

COMPREHENSIVE PROTEOMICS IDENTIFICATION OF IFN- $\lambda$  3-REGULATED ANTIVIRAL  
PROTEINS IN HBV-TRANSFECTED CELLS



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จิราเดช มัคเจริญ : การวิเคราะห์ผลของอินเตอร์เฟียรอนแลมบ์ดาสามต่อโปรตีนยับยั้งไวรัสในเซลล์ที่  
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อินเตอร์เฟียรอนแลมบ์ดาได้ถูกนำมาศึกษาทดลองทางคลินิกในผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรัง  
เนื่องจากว่ายาชนิดนี้มีฤทธิ์ต้านไวรัสและมีผลข้างเคียงน้อยเมื่อเปรียบเทียบกับอินเตอร์เฟียรอนแอลฟา ทั้งนี้เพราะ  
ตัวรับสัญญาณของอินเตอร์เฟียรอนแลมบ์ดานั้นพบเฉพาะในเซลล์เยื่อบุผิวเท่านั้น ในปัจจุบันวิธีสัญญาณต่างๆ ที่ถูก  
ควบคุมด้วยอินเตอร์เฟียรอนแลมบ์ดาได้มีการศึกษาไม่มากนัก โดยเฉพาะอย่างยิ่งการศึกษาด้วยวิธีทางโปรตีโอมิกส์  
ในงานวิจัยนี้คณะผู้วิจัยพบว่าอินเตอร์เฟียรอนแลมบ์ดา 3 สามารถยับยั้งการเพิ่มจำนวนของไวรัสตับอักเสบบี โดย  
ลดการแสดงออกของยีนของไวรัส และลดปริมาณสารพันธุกรรมของไวรัสภายในเซลล์ การศึกษาโปรตีโอมิกส์เชิง  
ปริมาณได้ถูกนำมาใช้เพื่อหาไกลที่เกี่ยวกับการลดลงของไวรัสในเซลล์ HepG2.2.15 ที่ถูกกระตุ้นด้วยยาชนิดนี้  
เป็นเวลา 24 ชั่วโมง เปรียบเทียบกับเซลล์ที่ถูกกระตุ้นด้วยอินเตอร์เฟียรอนแอลฟาทูเอและพีบีเอส (ตัวควบคุม) โดย  
ใช้เทคนิคการติดฉลากด้วยไอโซโทปที่แตกต่างกันของเดเมทิล ด้วยเทคนิคและวิธีการวิจัยที่ใช้ในการศึกษาครั้งนี้ทำ  
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จำนวนของไวรัสตับอักเสบบี

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Quantitative proteomics, Dimethyl labeling, Type III IFNs, Hepatitis B virus

Jiradej Makjaroen : COMPREHENSIVE PROTEOMICS IDENTIFICATION OF IFN- $\lambda$ 3-REGULATED ANTIVIRAL PROTEINS IN HBV-TRANSFECTED CELLS. ADVISOR: Trairak Pisitkun, M.D., Prof. Nattiya Hirankarn, M.D., Ph.D.

Interferon (IFN)- $\lambda$  is a relatively unexplored, yet promising anti-viral agent. IFN- $\lambda$  has recently been tested in clinical trials of chronic hepatitis B, with the advantage that side effects may be limited compared with IFN- $\alpha$ , as IFN- $\lambda$  receptors are found only in epithelial cells. To date, IFN- $\lambda$  downstream signaling pathway remains largely unelucidated, particularly via proteomics methods. Here, we report that IFN- $\lambda$ 3 inhibits HBV replication in HepG2.2.15 cells, reducing levels of both HBV transcripts and intracellular HBV DNA. Quantitative proteomic analysis of HBV-transfected cells was performed following 24-hour IFN- $\lambda$ 3 treatment, with parallel IFN- $\alpha$ 2a and PBS treatments for comparison using a dimethyl labeling method. The depth of the study allowed us to map the induction of anti-viral proteins to multiple points of the viral life cycle, as well as facilitating the identification of anti-viral proteins not previously known to be elicited upon HBV infection. This study also shows up-regulation of many effectors involved in antigen processing/presentation indicating that this cytokine exerted immunomodulatory effects through a number of essential molecules for these processes. Interestingly, immunoproteasome caps were up-regulated while cap components of the constitutive proteasome were down-regulated upon IFN- $\lambda$ 3 treatment, suggesting coordinated modulation towards the antigen processing/presentation mode. Furthermore, we reveal that IFN- $\lambda$ 3 restored levels of RIG-I and RIG-G, proteins known to be suppressed by HBV. Enrichment analysis demonstrated that several biological processes including RNA metabolism, translation, and ER-targeting were differentially regulated upon treatment with IFN- $\lambda$ 3 vs. IFN- $\alpha$ 2a. Our proteomic data suggests that IFN- $\lambda$ 3 regulates an array of cellular processes to control HBV replication.

