FUNCTIONAL EVALUATION OF FLAG-STAT1 IN U3A CELLS

by

TEY LEE HUNG

ADVANCED MEDICAL AND DENTAL INSTITUTE (AMDI)

UNIVERSITI SAINS MALAYSIA

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LIST OF ABBREVIATIONS

µg	Micro gram
µl	Microliter
ADAR1	Adenosine deaminase that act on RNA
APS	Ammonium persulfate
BME	Beta-mercaptoethanol
bp	Base pair
BSA	bovine serum albumin
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CO_2	Carbon dioxide
DBD	DNA binding domain
DMSO	Dimethyl suloxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dsRNA	double-stranded RNA
EDTA	Ethylenediaminetetra acetic acid
ET	Essential thrombocythemia
FD	Fast Digest
g	gram
g	gravity
GAS	IFN-gamma-activated sequence

HSV-1	Herpes simplex virus type 1
IFN	Interferon
IFNAR	Type I IFN to interferon alpha receptors
IL-12	Interleukin 12
IL-6	Interleukin 6
IRF	IFN regulatory factor
IRF9	Interferon regulatory factor 9
ISG	Interferon stimulated genes
IU	International units
JAK	Janus kinase
JH	JAK homology
kb	Kilo base
KCl	Potassium chloride
l	Liter
LB	Luria Bertani media
M	Molarity
MDA5	Melanoma differentiated-associated protein 5
MERS	Middle East respiratory syndrome
MKH_2HPO_4	Monopotassium phosphate
ml	Mililiter
mM	Milimolar
MyD88	Myeloid differentiation primary response 88
Na_2HPO_4	Sodium phosphate
NaCl	Sodium chloride
ng	Nano gram

NOD	Nucleotide-binding oligomerization domain
OD	Optical density
p300-CBP	E1A binding protein p300-CREB binding protein
PAGE	polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKR	Interferon-induces, dsRNA-activated protein kinase
PMF	Primary myelofibrosis
PRRs	Pattern-recognition receptors
PV	Polycythemia vera
PVDF	Polyvinylidene difluoride
RIG	Retinoic acid-inducible gene
RNA	Ribonucleic acid
SARS	Severe acute respiratory syndrome
SDS	Sodium dodecyl sulfate
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
TAD	Transcription activation domain
TAE	Tris-Acetate-EDTA
TBST	Tris-buffered Saline with Tween
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptors
TRIF	TIR-domain-containing adapter-inducing interferon-β
TYK2	Tyrosine kinase 2

UV	Ultraviolet
V	Volt
v/v	Volume per volume
VSV	Vesicular stomatitis virus

PENILAIAN FUNGSI FLAG-STAT1B DI SEL U3A

ABSTRAK

Wabak penyakit seperti SARS, Ebola dan Zika bukan sahaja membawa kesan negatif kepada ekonomi dan sosial global, juga kesihatan manusia dan nyawa yang berharga. Peranan imuniti adalah penting untuk perlindungan kesihatan. Interferon (IFN) jenis I yang terdiri daripada IFN-α dan IFN-β dihasilkan oleh imuniti semula jadi semasa jangkitan virus melalui interaksi host-patogen. IFN mencetuskan respons IFN yang menyumbang kepada akitivi antivirus melalui laluan isyarat intraselular *Janus kinase-signal transducer and activator of transcription (JAK-STAT)* termasuk komponen utamanya, STAT1. Manusia STAT1 terdapat dua isoform iaitu STAT1A yang panjang sepenuh dan variasi split STAT1B yang kekurangan sebahagian domain pengaktifan transkripsi. Kepentingan STAT1 dalam imuniti telah terbukti oleh tikus fenotip *Stat1*$^{-/-}$ yang lebih terdedah kepada jangkitan virus. Permahaman yang lebih lanjut terhadap laluan intraselular JAK-STAT dan STAT1 dengan respons interferon mampu menambah pengetahuan mengenai interaksi host-patogen dalam imuniti semula jadi. Ini mendorong projek kami sebagai kajian awal yang bertujuan menyiasat keupayaan rekombinan Flag-STAT1B di sel U3A dalam mengembalikan respons IFN melalui laluan JAK-STAT. Plasmid rekombinan p3xFlag-STAT1B yang dihasilkan telah menghantar kepada sel U3A.

IFN-β telah digunakan untuk mengekspresikan sel-sel. Penyelidikan ekspresi STAT1 dan interferon stimulated genes (ISGs) di sel U3A dilengkapi Flag-STAT1B telah dilaksanakan melalui immunoblot dan ImageJ digunakan untuk analisis kuantitatif. Kawalan posited adalah sel 2fTGH (STAT1A dan STAT1B) yang mendapat laluan JAK-STAT yang sempurna. Ekspresi ISGs dalam kajian ini menunjukkan pembaikan laluan isyarat JAK-STAT. Tetapi, ekspresi ISGs tidak dikompensasikan sepenuhnya kalau berbanding dengan sel 2fTGH. Hasil kajian ini adalah sel U3A dilengkapi STAT1B boleh memulihkan laluan isyarat JAK-STAT dengan sebahagian ekspresi ISGs semasa IFN-β dibagikan. Kajian ini mampu membawa kepada kemungkinan baharu untuk mekanisme STAT1 isoforms dalam laluan isyarat JAK-STAT dan respons IFN.

