PHOSPHORYLATION OF HUMAN INTERFERON REGULATORY FACTOR 9 DURING TYPE I INTERFERON RESPONSE

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by

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LIST OF SYMBOLS & ABBREVIATIONS

× *g*	relative centrifugal force
µl	microliter
bp	base pair
BSA	Bovine serum albumin
CCD	charged coupled device
cDNA	complementary DNA
CHAPS	3-[(3-**ch**olamidopropyl)dimethyl**a**mmonio]-1-**p**ropane**s**ulfonate
ChIP	Chromatin immunoprecipitation
CKI/II	casein kinase I / II
CLRs	C-type lectin receptors
DBD	DNA binding domain
DMSO	dimethysulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
dNTP	Deoxynucleoside triphosphate
DTT	Dithiotreitol
ECL	enzymatic chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
et al.	and others
FBS	Fetal bovine serum
HEK293T	Human embryonic kidney 293T cell line
HeLa	Human cervical cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase

IAA	Iodoacetamide
IAD	IRF-associated domain
IFNAR	Interferon alpha receptor
IFNβ	Interferon beta
IL6	Interleukin 6
IRFs	Interferon regulatory factors
ISGF3	Interferon-stimulated gene factor 3
ISGs	Interferon-stimulated genes
ISRE	Interferon-stimulated response element
JAK	Janus kinase
kb	kilobase pair
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
mRNA	messenger ribonucleic acid
NEK6	Never in mitosis A (NIMA)-related kinase 6
NLRs	NOD-like receptors
NMR	nuclear magnetic resonance
NP-40	Nonidet P-40
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKCε	Protein kinase C epsilon
PKD1	Protein kinase D1
PKR	Protein kinase R
PLK1	Polo-like kinase 1
qPCR	quantitative PCR
RNA	ribonucleic acid

SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SILAC	Stable isotope labeling by amino acids in cell culture
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TEMED	Tetramethylethylenediamine
TLRs	Toll-like receptors
TYK2	tyrosine kinase 2
v/v	volume per volume
w/v	weight per volume
β-ME	beta-mercaptoethanol

PEMFOSFORILAN FAKTOR PENGAWAL INTERFERON 9 MANUSIA SEMASA RESPON INTERFERON JENIS I

ABSTRAK

Respons interferon jenis I merupakan sejenis sistem imunisasi intrinsik yang bertindak ke atas jangkitan virus di mana ianya menyebabkan pengaruhan transkripsi beratus-ratus gen interferon (ISG) sejurus infeksi virus berlaku. Pengaruhan gen-gen ISGs dikawal selia oleh laluan proses kinase Janus-isyarat molekul pengantara dan pengaktif transkripsi (JAK-STAT), di mana faktor pengawal interferon 9 (IRF9), bersama-sama dengan STAT1 dan STAT2, membentuk kompleks pengaktif transkripsi yang dikenali sebagai ISGF3. Modifikasi-selepas-translasi melalui proses pemfosforilan adalah sejenis mekanisme kawalan yang lazim dalam laluan proses JAK-STAT. Tinjaun literatur juga menunjukan mekanisme kawalan pemfosforilan dalam mengawal-selia kesemua jenis famili IRF. Walaubagaimanapun, tiada kajian terdahulu yang menunjukkan bahawa IRF9 dikawal-selia secara proses pemfosforilan. Dalam kajian tesis ini, pemfosforilan IRF9 telah dibuktikan berlaku yakni sejurus selepas respons interferon jenis I. Sejumlah ekstrak protein yang melalui pemilihan selektif kolum fosfoprotein menunjukkan bahawa IRF9 boleh diekstrak keluar daripada pecahan sejumlah ekstrak protein. Tambahan pula, gel poli-akrilamida Phos-Tag™ menunjukkan bahawa terdapat dua jenis protein IRF9. Kedua-dua hasil penemuan ini menujukkan bahawa IRF9 adalah sejenis protein yang difosforilasikan. Untuk menentusahkan amino asid yang difosforilasikan, IRF9 telah disubjek kepada proses pemendakan-imuno dan seterusnya analisis spektrometri jisim dilakukan ke atas IRF9. Pemfosforilan IRF9 daripada ekstrak sel HEK293T didapati berlaku pada

amino asid serina 252 (S252) dan serina 253 (S253) setelah sel-sel tersebut didedahkan kepada IFNβ (yakni salah satu spesis molekul respons interferon jenis I). Kedua-dua lokasi spesifik amino asid tersebut juga didapati terletak dalam domain-berkaitan IRF (IAD) IRF9. Mutagenesis-lokasi-bersasar telah dilakukan terhadap kedua-dua amino asid tersebut untuk menentukan kepentingan pemfosforilan amino asid tersebut dalam konteks fisiologi. Mutasi fosfomimetik dan bukan-fosfomimetik IRF9 dinilai menerusi analisis aruhan beberapa gen-gen ISG dengan menggunakan teknik kuantitatif PCR (qPCR). Apabila dibandingkan dengan IRF9 jenis liar, hanya ekspresi gen *USP18* menunjukkan penurunan induksi setelah terdedah kepada IFNβ bagi kesemua mutasi IRF9. Manakala, tiada perbezaan ekspresi bagi gen *MxA* dan gen *OAS1*. Ini menunjukkan bahawa, fosforilasi S252 dan S253 terlibat dalam mengawal selia subset ISGs. Kajian tesis ini melaporkan pemfosforilan IRF9 buat pertama kalinya. Walaubagaimanapun, kerja-kerja kajian experimentasi yang lebih lanjut perlu dilakukan bagi memahami laluan pengawal-seliaan IRF9 secara pemfosforilan di dalam respons interferon jenis I dan selanjutnya.