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## LIST OF ABBREVIATIONS

2-DE	2-Dimensional gel electrophoresis
Ab	Antibody
ACN	Acetonitrile
ALT	Alanine aminotransferase
BCA	Bicinchoninic acid assay
bp	Base pair
BSA	Bovine serum albumin
cccDNA	Covalently closed circular DNA
cDNA	Complementary DNA
CHB	Chronic hepatitis B
CsA	Cyclosporine A
Ct	Cycle threshold
Ctrl	Control
ddCt	Delta delta cycle threshold
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
e <sup>-</sup>	HBeAg negative
e <sup>+</sup>	HBeAg positive
EC <sub>50</sub>	Half maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
FA	Formic acid
FDR	False discovery rate
GO	Gene ontology
GWAS	Genome-wide association studies
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B early antigen

HBsAb	Hepatitis B surface antibody
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x protein
HCC	Hepatocellular carcinoma
HCD	Higher-energy collisional dissociation
HLA	Human leukocyte antigen
IA	Iodoacetamide
ICAT	Isotope-coded affinity tag
IFNR	Interferon receptor
IFN- $\gamma$	Interferon-gamma
IFN- $\alpha$	Interferon-alpha
IFN- $\beta$	Interferon-beta
IFN- $\lambda$	Interferon-lambda
IL	Interleukin
ISG	Interferon-stimulated gene
iTRAQ	Isobaric peptide tags for relative and absolute quantification
LC	Liquid chromatography
M	Molar
MHC	Major histocompatibility complex
MS/MS	Tandem mass spectrometry
MTT	Thiazolyl blue tetrazolium bromide
NA	Nucleotide analogue
nLC	Nanoliquid chromatography
PegIFN- $\alpha$	Pegylated IFN- $\alpha$
pgRNA	Pregenomic RNA
ppm	Parts per million
PTM	Post-translational modification

qPCR	Quantitative polymerase chain reaction
rcDNA	Relaxed circular DNA
RT-PCR	Reverse-transcription polymerase chain reaction
SD	Standard deviation
SDC	Sodium deoxycholate
	Sodium dodecyl sulfate-polyacrylamide gel
SDS-PAGE	electrophoresis
SEM	Standard error of the mean
sgRNA	Subgenomic RNA
SILAC	Stable isotope labeling by amino acids in cell culture
SVR	Sustained virological response
TEAB	Triethylammonium bicarbonate
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TMT	Tandem mass tags
ULN	Upper limits of normal
WB	Western blotting assay

# CHAPTER I

## INTRODUCTION

Chronic hepatitis B (CHB) is a major health problem worldwide, affecting 257 million people throughout the world with a prevalence in Africa and South-East Asia (1). Chronic HBV-infected individuals mostly acquire the virus at a young age through vertical transmission or contact with the blood or other body fluids of an infected person. CHB eventually progresses to severe and high-mortality liver diseases including liver cirrhosis and hepatocellular carcinoma (HCC) resulting in 887,000 deaths annually (2, 3).