FUNCTIONAL EVALUATION OF FLAG-STAT1 IN U3A CELLS

ABSTRACT

Viral infections have had massive socio-economic impact, at the costs of human health and invaluable lives. However, effective and broad spectrum protection against viruses is still lacking despite best efforts. Antiviral immune defense is crucial for host protection in the event of viral infections. Type I interferons (IFN), are key mediators for antiviral immunity, and are induced following recognition of viral-associated molecular patterns. Type I IFNs released to the extracellular matrix then help establish antiviral state at the cellular level in autocrine and paracrine manner. Signaling by Type I IFNs is transduced via Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, leading to upregulation of interferon stimulated genes. STAT1 is a key component of the JAK-STAT signaling pathway, where two major isoforms: STAT1A and STAT1B are naturally expressed during Type I IFN response. STAT1 is essential in antiviral immunity as *Stat1*$^{-/-}$ mice are more susceptible to virus infection. However, the possibly of distinct roles of STAT1 isoforms in antiviral immunity warrants further investigation for more comprehensive understanding of the JAK-STAT pathway towards developing protection against viruses. This project thereby serves as groundwork in preparation for future research on STAT1 isoforms and their cellular functions. We aim to establish an essay

involving the expression of a functional recombinant Flag-STAT1B in U3A cells, a cell line lacking endogenous STAT1 that is derived from 2fTGH cells. Therefore, expression plasmid for recombinant Flag-STAT1B was constructed by sub-cloning. The expression plasmid was transfected into U3A cells and Flag-STAT1B was successfully expressed. When the transfected cells were challenged with IFN-β, recombinant Flag-STAT1B partially restored the previously dysfunctional JAK-STAT signaling pathway in U3A cells. Protein expression levels of several interferon stimulated genes were semi-quantitatively analyzed through immunoblotting and ImageJ. From our results, overexpression of STAT1B without STAT1A in U3A cells could not fully restore the JAK-STAT signaling pathway. While more research is necessary, our work suggest that the different STAT1 isoforms may have overlapping but yet distinct roles at the cellular level during Type I interferon response. Further investigation may lead to novel understanding about the mechanism of STAT1 isoforms and their impact on host antiviral immunity.

CHAPTER 1: INTRODUCTION

1.1 Background

Over the past two decade, viral outbreaks such as Zika, Ebola, Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS), avian flu virus has dominated headlines and captured the public attention. SARS epidemic outbreak origin from southern China have spread globally and over 8000 cases had reported in 37 countries from 2002-2013. Avian influenza virus have transmit from Asia to Africa and Europe since 2003 and re-emerging in China in 2017 involving avian influenza A(H7N9) virus. MERS cause by MERS coronavirus first reported in Saudi Arabia at 2012 had spread to several countries included Malaysia. Recent Ebola virus outbreak in West Africa and Zika virus disease outbreak that causes fetal neurological disease (World Health Organization, 2017; Centers for Disease Control and Prevention, 2017).

On the other hand, despite concerted effort from the biomedical community, proper cure from disease chronic viral infections like acquired immune deficiency syndrome and Hepatitis has not been found, though disease management has been improved. All of these showed the inadequate understanding of viral-host interaction. To prevent future emerging viral outbreaks and to manage persistent virus infections, it is necessary for further research on both viruses and human immune system, and their interactions.

Immunity system in human can be generally divided into innate and adaptive immunity. Innate immune response is less specific against pathogens. Cellular components of innate immunity are ready prior to infection and can be activated rapidly for onset of immune defense. On the other hand, adaptive immune response is slower but more specific, as

lymphocytes activation and antibody production are driven by encountered antigen. Both innate and adaptive immune system are communicative and heavily interlinked (Koyama et al., 2008).

1.2 Innate Immunity

Host innate immunity plays a vital role as rapid first line defense against viral infection before the slow onset of adaptive immune system. Host innate immunity on self can distinguish between "self" and "non-self" biomolecules. Immune cells involved innate immunity including natural killer cells, phagocytic cells and inflammatory cells. An innate immunity response is elicited upon recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) (Noakes and Michaelis, 2013). Toll-like receptors (TLRs) are either expressed on cell membrane or associated with intracellular vesicles that enable both extracellular and cytoplasmic recognition of the PAMPs. Retinoic acid-inducible gene I-like receptors (RIG-I-like receptors) and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) are involved in cytoplasmic PAMPs detection (Nakhaei et al., 2009). Distinct PAMPs derived from viruses, bacteria, mycobacteria, fungi and parasites are detected by specific PRRs. For example, viral DNA is recognized by TLR9 whereas viral RNA cache detected by TLR3, TLR7 and TLR8. Activation of PRRs will then leads to signaling cascade adaptor proteins and induce Type I Interferon (IFN) or pro-inflammatory cytokines production (Kawai and Akira, 2010, Nguyen et al., 2016). Type I IFN then induce IFN response via Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway.

1.3 Interferon (IFN)

IFN was first discovered in 1957, based on its ability to interfere with the life cycle and replication of influenza virus in chick embryo cells (Isaacs and Lindenmann, 1957). After that, subsequent studies revealed many different types of IFNs. IFNs have antiviral properties, plays a role in bridging innate and adaptive immunity and regulate cell cycle (Mossman, 2011). Human IFN could be classified into 3 classes: Type I, Type II and Type III. Type I IFN comprised of IFN-α and IFN-β are the principle cytokines mediating antiviral response and can be expressed by all nucleated cells upon viral infection (Koyama et al., 2008). IFN-γ is the only Type II IFN, secreted by natural killer cells and T cells responsible for immunoregulatory in innate and adaptive immunity (Saha et al., 2010). Type III IFNs, such as IFN-λ1, IFN-λ2 and IFN-λ3 has been discovery recently to possess antiviral properties as well. Induction of IFN-λ are independent on IFN regulatory factor (IRF) and nuclear factor-kappaβ (Iversen and Paludan, 2010). IFNs induce interferon stimulated genes (ISGs) via JAK-STAT signaling pathway.