PHOSPHORYLATION OF HUMAN INTERFERON REGULATORY FACTOR 9 DURING TYPE I INTERFERON RESPONSE

ABSTRACT

Type I interferon response describes the cellular innate antiviral response whereby hundreds of interferon-stimulated genes (ISGs) are upregulated following virus infection. This massive induction of ISGs is mediated by the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, where interferon regulatory factor 9 (IRF9), together with phosphorylated STAT1 and STAT2, forms the transcription activator, ISGF3. Post-translational modification by phosphorylation is an essential regulatory mechanism within the JAK-STAT pathway. Furthermore, studies have shown ubiquitous phosphorylation regulation for other human IRF family members. However, none have been described for IRF9. In this study, IRF9 was shown to be phosphorylated in response to type I interferon (e.g. IFNβ). Total protein lysate subjected to selective phosphoprotein enrichment column showed enrichment of IRF9 in the phosphoprotein fraction. Furthermore, Phos-Tag™ polyacrylamide assay showed the presence of two IRF9 protein phosphorylation states. Both of the observations indicated that IRF9 is a phosphoprotein. To determine the specific phosphorylated amino acid residue(s), IRF9 was immunoprecipitated and then subjected to tandem mass spectrometry analysis. The phosphorylation of IRF9 occurred at serine 252 (S252) and serine 253 (S253) under IFNβ-induced conditions in human embryonic kidney 293T (HEK293T) cells. These sites lie within the IRF-associated domain of IRF9. Site-directed mutagenesis was carried out on S252 and S253 to infer the physiological importance of both phosphorylation sites.

Phosphomimetic and non-phosphomimetic IRF9 mutants were evaluated on the induction of ISGs, using quantitative PCR. Interestingly, only the gene expression of *USP18* showed reduced induction under IFNβ-induced conditions for all IRF9 mutants compared to wildtype IRF9. In contrast, no difference was observed for the expression of *MxA* and *OAS1* genes between IRF9 mutants and wildtype IRF9 when induced by IFNβ. This indicated that phosphorylation of S252 and S253 could be involved in regulating a subset of ISGs. This thesis reported the phosphorylation of IRF9 for the first time. However, further experimental work will be needed to fully understand the possible phospho-regulatory pathway of IRF9 in the type I IFN response or beyond.

CHAPTER 1

INTRODUCTION

1.1. Rationale of the study

Interferon regulatory factor 9 (IRF9) is an important transcription factor in regulating the innate immue response. The mechanism of IRF9 is mediated through the Janus kinase-Signal transducer and activator of transcription (JAK-STAT) pathway. The literature on IRF9 regulatory mechanism is less extensive compared to other IRFs. There is a lack of post-translational modification studies on IRF9, its regulation at the transcriptional and translational levels. This is surprising, given the importance of IRF9 in regulating viral infection. Undoubtedly, IRF9 plays an essential role in the regulation of the innate antiviral immune response, in particular in maintaining host immunity against influenza virus (Jing and Su, 2019). A previous study had highlighted a genetic defect or a loss-of-function of the *IRF9* allele resulting in a life-threatening influenza pneumonitis (Hernandez et al., 2018). Another study highlighted a frameshift mutation that resulted in IRF9 deficiency, which causes the loss of control in multiple viral infections (Bravo García-Morato et al., 2019).

Post-translational modification by phosphorylation is an important mechanism in regulating protein function. While specific phosphorylation sites of IRFs have been reported in previous studies, no particular phosphorylation site was reported in IRF9. Early research conducted by Veals et al. (1993) indicated a phosphorylation event in IRF9. The authors proposed that the DBD region between 1-117 required a phosphorylation event for DNA-binding activity. The authors also hypothesized that the IRF9 DBD region is constitutively phosphorylated on the basis that IRF9 obtained

from cells without calf intestinal phosphatase treatment was able to bind to DNA. However, there was no specific phosphorylated amino acid identified and there has been no follow-up on the study ever since.

Preliminary studies, which include computational prediction (Appendix H), suggested the possibility of IRF9 being a phosphoprotein. However, there has been no report on its phosphorylation status and its regulation. As mentioned previously, the immune signaling cascade of type I IFN response is tightly regulated by phosphorylation reaction from the dimerization of IFN receptors that triggers the phosphorylation of Janus kinases, which eventually leads to the phosphorylation of STAT proteins in the cytoplasm. The phospho-STATs would then recruit IRF9 to form the ISGF3 complex. Given that IRF9 is a critical transcription factor in regulating the immune response, it is apt that we aimed to determine the phosphorylation status of IRF9 and its effect within the context of the innate antiviral immune response. The pleiotropy function of IRF9 in various pathogenesis has been recognized only recently, hence underscoring the importance of studying IRF9 regulation.

1.2. Research Questions

In this study, we seek to resolve the following research questions;

1. What is the phosphorylation status of IRF9 under type I IFN response?
2. Where does the phosphosite of IRF9 occur, if any?
3. How does the phosphorylation of IRF9, if occur, regulate the type I IFN gene activity?
4. How does the phosphorylation of IRF9, if occur, regulate its activity?

1.3. Hypothesis

Our previous preliminary findings (unpublished) suggested that IRF9 is phosphorylated following poly(IC) transfection. Poly(IC) is a double-stranded RNA, and is transfected to cells (*in vitro*) to mimic viral infection. Transfection of poly(IC) induces the activation of type I interferons, which then activates the ISGF3 complex of which IRF9 is a part of. We hypothesized that IRF9 is phosphorylated during type I interferon response and that the phosphorylation affects the activity of antiviral gene expression.

1.4. Objectives of the study

1.4.1. Main objective

The main objective of this study was to determine the phosphorylation state of interferon regulatory factor 9 during type I interferon response.

1.4.2. Specific objectives

1. To identify the phosphorylation status of IRF9 by phosphoprotein enrichment assay and Phos-Tag™ based mobility shift assays.
2. To determine the phosphorylation site(s) of IRF9 by tandem mass spectrometry.
3. To render the 3D model of IRF9 by homology modeling using Phyre2
4. To predict the potential kinase(s) using NetPhos 3.1 and Scansite 4.0.
5. To determine the effect of IRF9 phosphorylation by mutational analysis.

