treated with mTOR inhibitors (Rapamycin 1 μM, or PP242 2 μM) or activator (MHY1485 2 μM) for 12 h. Total RNAs were extracted to analyze expression of type I IFNs and their receptors in cells infected with PRRSV or not (A) and PRRSV-infected cells w/wo inhibitor or activator treatments (B) by specific primers using two-step RT-PCR. Control was normalized to 0, n=3, a=p<0.001, b=p<0.01, c=p<0.05 to the control. Rapa: rapamycin, MHY: MHY1485.

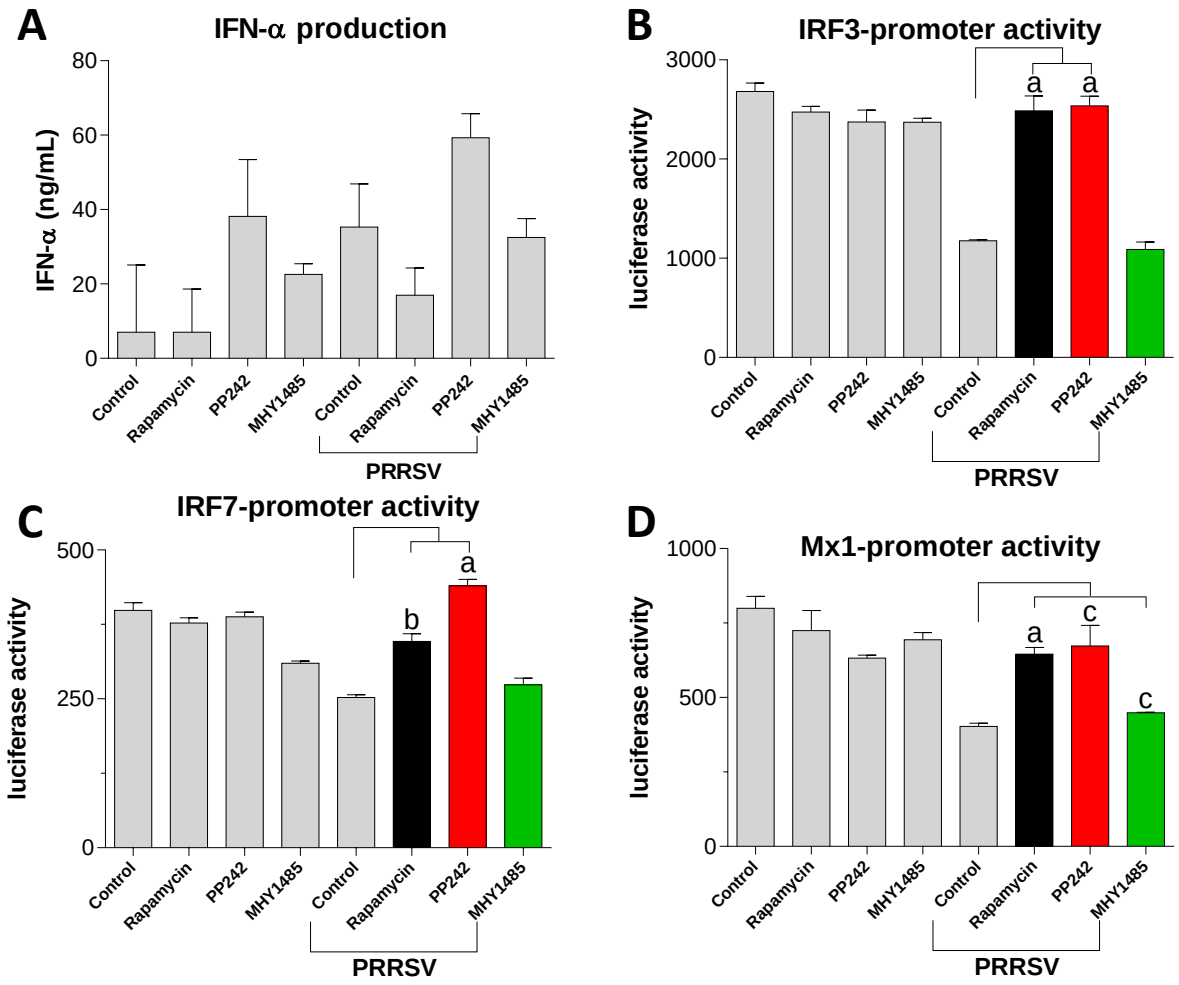
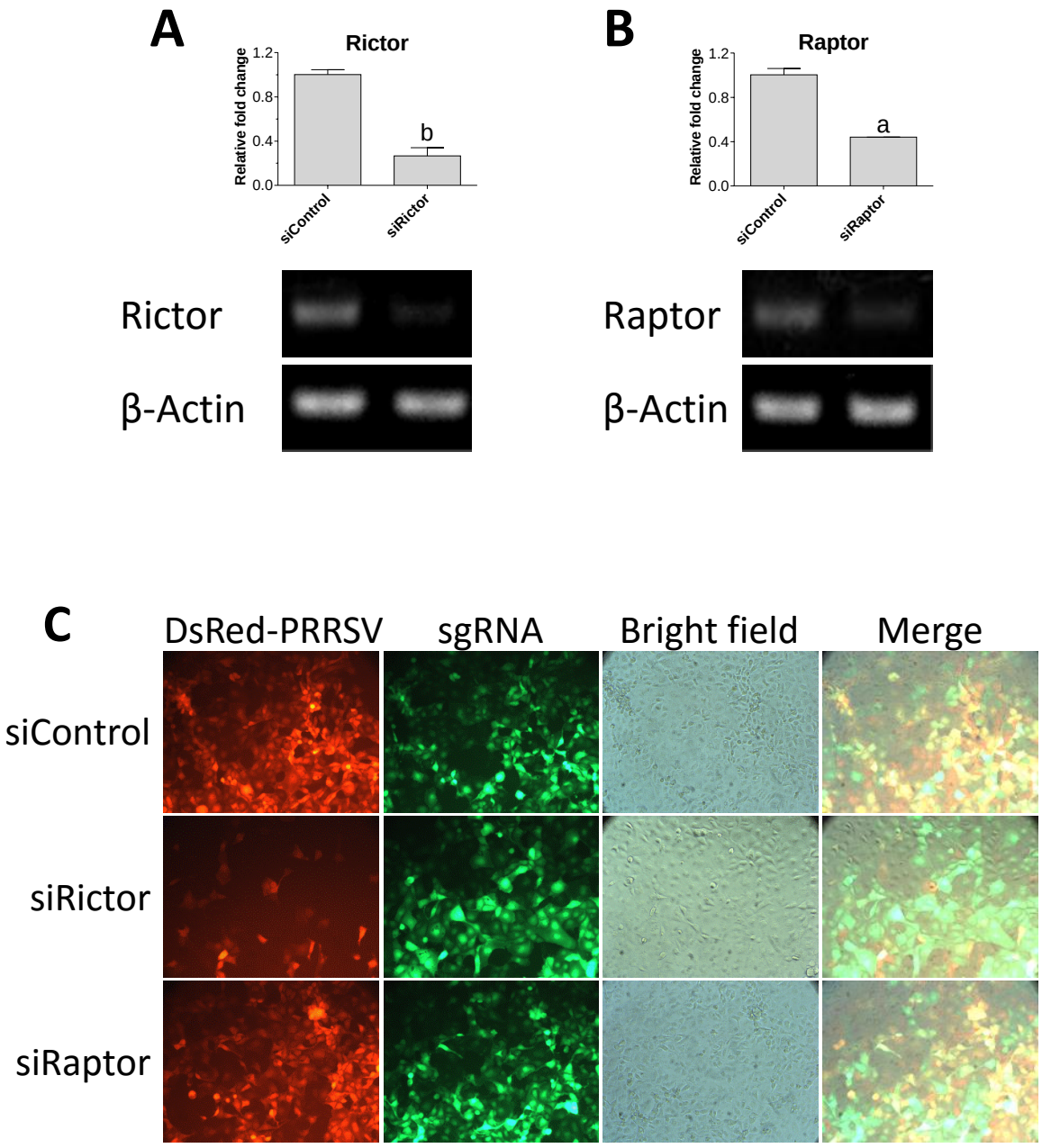


Figure 3.6 Disruption of mTOR signaling pathway promotes type I IFN production and signaling.