1.3.1 Induced expression of Type I interferon

Distinct viral constituents are recognized by various PRRs including TLRs and RIG-I-like receptors (Figure 1.1). TLR3 recognizes extracellular viral double-stranded RNA (dsRNA), TLR7 and TLR8 recognizes viral single-stranded RNA (ssRNA) whereas TLR9 detects viral DNA contains CpG-motifs (Blasius and Beutler, 2011, Kawai and Akira, 2011). Activated TLRs can induce Type I IFN production via signaling cascade involving TIR-domain-containing adapter-inducing interferon-β (TRIF) and myeloid differentiation

primary response 88 (MyD88) (Piras and Selvarajoo, 2014). Cytoplasmic viral ssRNA and dsRNA can also be recognized by RIG-I-like receptors such as retinoic acid-inducible gene I receptors (RIG-I) and melanoma differentiated-associated protein 5 (MDA5), eventually leading to IFN-α/β expression via downstream cascade involving mitochondrial antiviral signaling protein (Dixit and Kagan, 2013).

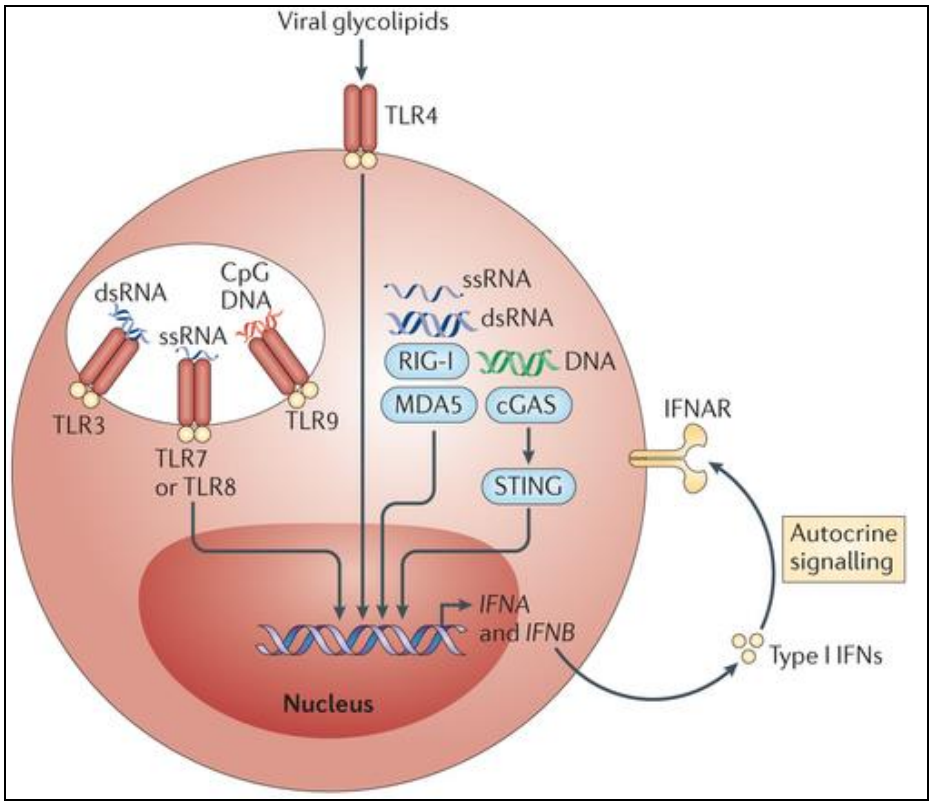

Figure 1.1: A schematic diagram of viral nucleic acids recognition by pattern recognition receptors. Viral nucleic acid can be detected by Toll-like receptors, RIG-I like receptors, NOD-like receptors and intracellular DNA receptors. Detection of viral constituents induces Type I IFN production and response to viral infection. [Figure is from (Crouse et al., 2015)].

1.3.2 Type I interferon response

ISGs is quiescent and has low basal expression in general for unstimulated cells. Expression of ISGs can be induced by Type I IFN via JAK-STAT intracellular signaling pathway (Figure 1.2). Collectively, expression of ISGs with establish antiviral response in host cells (Leaman et al., 1998). Type I IFN to interferon alpha receptors (IFNARs) receptors for Type I IFN ligand composed of IFNAR1 and IFNAR2 subunits, associated with kinases tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) respectively. Binding of IFNARs will trigger dimerization of receptors that further activates JAK1 and TYK2. Activated JAK1 and TYK2 then phosphorylate tyrosine residue on IFNARs and in turn mediate the recruitment of signal transducer and activator of transcriptions 1 (STAT1) and signal transducer and activator of transcriptions 2 (STAT2) via their SH2 domains (Schindler et al., 1992, Darnell et al., 1994). Next, phosphorylation of the recruited STAT1 and STAT2 will lead to formation of STAT1/STAT2 heterodimers. STAT1/STAT2 dimers associated with interferon regulatory factor 9 (IRF9) to form interferon stimulatory gene factor 3 (ISGF-3), a potent transcription factor that translocates into the nucleus. ISGFs binds to IFN-stimulated response elements (ISRE; AGTTTNNNTTTCC) in the promoter region of many ISGs, promoting transcription of those ISG (Schindler et al., 1992, Schindler et al., 2007). ISGs included *ADAR1, PKR, MX1, OAS1* and also gene involved in JAK-STAT signaling pathway such as *STAT2* and *IRF9*.