Current therapeutic agents for CHB are interferon (IFN)- $\alpha$  and nucleos(t)ide analogues (NAs) (4-6). IFN- $\alpha$  is a cytokine that possesses anti-viral and immunomodulatory effects, which promotes control and eradication of viral infection. The advantages of using IFN- $\alpha$  for CHB treatment are the decreased incidence of viral resistance following this treatment and the finite duration of therapy with a higher rate of seroconversion of viral antigens, i.e. HBeAg and HBsAg, compared with NAs. However, the drawbacks of IFN- $\alpha$  treatment are its inconvenient route of administration and its adverse effects such as influenza-like symptoms, nausea, vomiting, cytopenia, and psychiatric disorders. Regarding the latter agent, NAs against CHB, the lack of a 3'-hydroxyl group in these compounds allows competitive incorporation into the viral genome resulting in termination of viral replication (7). Although NAs can directly suppress HBV replication and have fewer unfavorable side effects compared with IFN- $\alpha$ , CHB patients usually require a long-term treatment of these agents, thus increasing the chance of the emergence of viral resistance. In addition, the low rate of HBeAg and HBsAg seroconversion is another limitation of NAs. Therefore, new drugs that overcome restrictions of current anti-HBV treatments are still needed.

Several lines of reasoning suggest that IFN- $\lambda$  could have a superior combination of efficacy and reduced side-effects. IFN- $\lambda$  or type III IFN is a cytokine in

the class II cytokine family that has recently been used in clinical trials of CHB treatment (8, 9). IFN- $\lambda$  has 4 subtypes, namely IFN- $\lambda$ 1, - $\lambda$ 2, - $\lambda$ 3, and - $\lambda$ 4. IFN- $\lambda$ 3 was shown to have superior anti-viral potency compared with other IFN- $\lambda$  subtypes (10). IFN- $\lambda$  receptor is composed of an IFNLR1 and IL10R2 dimer; hence upon ligand binding, the receptor activates both IFN and IL-10-like signaling pathways. The type I IFN-receptors also form a heterodimer (IFNAR1 and IFNAR2), however, the sequences of all these receptors do diverge, particularly at the C-terminus (11, 12). IFNLR1 is expressed only in epithelial cells including hepatocytes in contrast to IFN- $\alpha$  receptor, which is widely expressed in many cell types throughout the body; thus IFN- $\lambda$  treatment results in fewer side-effects when compared with IFN- $\alpha$  treatment (9, 13, 14). IFN- $\lambda$  has been shown to activate the JAK-STAT pathway, inducing formation of the ISGF3 transcription complex, leading to expression of interferon-stimulated genes (ISGs) in similar fashion to type I IFN, but with a different temporal profile compared with those induced by type I IFN (15-17). A limited number of reports regarding IL-10-like signaling pathways have been published, with evidence of STAT3/5 biological activities (18, 19). In fact, no reports have comprehensively investigated the downstream molecular signaling effects of IFN- $\lambda$ .

To better understand molecular mechanisms underlying direct anti-viral and immunomodulatory effects of IFN- $\lambda$ 3, a comprehensive catalog of protein effectors regulated by IFN- $\lambda$ 3 was compiled using quantitative proteomics analysis in the well-established HBV-transfected hepatoblastoma cell line model viz. HepG2.2.15 cells (20). HepG2.2.15 cells have been widely used as a model of chronic hepatitis B because they support HBV replication and virion secretion (21-27). These new findings could improve the treatment strategy of HBV infection and expand potential applications of IFN- $\lambda$ 3.