1.5. Antiviral immune response

The antiviral immune response is the first line of defense of host protection from viral infection. The host recognizes unique viral signatures known as pathogen-associated molecular patterns (PAMPs). The innate immune recognition of PAMPs occurs either in a cell-intrinsic or cell-extrinsic way via the pattern recognition receptors (PRRs) (Stetson, 2009). Cell-intrinsic viral recognition is activated in infected cells using PRRs such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Iwasaki and Medzhitov, 2010). NLRs, which sense bacterial peptidoglycan, also detect some viruses. Meanwhile, RLRs are cytoplasmic RNA helicases that detect viral RNA products. In contrast, cell-extrinsic viral recognition activation does not require cells to be infected. The cell-extrinsic viral recognition involves non-infected immune cells (e.g., macrophages and plasmacytoid dendritic cells) using PRRs such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (Iwasaki and Medzhitov, 2010). TLRs are membrane-associated molecules that detect pathogens at the cell membrane or during viral transit within the endosomes. CLRs are also membrane-associated molecules with a carbohydrate recognition domain for ligand binding in a Ca^{2+}-dependent manner (Brown et al., 2018). Cell-intrinsic and cell-extrinsic virus recognition will lead to the induction of various cytokines such as type I interferons.

Activation of the innate immune response occurs in a biphasic manner. In the early activation phase of antiviral innate immune response (Figure 1.1), viral nucleic acid recognition by TLRs induces the production of NF-κB-dependent pro-inflammatory cytokines, the IRF-dependent antiviral cytokines, and the mitogen-activated protein kinases (MAPKs) pathway (Kawai and Akira, 2010). Different TLRs detect different viral nucleic acids. TLR3 detects double-stranded RNA (dsRNA), TLR7 and TLR8

detect single-stranded RNA (ssRNA), and TLR9 detects dsDNA. Depending on the type of viral nucleic acids, each will trigger different activation pathways. TLR3 recruits TRIF proteins, which binds to the TRAF3-TANK-NAP1 protein complex and subsequent engagement with TBK1 and IKKi proteins. As a result, IRF3 is activated and gets translocated into the nucleus and binds to type I IFN gene promoters. This is done in concert with c-Jun, ATF2, and NF-κB proteins. TRIF also recruits TRAF6, which then recruits RIP-1, which then recruits TAK1 to engage the IκB kinase complex (IKKα-IKKβ-IKKγ). The IκB complex then engages with NF-κB to activate the transcription of cytokines. TLR7 and TLR8 recruit MyD88 protein, which then recruits IRAK proteins to induce the activation of the NF-κB-dependent cytokines via a similar pathway (Figure 1.1). TLR9 also recruits MyD88, which then recruits IRAK proteins to activate IRF7 via TRAF3. Translocation of IRF7 into the nucleus resulted in the transcription of type I IFNs.

Viral RNA recognition by RLRs (retinoic acid-inducible gene-I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA-5) RNA helicases) induces the production of NF-κB-dependent pro-inflammatory cytokines and the IRF-dependent antiviral cytokines (Figure 1.2). Both RIG-I and MDA-5 use the mitochondria antiviral signaling protein (MAVS) localized to the mitochondria to activate protein complexes; FADD-Caspase 8/10-RIP-1, TRAF3-TANK-NAP1, and the TRAF6 dependent pathway. The FADD-Caspase 8/10-RIP-1 activates the IκB complex, which activates NF-κB, activating the transcription of cytokines. The TRAF3-TANK-NAP1 complex activates the TBK1-IKKi complex, which activates the IRF3. The TRAF6-dependent pathway binds to MEKK1 to activate the MAP kinase pathway to activate the c-Jun.

As previously, c-Jun, together with ATF2, IRF3, and NF-κB complex, activates the transcription of antiviral cytokines (Figure 1.2).

Cytokines such as type I interferons produced in the early phase would trigger the late phase of antiviral innate immune response (Kawai and Akira, 2010). The late phase of antiviral innate immune response is mediated by the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, leading to the production of interferon-stimulated genes (ISGs). The biphasic manner in which ISGs are induced allows for (i) a tight regulatory process over the production of potent ISGs, and (ii) increased complexity in the activation pathway, which leads to signaling redundancies of which is helpful to overcome multiple viral evasions.