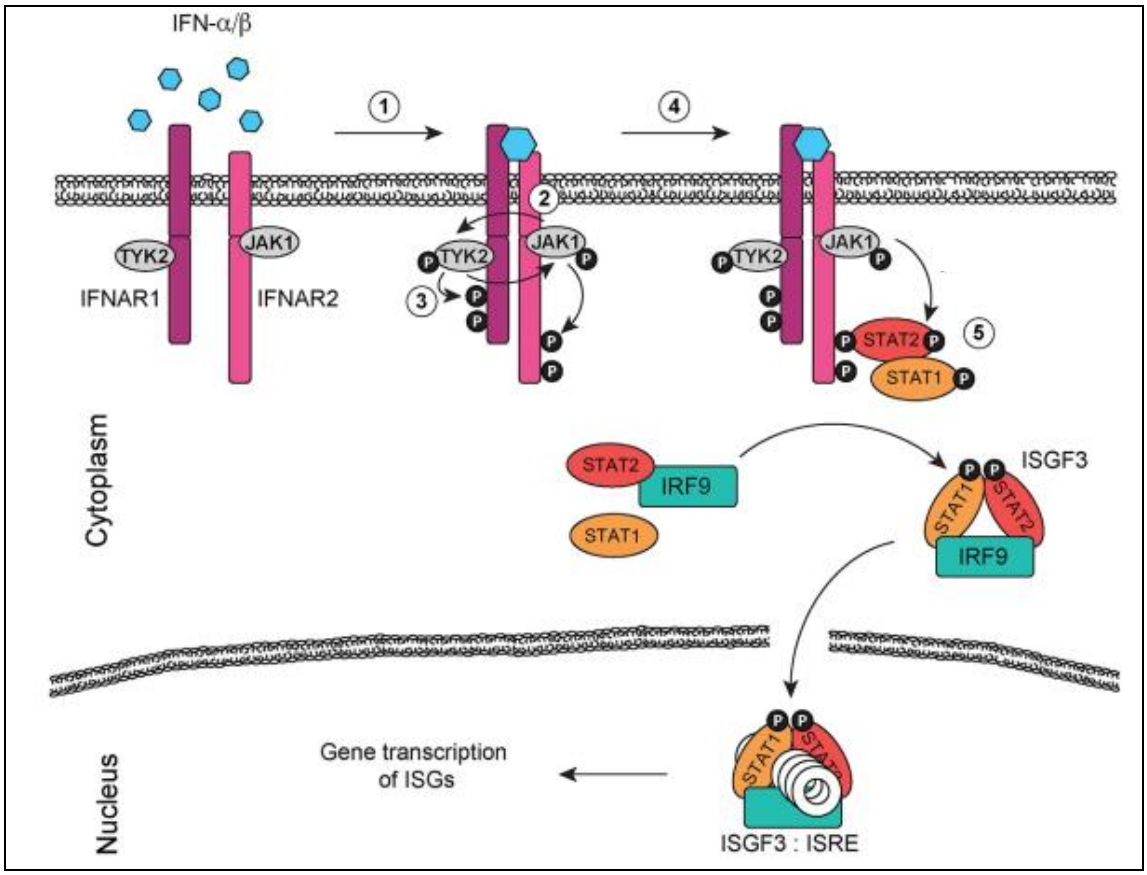

Figure 1.2: Schematic diagram depicting Type I IFN response through JAK-STAT signaling pathway. (1) Binding of Type I IFN stimulates dimerization of IFNAR1 and IFNAR2. (2) Activated IFNAR transphosphorylate TYK2 and JAK1. (3) Phosphorylated Janus kinases (JAKs) in turn phosphorylate tyrosine residues on IFNARs, (4) which provide docking site to STAT2. (5) STAT2 subsequently phosphorylated by JAKs. It recruits and activates STAT1 leading to STAT1/STAT2 dimerization. (6) STAT1/STAT2 dimers together with IRF9 formed ISGF3, translocate into nucleus and bind to IFN-stimulated response elements (ISRE) for ISGs expression. [Figure is from (Suprunenko and Hofer, 2016)].

1.4 JAK-STAT signaling

The JAK-STAT is an important intracellular signaling pathway in response to various cytokines. Different combination of JAKs and signal transducer and activator of transcriptions (STATs) are activated in response to different ligands (Figure 1.3). For example, IFN-R family activates STAT1, STAT2, STAT3 and STAT5 whereas IL-3R family only activates STAT5 (Figure 1.3). The pathway transduces information from the extracellular environment to the nucleus by STATs dimer translocation and stimulates corresponding gene expression in accordance to the extracellular signal. As such, JAK-STAT signaling is vital for fundamental cellular process such as cell proliferation, differentiation, apoptosis, immunity development, hematopoiesis and homeostasis (Rawlings et al., 2004, Pfitzner et al., 2004, Song et al., 2011, Coskun et al., 2013).

1.4.1 JAKs

The four members of JAKs family, JAK1, JAK2, JAK3 and TYK2 range in sizes between 120-140 kDa. JAKs are composed of seven conserved JAK homology (JH) domains (Figure 1.4A), where JH1 and JH2 are kinase domains responsible for phosphorylation of receptors and STATs. Meanwhile, the N-terminal domains (JH4-JH7) mediates interaction with receptors and JH3 binding to phosphorylated tyrosine (Schindler et al., 2007, Coskun et al., 2013).