MARC-145 cells were treated with mTOR inhibitors (rapamycin and PP242) and activator (MHY1485) for 24h, and then infected with DsRed-labelled PRRSV (MOI of 1) for another 24h. Cell culture supernatants were collected and PRRSV was inactivated with UV illumination. Supernatants were used to measure IFNs with an ELISA for detection of IFN-α subtypes (A), or a bioassay in MARC-145 cells stably transformed with an IRF3-, IRF7- or Mx1-promoter driven luciferase reporter system (B, C and D). n=3, a=p<0.001, b=p<0.01, c=p<0.05 to the control.


A
Rictor
Relative fold change
1.2
0.8
0.4
0.0
b
siControl
siRictor
Rictor
β-Actin
B
Raptor
Relative fold change
1.2
0.8
0.4
0.0
a
siControl
siRaptor
Raptor
β-Actin
C
DsRed-PRRSV
sgRNA
Bright field
Merge
siControl
siRictor
siRaptor

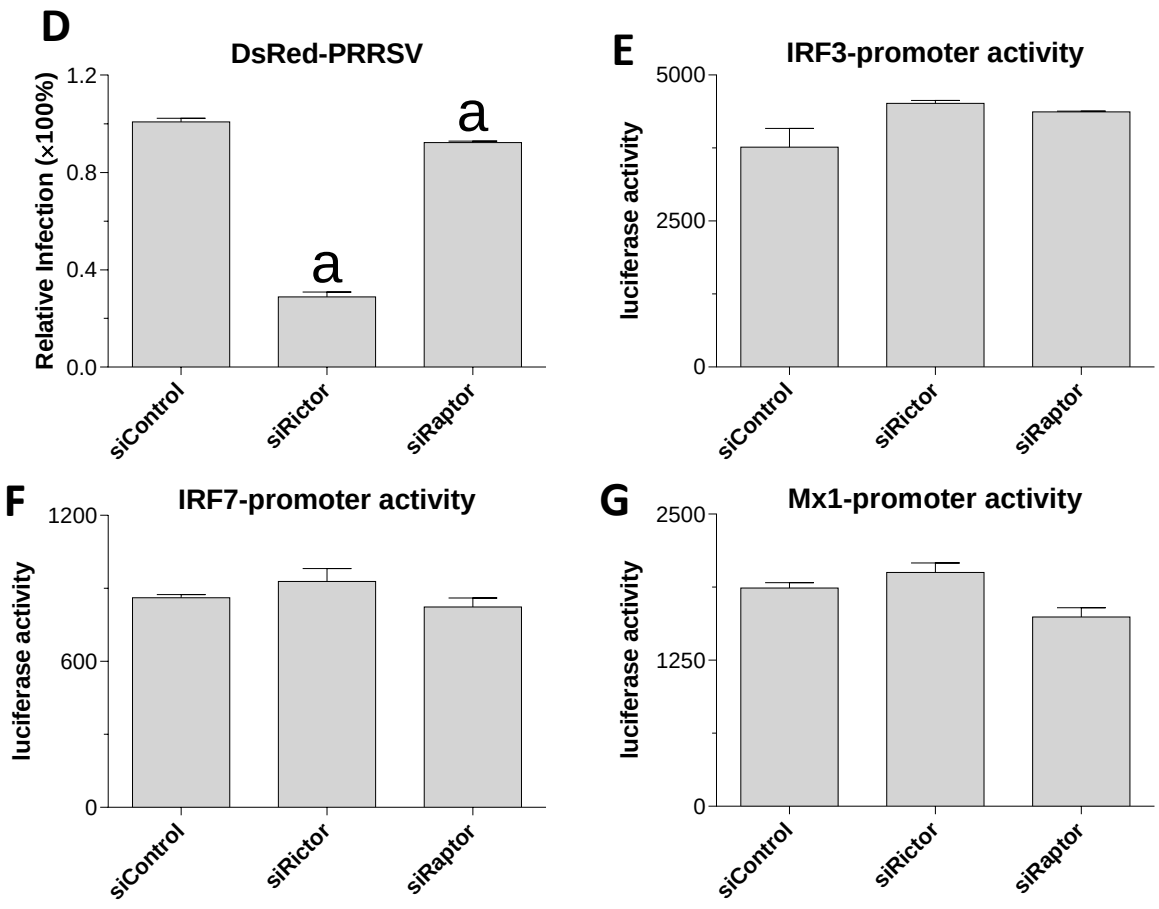


Figure 3.7 Blocking mTOR signaling by down-regulating Rictor or Raptor expression suppresses PRRSV replication in MARC-145 cells.

The gene expression of Rictor and Raptor was down-regulated using CRISPR/Cas9 system. Rictor expression was down-regulated by up to 70% (A), and Raptor expression near 60% (B). MARC-145-sgContro/sgRictor/sgRaptor cells were infected with DsRed-labelled PRRSV (MOI of 1), visualized and imaged with fluorescent microscopy (C), and quantified using a SpectraMax i3 at 36 hpi (D); or infected with DsRed-labeled PRRSV for 24 h. Then cell culture supernatants were collected and PRRSV was inactivated with UV illumination. Supernatants were used to detect IFN production and action with a bioassay in MARC-145 cells stably transformed with an IRF3-, IRF7- or Mx1-promoter driven luciferase reporter system (E, F and G). n=3, a=p<0.001, b=p<0.01, c=p<0.05 to the control.