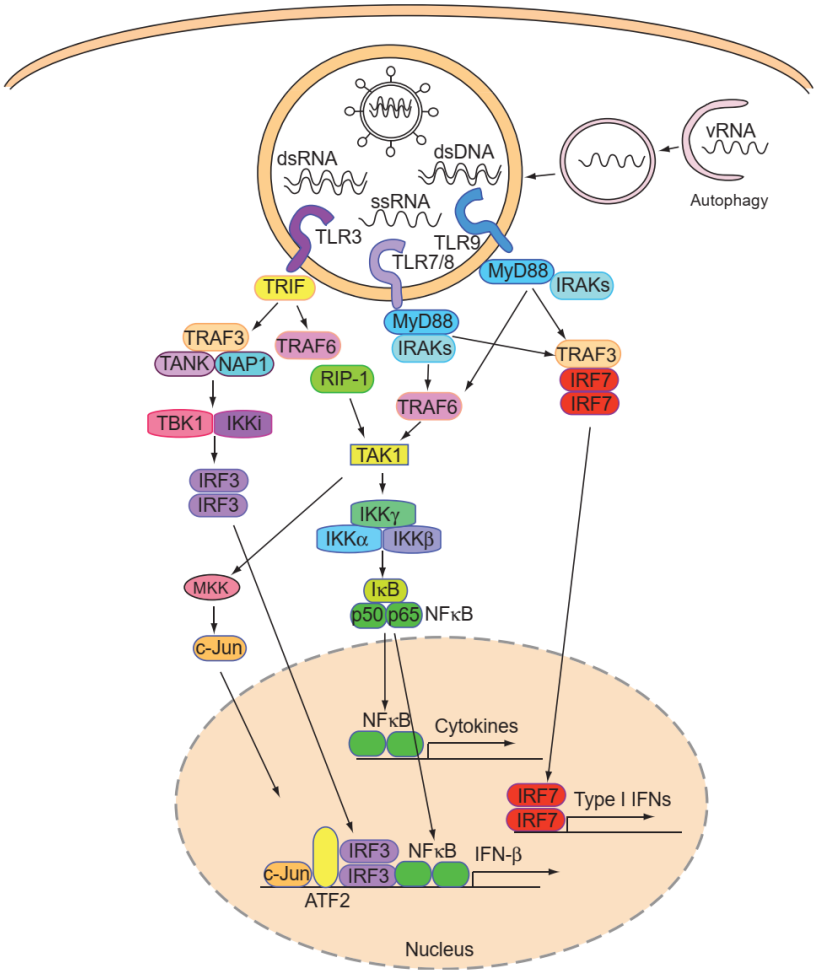

Figure 1.1. Viral nucleic acids recognition and signaling by TLRs. In the early phase signaling of the antiviral innate immune response, viruses get endocytosed and digested within the endosomes. Viral nucleic acids would then become accessible to TLRs for recognition. Each TLR recognizes a different variant of nucleic acids. For example, TLR3 detects dsRNA, TLR7 and TLR8 detect ssRNA, and TLR9 detects dsDNA. The TLR signaling leads to the downstream induction of cytokines. For instance, NF-κB activates the transcription of cytokines. IRF7 homodimer in the nucleus activates the transcription of type I IFNs. Similarly, IRF3 homodimer associates with c-Jun, ATF2, and NF-κB to activate the transcription of IFNβ. This figure was taken from Fields et al. (2013).

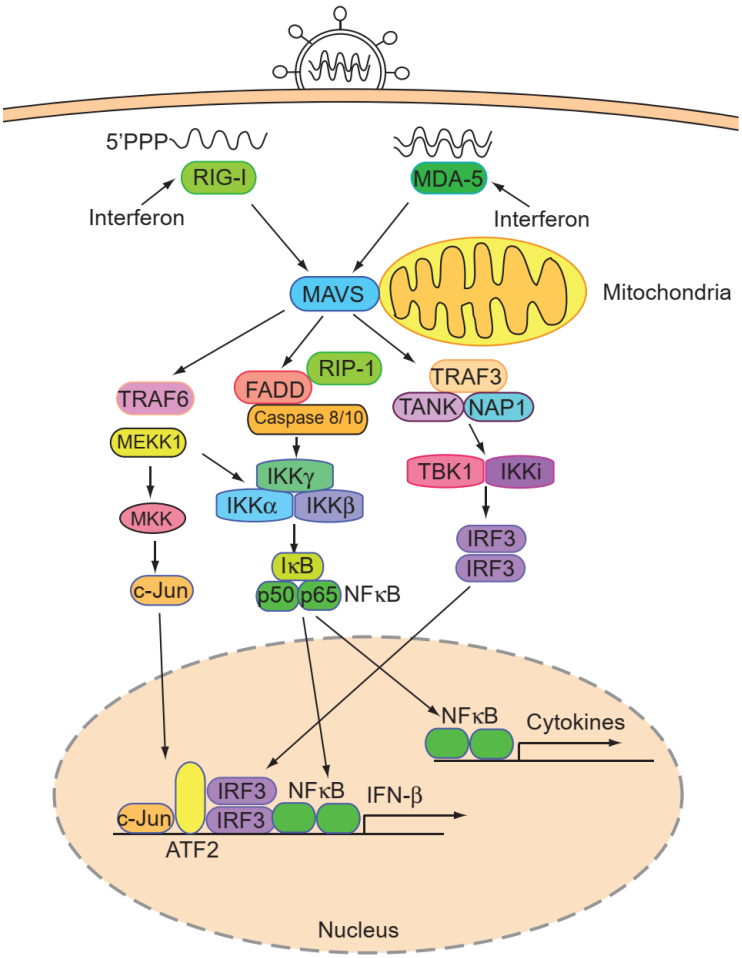

Figure 1.2. Viral nucleic acid recognition and signaling by RLRs. RIG-I detects ssRNA that contains 5'-triphosphate. MDA-5 detects dsRNA. Detection of viral RNAs induces conformational changes to RIG-I and MDA-5, allowing them to bind to the MAVS adaptor protein. Activation of the MAVS pathway activates NF-κB, the MAP kinases, and IRF3, leading to the transcription of IFNβ and cytokines. The figure was taken from Fields et al. (2013).

1.5.1. Innate antiviral cytokines: The interferons

Interferon (IFN) was discovered by Isaacs and Lindenmann in 1957 while studying the mechanism of viral interference (Isaacs et al., 1957). The term "interferon" – coined by the authors – was an acknowledgment of the substance's interfering activity of the influenza infection. The discovery of interferons paved the way for the study of innate antiviral immune responses in humans. There are three types of IFNs – Type I, II, and III. Type I IFNs are consists of IFNα and IFNβ, type II IFN is also known as IFNγ, while type III IFN is also known as IFNλ. Each type of IFN binds to different receptor complexes (Figure 1.3). Type I IFN binds to IFN alpha receptor 1 and 2 (IFNAR1 and IFNAR2), type II IFN binds to IFN gamma receptor 1 and 2 (IFNGR1 and IFNGR2), and type III IFN binds to IFN lambda receptor 1 (IFNLR1) and interleukin 10 receptor 2 (IL10R2) (Borden et al., 2007). IFNs are expressed ubiquitously in every human cell, and their synthesis is transient and requires stimulation by either virus, products of microbial origins, or chemical substances (Borden et al., 2007). In the absence of an infection, IFNs expression is kept silent (Levy et al., 2011).

A recent study by Altman et al. (2020) found that type I IFNs are kept in endosomes for days. These IFN silos remain dormant due to type I IFN negative regulators, ubiquitin-specific peptidase 18 (USP18), and interferon-stimulated gene 15 (ISG15) (Altman et al., 2020). The study sheds light on the phenomena of a long-lasting effect of type I IFNs, as evident from a sustained high level of ISGs. For example, Hermesh et al. (2010) reported that after virus infection resolution, the bone marrow leukocytes continue to express antiviral genes despite no type I IFN in the bone marrow or serum. Type I IFN – as a result of acute infection – primes the bone marrow leukocytes through mediation by other cells for protection against future infection, and that the

absence of type I IFN post-infection was hypothesized to reduce the risk of overwhelming bone marrow leukocytes with cytokines (Hermesh et al., 2010).