1.4.2 STATs

STATs family comprised of seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. Sizes of STATs range from 750-900 amino acids and consist of seven conserved domains (Figure 1.4B). All the conserved STAT1 domains are known for distinct functions, where: the N-terminal (NH_2) domain is important in STATs dimerization at inactive state; the hydrophilic surface of coiled-coil domain is critical for regulators binding; the DNA-binding domain determines the DNA binding specificity in the nucleus; the SH2 domain crucial in STAT recruitment to the activated receptors and dimerization; the tyrosine activation domain at around 700 amino acid residues; and C-terminal transcription activation domain (TAD) involved in driving transcription through association with transcriptional regulators (Hoey and Schindler, 1998, Schindler et al., 2007).

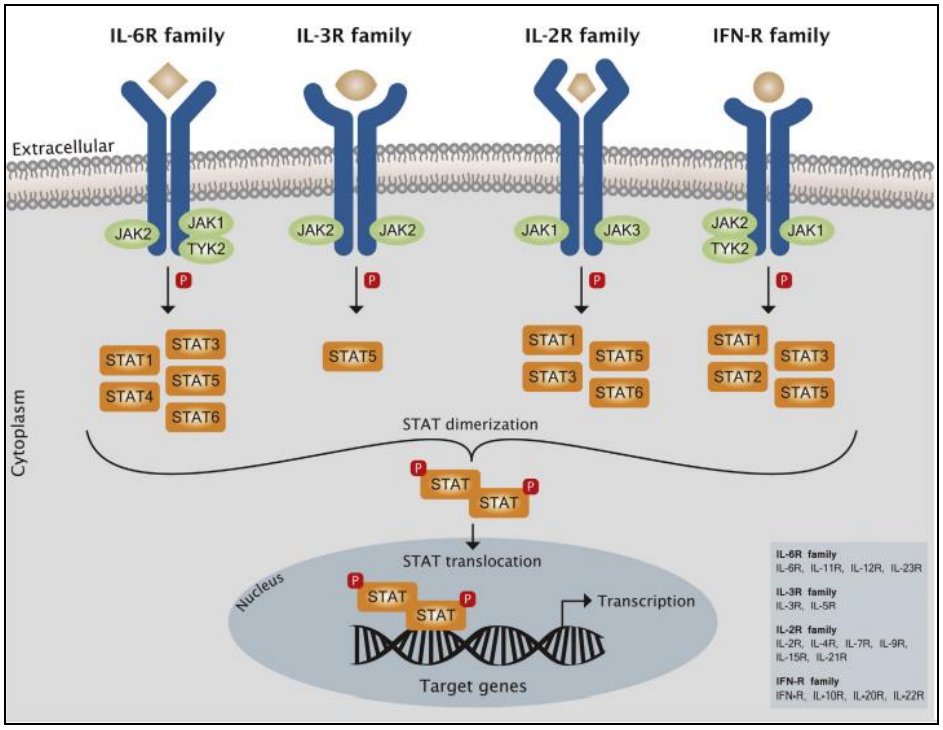

Figure 1.3: JAK-STAT signaling pathway. Different JAKs and STATs are involved in mediating signals from different cytokines. STATs dimer or associated with regulator translocate into nucleus, binds to IFN-stimulated response elements (ISRE) and simulate interferon stimulated genes (ISGs) transcription. [Figure is from (Coskun et al., 2013)].

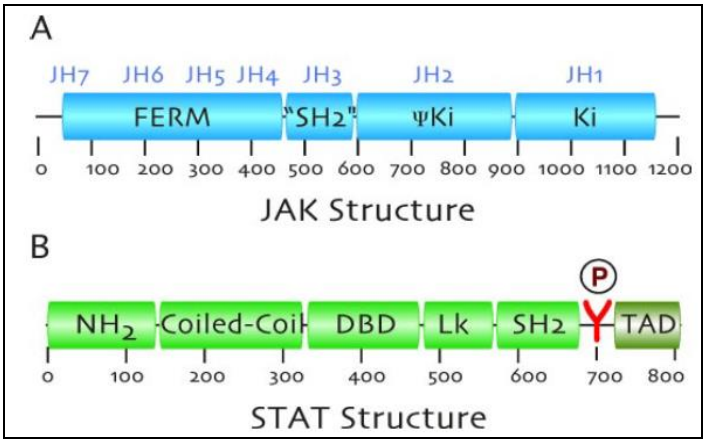

Figure 1.4: Conserved domain for JAKs and STATs. (A) General structure of JAKs, consists of seven JH domains, JH1-7. (B) Structure of STAT with seven conserved domains including amino-terminal (NH_2) domain, coiled-coil domain, DNA binding domain (DBD), linker domain (LK), SH2 domain, tyrosine activation domain and transcription activation domain (TAD). [Figure is from (Schindler et al., 2007)].

1.5 STAT1

Transcriptional activation studies following exposure to IFN established a multitude of expressed genes (ISGs). A consensus sequence (ISRE) was discovered at the promoter region of ISGs, and the corresponding transcriptional factor was identified as ISGF-3. Protein purification revealed that ISGF-3 is a protein complex with four proteins with sizes 113, 91, 84, 48 kDa, respectively. The proteins were named subsequently STAT1A (p91), STAT1B (p84) and STAT2 (p113) due to their dual function as signal transducers and activators of transcription, whereas p48 was later identified as IRF9. STAT1 and STAT2 were the first identified STAT proteins (Shuai et al., 1992, Schindler et al., 1992).