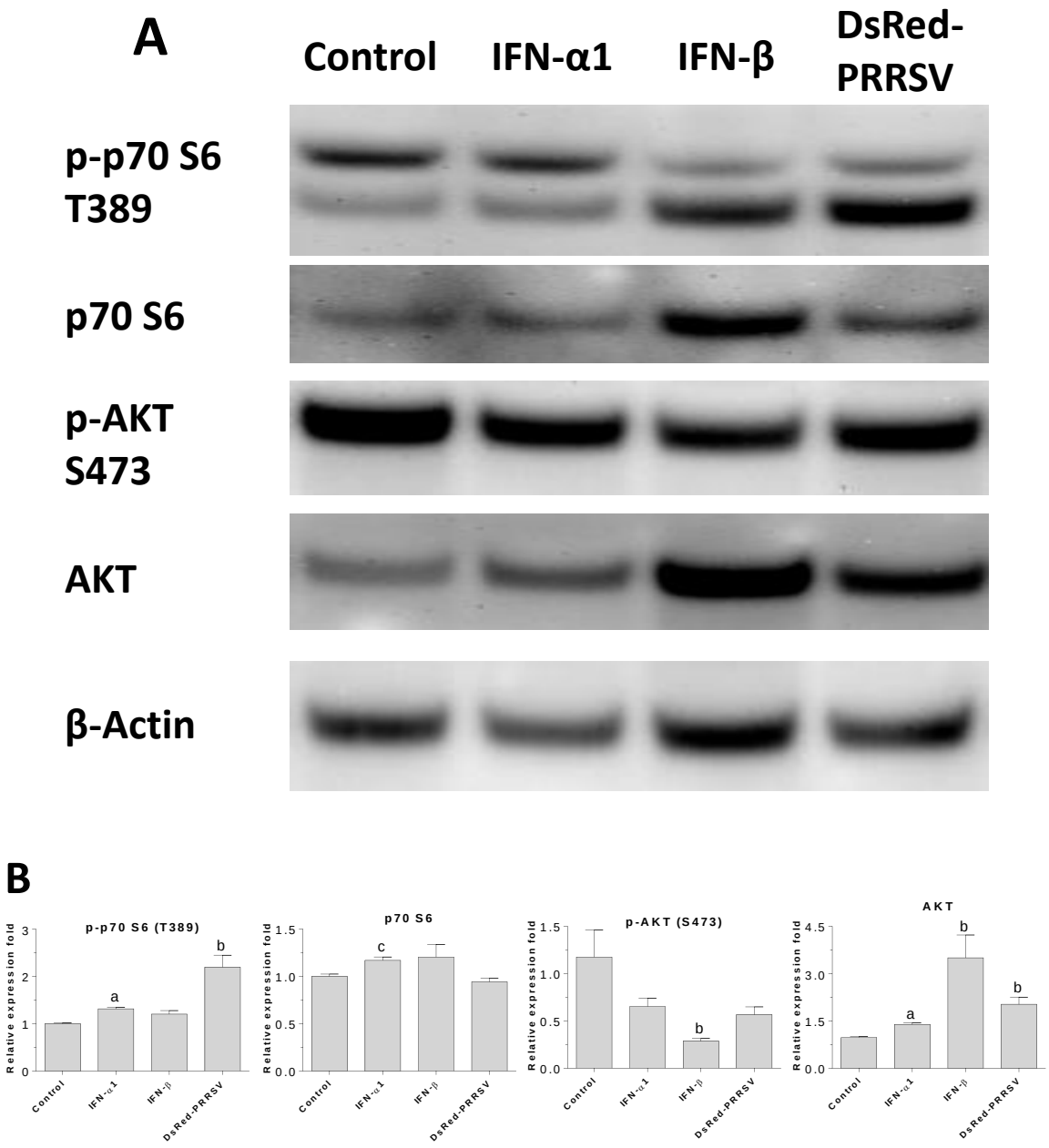


Figure 3.8 Phosphorylation and expression of AKT and p70 S6 kinase, two key components in mTOR signaling, are differentially regulated by IFN-α1, IFN-β and PRRSV infection.

MARC-145 cells were treated with IFN-α1 and IFN-β, or infected with DsRed-labelled PRRSV (MOI of 1) for 24 h. Cell lysates were resolved in SDS-PAGE to detect target proteins with western blotting (A). The picture shown is a representative blot of three repeats for each protein. Protein expression was quantified with ImageJ (B). n=3, a=p<0.001, b=p<0.01, c=p<0.05 to the control.

Chapter 4 - Conclusions and discussion

In mammalian cells, mTOR signaling is key in orchestrating various cellular activities and affects host antiviral responses [43, 44, 49, 123, 124]. Type I IFNs are a group of cytokines critical in mediating innate and adaptive immunity against viral infection [1, 16, 125]. Our findings showed that, in addition to its role in regulation of cell metabolic/immune status, the mTOR pathway is involved in the antiviral response through modulation of type I IFN expression and signal processing.

MΦs were first described by Metchnikoff and are ubiquitously presented in most, if not all, animal tissues, and play a central role in innate immunity and regulating adaptive immunity [97, 98]. Their regulatory functions are mechanistically dependent on proper polarization into distinct activation statuses [93, 100]. PRRSV primarily attacks alveolar MΦs and mDCs in its natural host, pigs, establishing chronic and persistent infection [73, 126]. Sang et al. showed that many pathways are significantly modulated in PRRSV-infected MΦs at different activation statuses [93, 94]. It has been shown that mTOR signaling involves regulating MΦ polarization [13, 56]. RNA-Seq analysis indicated that a large number of genes in the mTOR pathway were significantly and differentially regulated, including mTOR, Rictor, Raptor, AKT3, IKBKB, EIF4E, RPS6KB2, EIFEBP2, ULK1 and ULK2 in PRRSV-infected MΦs at different activation statuses (Figure 3.1). The expression of Rictor, Raptor and mTOR is directly related to formation of mTOR complexes. ULK1 is critically important in IFN response mediated by the mTOR signaling pathway [31]. RNA-Seq results reveal that the mTOR signaling pathway closely involves antiviral regulation in MΦs.