1.5.1(a) Type I Interferons

As introduced previously, type I IFNs include IFNα and IFNβ. There are 13 known subtypes of IFNα, a single IFNβ, and IFNε/κ/δ/ζ/τ/ω. Almost all cells in the body can produce type I IFNs (McNab et al., 2015). Type I IFNs are potent cytokines that elicit an immune response and various other modulations of cell physiology (Stetson, 2009). Type I IFNs regulates the development of the adaptive immune response through direct and indirect means. The indirect way includes upregulation of cytokines and chemokines that affect immune cell activation, growth, and trafficking (Levy et al., 2011). Meanwhile, direct way includes affecting the maturation of dendritic cells, natural killer cells, and lymphocytes.

1.5.1(b) Type II Interferons

In type II IFN signaling, there is only one IFN subtype which is IFNγ. Unlike type I and type III IFNs, IFNγ binding to its receptors occurs in a homomeric configuration (Walter et al., 1995). IFNγ is mainly produced by natural killer cells (Gill et al., 2011). IFNγ binds to a different set of receptors for their signaling; two IFNγ receptor 1 (IFNGR1) and two IFNγ receptor 2 (IFNGR2) subunits (Figure 1.3).

1.5.1(c) Type III Interferons

Type III IFNs includes IFNλ1, IFNλ2, and IFNλ3 subtypes. Similar to type I IFNs, signaling of type III IFNs follows a monomeric configuration of cytokines (Figure 1.3), requiring one unit each of its low- and high-affinity receptors (de Weerd and Nguyen, 2012). Type III IFNs shares many similarities with the interleukin-10 (IL-10) cytokine family, thus utilizing IL-10R2 as one of its receptors (Figure 1.3).

1.6. The JAK-STAT pathway in IFN response

The JAK-STAT pathway in IFN response (Figure 1.3) describes the signaling pathway of activation of ISGs (Stark and Darnell, 2012). The pathway is heavily regulated by a cascade of phosphorylation reactions. In the first phase of the innate antiviral immune response, pathogen recognition receptors detect viral pathogens (Akira et al., 2006). The detection of viral pathogens leads to the inducement of type I IFNs. In the canonical JAK-STAT pathway, type I IFNs binding to its receptors (IFNAR1 and IFNAR2) lead to its dimerization, which then phosphorylates the IFNAR1-bound tyrosine kinase 2 (TYK2) (Majoros et al., 2017). TYK2 then phosphorylates IFNAR2-bound Janus kinase 1 (JAK1). JAK1 then phosphorylates STAT1 and STAT2 at amino acid tyrosine positions 701 and 690, respectively (Shuai et al., 1993, Improta et al., 1994). pSTAT1 Y701 and pSTAT2 Y690 dimerizes and binds to IRF9, forming the ISGF3 complex (Borden et al., 2007). ISGF3 then enters the nucleus and binds to the promoter region of the interferon-stimulated response element (ISRE), activating the transcription of hundreds of interferon-stimulated genes (Borden et al., 2007).

The signaling pathway following type III IFN binding runs through a similar pathway as the type I IFN signaling and shares many similar ISGs expressed in type I IFNs (Doyle et al., 2006). In contrast, type II IFN binding recruits only phosphorylated STAT1, forming a homodimer known as IFNγ-activation factor (GAF). The GAF complex then translocates into the nucleus and binds to the IFNγ-activated site (GAS) promoter element. Activation of GAS promoter elements induces a different set of ISGs (Schroder et al., 2004). The consensus sequence of ISRE and GAS elements are AGTTTCNNTTTCN and TTCCNGGAA, respectively (Michalska et al., 2018).

The main components of the JAK-STAT pathway will be explored further in the following sub-sections. These include its receptors IFNAR1 and IFNAR2; its receptor-associated kinase protein JAK1 and TYK2; two of the ISGF3 components STAT1 and STAT2; and relevant ISGs. IRF9 is also part of the ISGF3 complex. Since IRF9 is the main focus of this thesis, one separate section will be devoted to it.