STAT1 activation by phosphorylation at tyrosine 701 can be induced by various ligands such as IFN, platelet-derived growth factor, IL-6 and IL-12. Activated STAT1 will then form heterodimer under most circumstances, except in response to IFN-γ that leads to STAT1 homodimerisation in turn binding to IFN-gamma-activated sequence (GAS) in the nucleus (Wegenka et al., 1994). There are two natural isoforms of STAT1, the full length STAT1A (750 amino acids) and smaller split variant STAT1B (712 amino acids). STAT1B lacks the last 38 residues within the TAD, compared to STAT1A (Figure 1.5). The variation arise from alternative splicing event, where *STAT1A* mRNA has 25 exons while *STAT1B* mRNA only has 23 spliced exons (Darnell et al., 1994, Chapgier et al., 2009). STAT1B that lacks part of the TAD is unable to bind to E1A binding protein p300-CREB binding protein (p300-CBP) coactivator family (Zhang et al., 1996).

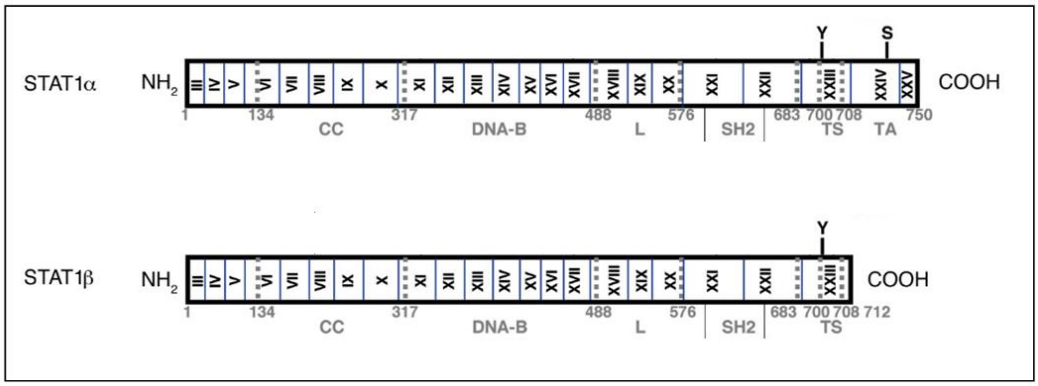

Figure 1.5: Schematic diagram depicting of STAT1A and STAT1B. [Figure is from (Chapgier et al., 2009)].

1.5.1 STAT1 in innate and adaptive immunity

STAT1 is one of the key mediators in IFN response for antiviral immunity. The importance of STAT1 in immunity is apparent from the analysis of *in vivo* STAT1 deficient mice or by clinical cases. *Stat1*$^{-/-}$ mice showed compromised innate immune response and are susceptible to viral infection such as vesicular stomatitis virus (VSV) infection and herpes simplex virus type 1 (HSV-1), compared to *Stat1*$^{+/+}$ mice (Durbin et al., 1996, Pasieka et al., 2008). Patients with fatal combined immunodeficiency that displayed progressive destruction of lymphocytes and autoimmune disorders has been associated with heterozygous mutation of *STAT1* with reduced STAT1 expression level (Sharfe et al., 2013). In another study, heterozygous *STAT1* gain-of-function mutation is associated with development of systemic or atypical viral infection (38%), chronic mucocutaneous candidiasis (98%) and bacterial infections (74%) (Toubiana et al., 2016).

1.5.2 STAT1 in haematopoiesis

Haematopoiesis or haemopoiesis describe the formation of blood cells, including formation of erythrocytes (erythropoiesis), platelet (thrombocytosis), lymphocytes (lymphocytosis) and granulocytes and monocytes (myelopoiesis). Site of haematopoiesis varies at different stage of growth, including yolk sac, liver, spleen and bone marrow at fetal state; all bone marrows during infancy; and at bone marrow of only central bones during adulthood. Besides, thymus, spleen and lymph nodes are also involve in maturation and proliferation of blood cells. All blood cells are derived from pluripotent haematopoietic stem cell (HSC). Differentiation, maturation and proliferation of different lineage progenitor cells and mature blood cells are regulated by haematopoietic growth factors (Hoffbrand and Moss, 2011).

STAT1 in hematopoiesis regulation have been demonstrated in various STAT1 deficient models. STAT1 deficient mice showed 1.6 fold reduction in of total body colony forming unit-erythroid, delayed differentiation and increased apoptosis of early erythroblasts in bone marrow (Halupa et al., 2005). The importance of STAT1A and STAT1B in zebrafish embryonic hematopoiesis was studied by Song, et al. (2011), which revealed distinct roles for the isoforms in hematopoiesis. Knockdown of *stat1b* in zebrafish embryo led to increased *scl* (hematopoietic progenitor marker), denser *gata1* (erythrocyte marker) and increased hemoglobin-producing cells compared to *stat1a* knockdown and control without *stat1* knockdown (Song et al., 2011).

1.5.3 STAT1 in myeloproliferative neoplasms (MPNs)

Myeloproliferative neoplasms (MPNs) are hematological malignancies characterized with overproduction of blood cells either erythrocyte, leukocyte, thrombocyte or granulocyte which can brings significant morbidity and mortality (Tefferi and Vardiman, 2008). MPN patient suffer from fatigue, pruritus, night sweats and bone pain (Mesa et al., 2007).