Protein kinase B, i.e. AKT, is a key component in mTOR signaling. Phosphorylation of AKT at T308 by PI3K/PDK1 or at S473 by mTORC2 acts as a main mechanism to regulate the activation of mTORC1 [59, 127-130]. Reciprocally, activation of mTORC1 by phosphorylated AKT at T308, enhances production of S6K that serves as a feedback mechanism to repress mTORC2 activity [59]. IFN-α1 and IFN-β increased mTORC1 activity accompanied by p70 S6 activation; however, they decreased mTORC2 activity through suppression of AKT phosphorylation at S473 (Figure 3.8). Compared with IFN-α1, IFN-β did not significantly stimulate expression and activation of mTORC1/p70 S6 kinase, but significantly suppressed mTORC2/AKT activation, which could partly link to stronger anti-PRRSV activity of IFN-α1 [92]. Blocking mTORC1 function with rapamycin treatment for short times did not confer protection against PRRSV. In contrast, non-selective PP242 and prolonged rapamycin treatments, which inhibit mTORC1 and mTORC2 activities, rendered significant suppression on PRRSV infection (Figures 3.2, 3.3 and 3.4). Furthermore, gene silencing of Rictor in mTORC2, but not Raptor in mTORC1, showed significant suppression on PRRSV infection. Our data obtained from both pharmaceutical treatments and genetic manipulation indicated that signaling mediated by mTORC2 and not mTORC1 is associated more with cell antiviral responses, and suggest that suppression of mTORC2 activity may provide a selective target to regulate anti-PRRSV immune responses. Compared with studies on mTORC1, little is known about the biological functions mediated by mTORC2. Results here imply a potential immune regulation role of mTORC2 rather than the primary metabolic regulation mediated by mTORC1 [36]. Clearly, further studies are required to understand signaling cascades and molecular mechanisms of mTORC2 in regulation of antiviral immunity, in particular, its potency to be targeted for antiviral regulation *in vivo*.

The p70 S6 kinase (a product of the RPS6KB2 gene) is a key component in the mTORC1-signaling pathway. The biological function of this kinase is multifunctional, which includes regulation of mRNA processing, translation initiation and elongation, ribosome function, nascent protein folding, adipocyte differentiation, synaptic plasticity, and cell survival signaling [131, 132]. However, it has not been well studied in antiviral response. We showed here that PRRSV infection robustly increased phosphorylation but not expression of p70 S6 kinase (Figure 3.8). The role of p70 S6 kinase phosphorylation in immune regulation thus requires further studies.

Cell-dependence was observed throughout our studies to regulate the antiviral response via mTOR pathway. In general, MARC-145 cells, is an established cell line containing cells more uniform and nearly 100% permissive to PRRSV; in contrast, porcine primary cells including MΦs and mDCs comprise diverse cell subsets and only partially permissive to PRRSV *in vitro* [72, 73]. Therefore, we generally have a more significant and reproducible observation in MARC-145 cells than in porcine primary cells. For examples, both mTOR inhibitors rapamycin and PP242 showed increased suppressive effects against PRRSV infection in MARC-145 cells; however, the suppressive effect also was clearly demonstrated in porcine primary cells even with reasonably different kinetics (Figures 3.2B, 3.3A, 3.4A and 3.4B). In addition, porcine primary cells are also more sensitive to mTOR inhibitors and an activator, and relatively a lower dose and shorter time treatments are critical. Conversely, Sun et al. reported that rapamycin, also as an autophagy inducer, could promote PRRSV entrance during the early phase of viral infection [115]. However, we showed here that suppression of the mTOR signaling pathway, in particular that mediated by mTORC2 with PP242 and prolonging rapamycin treatments, significantly inhibited PRRSV infection in both MARC-145 and porcine cells, revealing that IFN-mTOR

interaction plays a more important role in antiviral regulation than modulation on MΦ autophagy process. It is approachable at least for regulation of anti-PRRSV infection *in vitro* in both MARC-145 and porcine cells including MΦs and mDCs.