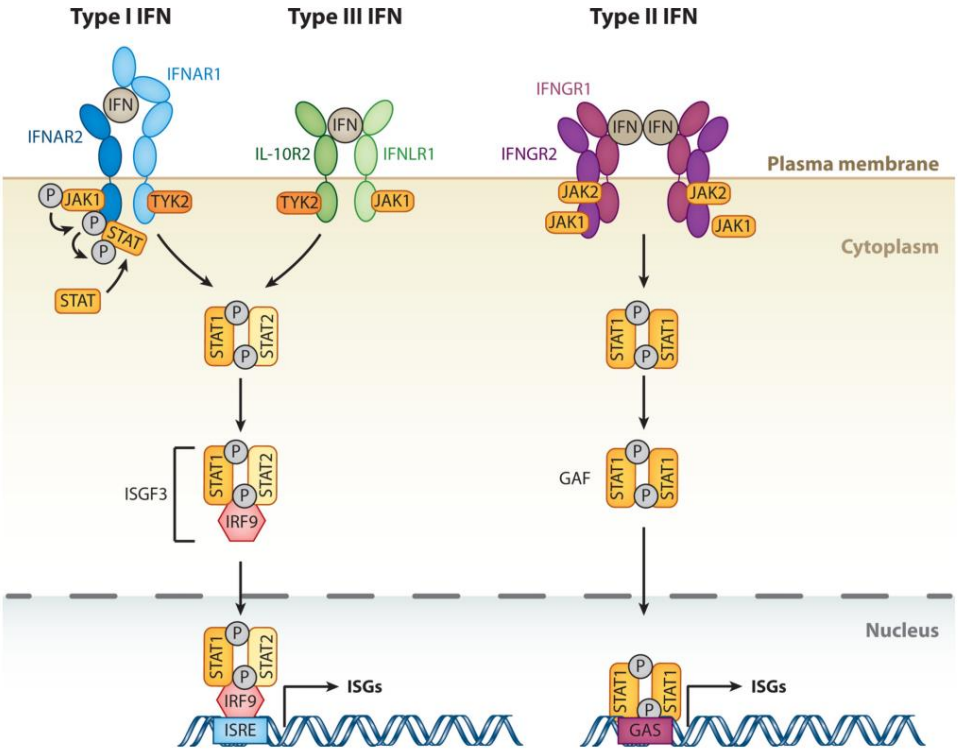

Figure 1.3. The JAK-STAT pathway in IFN response. Type I IFNs bind to IFNα receptor 1 (IFNAR1) and 2 (IFNAR2) heterodimers. Type II IFNs bind to IFNγ receptor 1 (IFNGR1) and 2 (IFNGR2). Type III IFNs bind to interleukin-10 receptor 2 (IL-10R2) and IFNλ receptor 1 (IFNLR1). The binding of type I and III IFNs to their respective receptors resulted in the phosphorylation of Janus kinase 1 and tyrosine kinase 2, resulting in the recruitment of STAT1 and STAT2. This triggers phosphorylation of STAT1 and STAT2, forming the heterodimer complex. pSTAT1-pSTAT2 then recruits IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) complex. Meanwhile, binding of type II IFNs triggers phosphorylation of STAT1 to form the IFNγ-activation factor (GAF). ISGF3 and GAF complex translocates to the nucleus and binds to ISRE and GAS promoter elements, respectively. The binding of both complexes to their respective promoter elements results in the expression of interferon-stimulated genes. The figure was taken from Schneider et al. (2014).

1.6.1. Interferon alpha receptors 1 and 2

The extracellular part of IFNAR1 is composed of four fibronectin III-like domains (SD1 to SD4) (Thomas et al., 2011). SD4 is not necessary for the formation of the ternary complex (Lamken et al., 2005). On IFNAR1, IFN binding occurs at the hinge between SD2 and SD3, with the SD1 act as a top "cap" (Thomas et al., 2011). The intracellular part of IFNAR1 is associated with tyrosine kinase 2 (TYK2). Phosphorylation of IFNAR1 on Tyr466 triggers recruitment of the unphosphorylated STAT2, which then gets phosphorylated by TYK2 (Yan et al., 1996). IFNAR1 is also phosphorylated at Ser535 and Ser539, resulting in its degradation through ubiquitination (Kumar et al., 2003). The low-binding affinity of IFNAR1 is important in functional plasticity, in contrast with the high-binding affinity of IFNAR2 (Thomas et al., 2011). Functional plasticity refers to the ability of the receptors to bind to different ligands of type I IFN that results in the activation and repression of various genes (Schreiber and Piehler, 2015).

The extracellular part of IFNAR2 is composed of two fibronectin III-like domains (D1 and D2) (Thomas et al., 2011). The intracellular part of IFNAR2 is associated with Janus kinase 1 (JAK1). On IFNAR2, type I IFN binding occurs at the D1 domain only (Figure 1.4). Tang et al. (2007) reported acetylation of IFNAR2 at lysine 399 and phosphorylation at serine 400 following type I IFN response. Both acetyl-Lys399 and phosphor-Ser400 were shown to be the docking site of IRF9 (Tang et al., 2007). Mutation of IFNAR2 at K399R lost the ability to bind to IRF9. Upon IFN binding, IFNAR1 and IFNAR2 form a ternary complex (Figure 1.4). Dimerization of IFNAR1 and IFNAR2 results in the activation by phosphorylation of the cytosolic-associated JAK1 and TYK2 kinases, which triggers a cascade of downstream phosphorylation

signaling mediated through STAT proteins (Schreiber and Piehler, 2015). It was thought that the formation of the stable ternary complex allows for proximity, which enables transphosphorylation between JAK1 and TYK2 (Thomas et al., 2011). The effects of JAK and TYK2 phosphorylation will be explored further in the following sub-section.

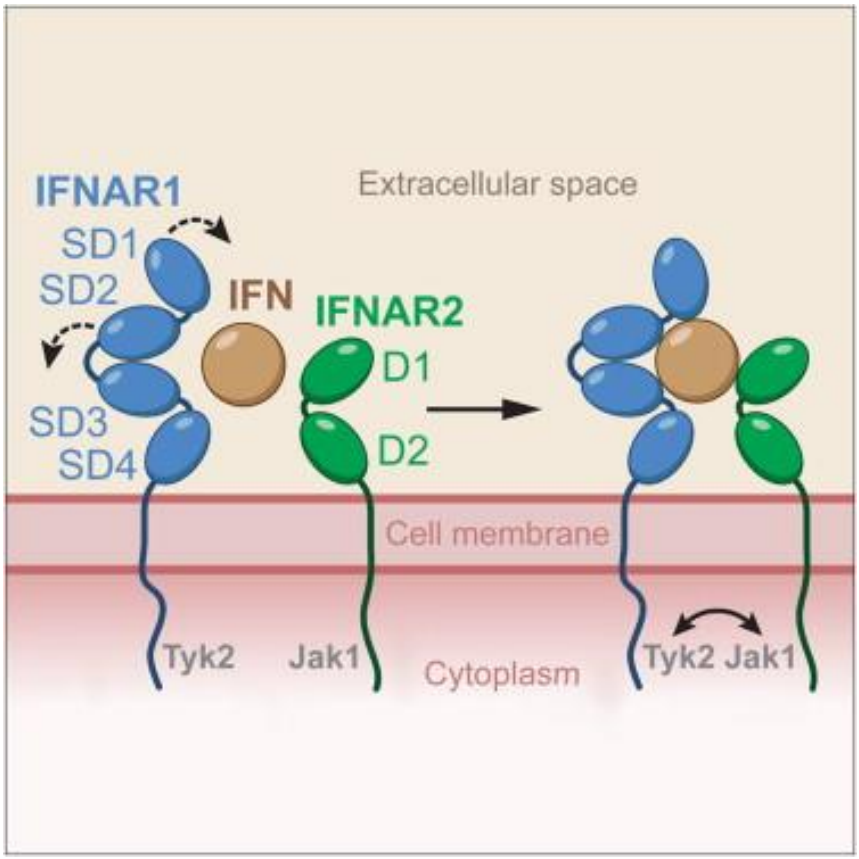

Figure 1.4. Schematic representation of IFNAR1 and IFNAR2 upon type I IFN binding. IFNAR1 has four extracellular domains: SD1 – SD4. TYK2 is associated with IFNAR1. IFNAR2 has two extracellular domains: D1 and D2. JAK1 is associated with IFNAR2. Upon IFN binding, a ternary complex of IFNAR1 and IFNAR2 is formed. The figure was taken from Thomas et al. (2011).