Hematopoietic tumor disorders classified under MPNs include chronic myelogenous leukemia (*BCR-ABL1*-positive), chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia (not otherwise specified), myeloproliferative neoplasms (unclassifiable) and mastocytosis (Vardiman et al., 2009). Mutation or genetic changes in hematopoietic stem cell is the main reason for MPNs, by causing gain of multilineage differentiation ability and myelopoiesis. *JAK2*, *MPL*, *TET2*, *ASCL1*, *IDH1*, *IDH2*, *CBL*, *IKZF1*, *LNK* and *EZH2* are known mutation associated with MPNs (Tefferi and Vainchenker, 2011). In PV, ET and PMF, the most prevalent mutation recognized in these three MPNs is *JAK2V617F*, found in about 96% of PV and 55% of ET and 65% of PMF (Tefferi, 2010).

High IFN and STAT1 plays a role in promoting ET phenotype in *JAK2V617F* positive MPNs. *JAK2V617F* mice displayed higher IFN level than non-transgenic mice (Chen et al., 2010), and *JAK2V617F*/*Stat1*$^{+/+}$ mice had higher megakaryopoiesis and reduced erythropoiesis compared to *JAK2V617F*/*Stat1*$^{-/-}$ mice (Duek et al., 2014). However, the molecular mechanism of IFN/STAT1 in promoting ET phenotype is not well understood.

1.5.4 STAT1 in cancer

Majority of the studies on the role of STAT1 in cancer was done using animal models. STAT1 is thought to be tumour suppressive, but recent a study revealed that STAT1 may promote tumourigenesis (Meissl et al., 2015). The expression of STAT1 leads to favorable prognosis in several cancers, such as hepatocellular carcinoma, breast cancer, colorectal carcinoma and pancreatic cancer (Gordziel et al., 2013, Sun et al., 2014). Overexpression of STAT1 was found to inhibit proliferation of HepG2 cells (human hepatocellular cancer cells) (Chen et al., 2013) while *Stat1*$^{-/-}$ female mice develop spontaneous mammary adenocarcinomas . Furthermore, *STAT1*$^{-/-}$ mammary cancer cells underwent apoptosis after introduction of functional STAT1 (Chan et al., 2012).

However, STAT1 may also confer promotion to tumour cells. CD95 stimulation of cancer cells leads to increased production of Type I IFN, which causes STAT1 activation and results in cancer therapy resistance. *STAT1* knockdown or knockout cells have reduced capability in sustaining the stemness of cancer cells by CD95 ligand and Type I IFN (Qadir et al., 2017). In addition, overexpression of STAT1 was found to impair apoptosis via IL-6 and IL-8 production (Efimova et al., 2009); whereas increased STAT1 and CD74 in triple-negative breast cancer was shown to promote tumour invasion and metastasis (Greenwood et al., 2012).

1.6 Aim of study

Our lab is interested in the JAK-STAT signaling pathway during Type I IFN response. For that, we have acquired 2fTGH cells and its derivative U2A, U3A, U5A, U6A cells lines (deficient in IRF9, STAT1, STAT2 and IFNAR, respectively from Prof. George Stark (Cleveland clinic, USA). My project is a preliminary study aiming to determine the feasibility of using U3A cells and restored recombinant STAT1 as a model system for STAT1 research. In particular, I need to determine if recombinant STAT1 protein functions similarly to endogenous STAT1 protein during Type I IFN response.

1.6.1 General objective

Plasmid construction and to characterize the function of overexpressed Flag-STAT1B in restoring the JAK-STAT signaling pathway in response to Type I IFN, using U3A cells.

1.6.2 Specific objectives

1) To construct recombinant plasmid expressing Flag-STAT1B by sub-cloning *STAT1B* into p3xFlag-CMV^{TM}-7.1 vector plasmid.
2) To induce Flag-STAT1B overexpression in U3A cells.
3) To evaluate the restoration of JAK-STAT signaling pathway in U3A cells, expressing Flag-STAT1B when induced by IFN-β.

CHAPTER 2: MATERIALS AND METHODS

2.1 Preparation of expression plasmids

2.1.1 *Escherichia coli*

2.1.1.1 *Escherichia coli* strain

Genotype of TOP 10 *Escherichia coli*: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G

2.1.1.2 Bacteria growth medium

* Ampicillin (Roscillin®, Ranbaxy Laboratories Limited)

* LB AGAR (LENNOX) (Laboratories CONDA; pH 7.0 ± 0.2 at 25 ºC; 15 g Bacteriological Agar, 5 g NaCl, 10 g Tryptone, 5 g Yeast Extract)

* LB BROTH (LENNOX) (Laboratories CONDA; pH 7.0 ± 0.2 at 25 ºC; 5 g NaCl, 10 g Tryptone, 5 g Yeast Extract)

Preparation of LB broth:

For 1 liter of LB broth, 20 g of LB BROTH (LENNOX) was dissolved in distilled water to make up to 1 liter. The LB broth was autoclaved before use. It was kept in room temperature for further use.

Preparation of LB agar plate with Ampicillin:

A total 5.25 g of LB AGAR (LENNOX) was dissolved in 150 ml of distilled water, and then autoclaved. The LB agar solution was allowed to cool down before 150 µl of 100 mg/l Ampicillin was added to a final concentration of 100 µg/ml. The LB agar (with Ampicillin) was then poured into petri dish (8- 9 plates) for solidification at room temperature. The

process normally took around 40 minutes. Solidified LB agar/Ampicillin plates were sealed with parafilm and kept at – 42 ºC until further use.