IFNs are critical antiviral cytokines [15, 19]. Most highly pathogenic viruses such as PRRSV have evolved diverse mechanisms to evade host IFN-mediated antiviral responses [19, 68]. For example, PRRSV primarily inhibits type I IFN signaling pathway by targeting inhibition of events relevant to IRF3, NF-κB, NEMO, and ISGs, resulting in pathogenic and persistent infection [75]. Type I IFNs are the most important antiviral IFN subgroup and consist of multiple subtypes [15, 21]. Expression and antiviral function of porcine type I IFN subtypes have been well studied [23, 92, 102]. We found that PRRSV infection suppressed gene expression of many type I IFNs including most anti-PRRSV effective subtypes. Comparing rapamycin and MHY1485, the inhibitor PP242 significantly increased gene expression of most of type I IFNs and also strengthened IFN signaling transduction as shown by high receptor gene expression levels (Figure 3.5B). This suggests that the mTOR signaling pathway, especially mTORC2 signaling, has a predominant role in transcription of genes related to type I IFN production and signaling. In addition, we showed that suppression of the mTOR pathway using PP242 induced production of IFN-α proteins, which are highly active against PRRSV and potentially provide a mean to counteract viral suppression on IFN production (Figure 3.6D) [92]. IRF3 and IRF7 are major transcription factors that bind interferon-stimulated response elements (ISRE) to mediate IFN induction. IRF3 is more potent in activating IFN-β genes than IFN-α genes [133, 134]. Not only functioning in stimulation of type I IFN production, IRF3 and IRF7 are also up-regulated by IFNs produced upon viral infection to form a positive regulatory loop in type I IFN signaling. Therefore, IRF3 and IRF7 are maker genes of ISGs indicating activation of IFN production

signaling. In addition to the difference in increasing type I IFN production, PP242 also showed a better reverse effect on IRF7 activation, compared with rapamycin, which partly explains why PP242 conferred better repression of PRRSV infection. In this context, interferon-induced dynamin-like GTPase genes Myxovirus 1 (Mx1) is a typical effector gene of ISGs to directly restrict viral infection, and serves as an indicator for type I IFN action signaling [135, 136]. Using promoter-reporter systems composed by promoters of these key ISGs, we measured the effect of modulation of mTOR pathways in IFN production and action. Together with direct analyses of type I IFN gene expression and protein secretion, our data showed that interfering with mTOR signaling, in particular through mTORC2, significantly potentiates type I IFN signaling, and even completely reverses PRRSV-suppression on type I IFN production. Therefore, polarization of cell statuses through modulation of mTOR pathway may provide an alteration to potentiate IFN response for antiviral regulation.

In conclusion, we have characterized the interaction of mTOR- and IFN-signaling pathways in following aspects in PRRSV infection in cells:

(1) Suppression of mTOR signaling using PP242, an inhibitor targeting both mTORC1 and mTORC2, represses PRRSV infection;

(2) Rapamycin, an inhibitor primarily targeting mTORC1, only significantly suppresses PRRSV infection during a prolonged treatment phase, when mTORC2 activity is also affected;

(3) Gene silencing of Rictor (a component of mTORC2) but not Raptor (a component of mTORC1) strikingly inhibits PRRSV infection;

(4) Production and signaling of type I IFNs, in particular of IFN-α, are potentiated by suppression of mTOR signaling, which contributes to restriction of PRRSV infection and counteracts PRRSV-suppression of IFN production;

(5) Compared with mTORC1, mTORC2 signaling plays a more predominant role in the regulation of anti-PRRSV response;

(6) The mTOR signaling pathway affects type I IFN signaling and type I IFNs also regulate expression and phosphorylation of key components in the mTOR signaling pathway, including AKT, and P70 S6 kinase, in a subtype-dependent manner.

Taken together, these studies establish platform to investigate inter-systemic interaction between two key pathways of immune and metabolic systems. Further validation of these results in animals may lead to identification of potential targets for antiviral potentiation through coordinative regulation of cell growth and activation statuses.

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