1.6.2. Janus kinase 1 and tyrosine kinase 2

There are four known Janus kinases; JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). While JAK1, JAK2, TYK2 are widely expressed, JAK3 is mainly expressed in hematopoietic cells (Ghoreschi et al., 2009). As illustrated in Figure 1.3, JAK1 is associated with IFNAR2, IFNLR1, IFNGR1; JAK2 is associated with IFNGR2, and TYK2 is associated with IFNAR1 and IL-10R2. All four JAK families of proteins share seven JAK-homology (JH1-JH7) domains (Figure 1.5). The enzymatically active kinase domain (Ki) resides in the JH1. The enzymatically inactive pseudokinase domain (ΨKi) resides in the JH2. The Src-homology 2 (SH2) domain resides in the JH3 and part of JH4. The four-point-one, ezrin, radixin, moesin (FERM) domain resides in the JH4, JH5, JH6, and JH7. The FERM mediates interaction with IFNARs (Ghoreschi et al., 2009). TYK2 was the first of the JAK family to be studied (Velazquez et al., 1992). TYK2 is critical in type I IFN but is not required in type II IFN signaling (Darnell et al., 1994). TYK2 was determined to be phosphorylated on tyrosine 1054 and 1055, following type I IFN treatment (Gauzzi et al., 1996). In an *in vitro* kinase assay, the TYK2 Y1054F/Y1055F substitutions failed to show phosphorylation activity under type I IFN response compared to wild-type TYK2 indicated to its role in the IFN response (Gauzzi et al., 1996). Truncation studies show JAK1 interacts at the inner side of the IFNAR2 via the 166 N-terminal amino acid (Usacheva et al., 2002). The domains of JH3 and JH5 also played a role in the IFNAR2 binding (Usacheva et al., 2002). As mentioned previously, type I IFN binding triggers the transphosphorylation of JAK1 and TYK2. This results in the phosphorylation of tyrosine residues within the intracellular receptor chains (Garrido-Trigo and Salas, 2020). The phosphorylated tyrosine residues eventually become STATs docking sites, mediated via its SH2 domains.

Figure 1.5. Schematic diagram of the Janus kinase family of protein. The Jak homology (JH1-JH7) domains are shared among all four JAKs (JAK1, JAK2, JAK3, and TYK2). The Four-point-one, Ezrin, Radixin, Moesin (FERM) of the N-terminal at the JH4-JH7 domains is responsible for stable interaction with membrane receptor motifs. The Src-homology 2 (SH2) domain is located at the JH3 and (part of) the JH4 domain. The enzymatically inactive ΨKi at JH2 is a pseudokinase domain. The enzymatically active Ki at JH1 of the C-terminal is a tyrosine kinase. This figure was adapted and modified from Schindler and Plumlee (2008).

1.6.3. Signal transducers and activators of transcription 1 and 2

There are seven STAT proteins known in humans: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. All STATs share seven structurally conserved domains (Figure 1.6). The amino-terminal domain (NH_2) promotes the formation of dimers between unphosphorylated STATs. The coiled-coil domain associates with regulatory proteins and is involved in the control of the nuclear-cytoplasmic shuttling. The DNA-binding domain (DBD) mediates binding to GAS elements. Except for STAT2, all activated STAT homodimers bind to GAS elements (Schindler and Plumlee, 2008). The linker domain is responsible for active dimerization to the DNA binding motif. The Src-homology 2 (SH2) domain mediates the recruitment of STAT to receptor chains and is the most conserved motif among STATs. The transcriptional activation domain (TAD) resides in the C-terminal region, and its sequence is the most highly variable among STAT family members.

There are two isoforms of STAT1, the larger STAT1a (91 kDa) isoform, and the splice variant STAT1b (84 kDa) isoform. STAT1b is missing 38 amino acid residues in the C-terminal region, therefore a truncated TAD (Schindler et al., 1992). Both isoforms restored the gene expression of type I IFNs, but only STAT1a restored the gene expression of type II IFNs (Müller et al., 1993). The *STAT1* gene is strongly activated by ISGF3 and GAF transcriptional activity in response to type I IFN and type II IFN signaling, respectively (Cheon and Stark, 2009). With a molecular weight of 113 kDa, STAT2 is the largest unit in the ISGF3 complex and the largest among STATs family members. STAT2 is only activated by type I and III IFNs (Blaszczyk et al., 2016). In contrast to STAT1 and other STATs, no STAT2 protein isoform have been found (Lim and Cao, 2006). Despite having a DBD and in contrast with other STATs, STAT2 does

not appear to stably bind to DNA (Bluyssen and Levy, 1997). Rather, an interaction with IRF9 is crucial for DNA-binding activity, while binding of STAT1 further stabilizes the ISGF3 complex through additional DNA contact (Martinez-Moczygemba et al., 1997). Interaction with IRF9 is mediated via the coiled-coil domain of STAT2 (Martinez-Moczygemba et al., 1997). A dominant nuclear export signal (NES) resides within the TAD of STAT2 (Banninger and Reich, 2004). The NES of STAT2 associates with nuclear export proteins to enable translocation of STAT2 protein from the nucleus to the cytoplasm. As such, the NES of STAT2 complements the nuclear localization signal in IRF9 to facilitate nuclear-cytoplasmic shuttling (Banninger and Reich, 2004).

Tyrosine phosphorylation is critical for the activation of STATs, as exemplified with a dedicated tyrosine activation domain (Lim and Cao, 2006). A major phosphorylation site in STAT2 is at Y690 within the TAD, which is critical for ISGF3 complex formation and STAT1 phosphorylation (Improta et al., 1994, Qureshi et al., 1996). Phosphorylation of Y690 STAT2 is in a homologous state to the phosphorylation of Y701 of STAT1, whereby both are occurring in response to type I IFN signaling (Improta et al., 1994). Additional phosphorylation sites have been reported on both STAT1 and STAT2. Phosphorylation of STAT2 at serine 734 was found to negatively regulate the type I IFN response (Steen et al., 2016). The antagonistic effect of STAT2 serine phosphorylation towards type I IFN response is in contrast with serine phosphorylation in STAT1 (Steen et al., 2016). For example, serine phosphorylation at 727 of STAT1 is required for ISGs transcriptional activation in response to type I IFN signaling (Pilz et al., 2003).