2.1.1.3 Preparation of *Escherichia coli* glycerol stock

One ml of cryopreserved *Escherichia coli* was inoculated into 9 ml of LB broth and incubated overnight at 37 ºC in shaking incubator (INFORS HT Minton) at 200 rpm. Overnight bacteria culture, every 200 µl of the *Escherichia coli* culture was mixed well with 100 µl of glycerol and froze in dry ice before stored at – 80 ºC. They were kept as cryopreserve *Escherichia coli* stock.

2.1.1.4 Preparation of competent *Escherichia coli*

* $CaCl_2$ (R&M Chemicals)
* Glycerol (R&M Chemicals)

One ml from the overnight bacteria culture (Section 2.1.1.3) was transferred into fresh 100 ml LB broth to make chemical competent cells. OD of the bacteria culture was measured using spectrophotometer (Eppendorf) at wavelength 600 nm until the OD_{600} reached approximately 0.5. At 2 hours incubation in shaking incubator (INFORS HT Minton) at 37 ºC, the OD_{600} reading was 0.5598. The 100 ml bacteria culture was splitted into two 50 ml volume in centrifuge tubes and centrifuged at 4 ºC, 2400 x *g* for 10 minutes. Supernatant was discarded and the pellet was resuspended with pre-chilled 15 ml of 0.1M $CaCl_2$. The resuspended bacteria was incubated in ice for 15 minutes before centrifuged again at 4 ºC, 2400 x *g* for 10 minutes were repeated two more times. After the third

centrifugation, the bacteria pellet was resuspended in 2 ml of 0.1 M of $CaCl_2$ with 20% (v/v) glycerol. Two hundred µl of chemical competent *Escherichia coli* was aliquoted into each pre-chilled microcentrifuge tube and quickly frozen in dry ice. The chemical competent cells were stored at – 80 ºC.

2.1.2 Primer Design

All the primers were ordered from Integrated DNA Technologies. *STAT1B* specific primers are p3xFlag-STAT1B_F (forward primer) and p3xFlag-STAT1B_R (reverse primer).Both primers were designed with introduction specific restriction enzyme (RE) site, *Eco*R1 and *Bam*H1 RE site for p3xFlag-STAT1B_F and p3xFlag-STAT1B_R primers respectively. These primers will be used in amplification of *STAT1B* and PCR colony. The customized RE site in primers are important for sub-cloning.

2.1.2.1 Oligonucleotides for sub-cloning

Name	Sequence (5' → 3')	Tm (ºC)
p3xFlag-STAT1B_F	CACTC**GAATTC**CATGTCTCAGTGGTAC	58.8
p3xFlag-STAT1B_R	CTC**GGATCC**TTACACTTCAGACACAG	58.4

*Restriction site in **Bold**

2.1.2.2 Oligonucleotides for sequencing

Name	Sequence (5' → 3')	Tm (ºC)
CMV_FSP	GTAGGCGTGTACGGTGGGAGG	61.8
STAT1B_FSP409	GACAAACAGAAAGAGCTTGAC	51.8
STAT1B_FSP3016	CAGAGGCCGCTGGTCTTGAAG	60.4
STAT1B_FSP3610	GGTCCTAACGCCAGCCCCGAT	64.1

2.1.3 Polymerase chain reaction (PCR)

* dNTPs (Promega)
* PCR buffer (Thermo Scientific)
* Pfu polymerase (Thermo Scientific)
* Taq polymerase (KAPA Biosystem)

Polymerase chain reaction was done using MyCycler Thermal Cycler (BIO-RAD). Primers specific to a gene will annealed to the complementary DNA sequence of the target gene is present in PCR sample. DNA polymerase could then synthesis the complementary strand from 5' to 3'and thus target gene was amplified.

PCR conditions were:

1.	Initializing step (95 ºC)	300 seconds
2. (x 30)	Denaturation step (95 ºC)	30 seconds
	Annealing step (Mentioned in Section 3.1.2.1 and 3.1.2.2)	30 seconds
	Elongation step (72 ºC)	180 seconds
3.	Finalizing step (72 ºC)	300 seconds
4.	Hold (4 ºC)	∞

2.1.3.1 Gradient PCR

A master mix solution was prepared as appropriate, according to Table 2.1. The polymerases were added to the reaction mixture just before PCR. Annealing temperature for amplification of *STAT1B* was optimized by using gradient PCR, in the range of 53 ºC to 63 ºC. The conditions of gradient PCR was set up as stated in Section 2.1.3. The annealing temperatures: 53.0 ºC, 53.7 ºC, 54.9 ºC, 56.7 ºC, 59.1 ºC, 61.0 ºC, 62.2 ºC and 63.9 ºC were tested.

Table 2.1: Master mix preparation in gradient PCR

Components	**Working Concentration**	**Volume per reactions (µl)**	**Volume for 9 reactions (µl)**
PCR buffer (10x)	1x	5	45
Distilled water		40.86	367.7
dNTPs (10 mM)	0.2 mM	1	9
p3xFlag-STAT1B_F (20 µM)	0.4 µM	1	9
p3xFlag-STAT1B_R (20 µM)	0.4 µM	1	9
pOTB7-STAT1B (25 ng/µl)	11 ng	0.44	4
Taq polymerase	0.5 U	0.2	1.8
Pfu polymerase	1U	0.5	4.5
Total		**50.0**	**450.0**