Figure 1.6. Schematic diagram of STAT family of protein domains. All seven STATs share seven structural domains. These are the amino-terminal domain (NH_2), the coiled-coil domain, the DNA-binding domain (DBD), the linker region (Link), the Src-homology domain 2 (SH2), the tyrosine activation domain (Y), and the transcriptional activation domain (TAD). This figure was adapted and modified from Schindler and Plumlee (2008).

1.6.4. Interferon-stimulated genes

ISGs are genes that are induced upon IFN signaling. Activation of ISGs transcription through the JAK-STAT pathway effectively renders the cell in an antiviral state. The components of ISGF3 are ISGs themselves (Schneider et al., 2014). This provides a means for amplification of IFN response (Borden et al., 2007) and at the same time regulates its expression as some of the ISGs, such as USP18, act as a negative regulator of IFN response. In this thesis work, only five ISGs (USP18, MxA, OAS1, PKR, and IL6) were included in qPCR data analysis. These were selected due to their significant roles in regulating the innate immune response. For a comprehensive compendium of ISGs, the reader is directed to this website; http://www.interferome.org/interferome/home.jspx.

1.6.4(a) Ubiquitin-specific peptidase 18

Ubiquitin-specific peptidase 18 (USP18) – also known as UBP43 – is a cysteine protease of the ubiquitin-specific protease family of enzymes (Malakhov et al., 2002). USP18 removes the ubiquitin-like protein interferon-stimulated gene 15 (ISG15) from target proteins (Malakhov et al., 2002). USP18 binds to IFNAR2 to negatively regulates IFN signaling, and this suppression mechanism is independent of its ISG15 peptidase activity (Malakhova et al., 2006). Specific binding of USP18 to IFNAR2 induces conformational changes to IFNAR2 that prevent the low-affinity IFNα from eliciting an immune response (François-Newton et al., 2011). In contrast, the high-affinity IFNβ is still able to bind to IFNARs and thus initiate IFN signaling. Because of the specific binding of USP18 to IFNAR2, inhibition by USP18 is restricted to type I IFN signaling (François-Newton et al., 2011, Schneider et al., 2014). A further study conducted by Arimoto et al. (2017) revealed that the USP18 inhibition of IFNAR2

required STAT2 to act as an adaptor molecule. Interfering with USP18 and STAT2 interaction suppresses the USP18-mediated inhibition of type I IFN signaling. It was shown that the coiled-coil domain (CCD) and the DNA-binding domain (DBD) of STAT2 are critical for interaction with USP18. The authors also noted that the CCD and DBD of STAT2 are both critical for interaction with IFNAR2 as well, demonstrating the link between the STAT2-USP18-IFNAR2.

1.6.4(b) Human myxovirus resistance protein 1

The human myxovirus resistance protein 1 (MxA) is one of the main antiviral effectors induced by type I IFN and type III IFN, but not type II IFN response (Holzinger et al., 2007). A viral ribonucleoprotein complex (vRNP) is consist of nucleoprotein (NP) molecules and one RNA-dependent RNA polymerase (RdRp) complex (which contains polymerase basic protein 1 (PB1) and 2 (PB2), and polymerase acidic (PA) protein) (Verhelst et al., 2012). In an influenza A virus infection model, MxA was shown to inhibit viral infection by disrupting the assembly of vRNP, more specifically inhibiting the interaction between PB2 and NP proteins (Verhelst et al., 2012).

1.6.4(c) 2’-5’-oligoadenylate synthetase 1

2’-5’-oligoadenylate synthetase 1 (OAS1) triggers the dimerization of latent ribonuclease L enzyme (Cole et al., 1996, Silverman, 2007). Active RNase L degrades viral RNA, thereby blocking further infection (Silverman, 2007). OAS1 was known to be regulated by un-phosphorylated ISGF3 (U-ISGF3) (Cheon et al., 2013), and this was further shown in hepatitis C virus (HCV)-infected hepatocytes (Sung et al., 2015).

1.6.4(d) Protein kinase R

Protein kinase R (PKR) is a double-stranded RNA-dependent serine/threonine kinase that phosphorylates the α-subunit of eukaryotic translation initiation factor 2 α (eIF2α) upon activation (Sadler and Williams, 2007). PKR is activated as a homodimer following the binding to a viral dsRNA via its dsRNA-binding motifs (Feng et al., 1992). The phosphorylation of eIF2α – as a result of PKR activation – leads to the inhibition of translation, thereby limiting viral protein synthesis (Rojas et al., 2010). Unlike OAS1, PKR is regulated only by ISGF3 in HCV-infected hepatocytes (Sung et al., 2015).

1.6.4(e) Interleukin 6

Interleukin 6 (IL6) has been known as a pro-inflammatory cytokine regulating apoptosis, proliferation, angiogenesis, and differentiation (Tanaka et al., 2014). IL6 was found to be upregulated following IFNβ inducement (Nan et al., 2018). It was hypothesized that upregulation of *IL6* gene expression is facilitated by the synergistic interaction between IRF9 and STAT2, whereby STAT2 acted as a bridge that stabilizes a complex with IRF9 and p65 resulting in the increase of IL6 transcription (Nan et al., 2018).

1.7. Interferon regulatory factors

Interferon regulatory factors (IRFs) – essentially transcription factors – are proteins that mediate the transcription of virus-, bacteria-, and IFN-induced signaling pathways (Paun and Pitha, 2007). There are eleven IRFs found in vertebrates and nine in humans. These are aptly numbered from 1 – 11. Only the tenth IRF (IRF10) and eleventh IRF (IRF11) are not found in humans (Huang et al., 2010). Additionally, homologs of