## CHAPTER II

### LITERATURE REVIEW

#### **Introduction, epidemiology and route of transmission of HBV infection**

Hepatitis B virus (HBV) can cause acute and chronic liver inflammation. Although the safe and effective vaccine which has been available since 1982 has decreased the number of infected people (28), it is estimated that 2 billion people or one-third of the world's population have been affected with this virus worldwide. The prevalence of infection varies greatly across the globe. In highly endemic areas such as Africa and South-East Asia, more than 8% of the population have seropositive to hepatitis B surface antigen (HBsAg) which is used as a marker for HBV infection. The infection mainly occurs in a neonate born from HBV-infected mother at birth during the delivery process or in a child under the age of five through contact with the HBV-infected household. However, the perinatal and early childhood transmission are the minor route of infection in low endemicity where the prevalence is less than 2% such as Northern America and Western Europe. The major sources of transmission in these areas are unprotected sexual promiscuity, received blood or blood products without screening before transfusion, sharing HBV-contaminated needle in drug-abused user or tattooing and occupational exposure of healthcare workers including a doctor, dentist and nurse. Most infected persons are acquired HBV in adolescence or adulthood (29, 30). Approximately 95% of healthy adults affected with HBV can spontaneously and efficiently control viral replication and eliminate virus out of the body and become resolved and self-limiting infection with or without acute hepatitis symptoms including fatigue, low fever, nausea, vomiting, appetite loss, muscle pain, joint aches, pain on the right upper abdomen and jaundice. In contrast, 5% of infected adults and 90% of people exposed to HBV at birth or early childhood fail to clear the virus and establish chronicity (30, 31). It strongly indicates that mature immunity is essential for HBV clearance. World Health Organization (WHO) reported in 2018 that there are 257 million chronically infected people worldwide and 887,000 of these annually die from HBV-

related complications such as liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Hepatitis B virus infection; therefore, remains one of the seriously major health problems worldwide (28-30).

### **Characteristic and molecular virology of HBV**

HBV, small DNA virus, is a member of the family hepadnaviridae and genus orthohepadnavirus. In addition to unique genomic organization, HBV differs from other DNA virus because reverse transcription is one of the critical steps in HBV replication (32). Hepatotropism and host-range specificity are also the outstanding characteristics of HBV. This virus is divided into at least 8 genotypes (A-H) based on the difference of its whole sequence genome greater than 8%. The spread of genotype is distinct geographic spread: for example; genotype A and D are predominant in America and Europe, genotype B and C are most common in high prevalent areas in Africa and South-East Asia (28-33). The structure of HBV enveloped by HBsAg is sphere particle about 42 nm in size and contains icosahedral nucleocapsid composed by hepatitis B core antigen (HBcAg) inside to protect the viral genome from degradation of exogenous nuclease. The partially double-stranded DNA or relaxed circular DNA (rcDNA) and viral polymerase which is covalently linked to the HBV genome are packed within capsid. HBV genome consists of complete minus strand about 3.2 kb in length and incomplete plus strand with variable length ranging 20-80% of the minus-stranded length (34). The two sequences called direct repeat (DR) 1 and DR2 are the regions linking both strands. The full-length minus strand is composed of 4 Open Reading frames (ORFs). The preS/S ORF encoding three surface proteins (HBsAg) including large proteins, middle proteins and small proteins. The PC/C ORF encoding core proteins (HBcAg) and secreted, non-structural proteins (HBcAg). The P ORF encoding viral polymerase including DNA polymerase, reverse transcriptase and RNase H. The X ORF encoding the regulatory HBx protein involving in cell cycle progression, DNA damage repair, signal transduction, apoptosis and up-regulating HBV genes expression by transactivating its promoters or transactivating cellular genes to modify the environment to suit for viral replication. Also, there are several studies reported that HBx associated with hepatic carcinogenesis owing to inhibiting tumor suppressor genes (35, 36).

## HBV life cycle

After entry of HBV, virus specifically attaches to the specific receptor, sodium taurocholate cotransporting polypeptide (NTCP), on the hepatocyte surface and penetrate into the cell cytoplasm (37, 38). Many studies reported that the preS1 domain on large surface protein is necessary for receptor binding (39, 40). Following penetration, the nucleocapsid is released from the envelope and then transported to the nuclear membrane by nuclear localization signal from HBcAg on viral capsid. The release of HBV genome into the cell nucleus through nuclear transport receptors Imp- $\beta$ /Imp- $\alpha$  in importin pathway is taken place at nuclear membrane because of the interaction between viral capsid and nuclear pore complex (40). Inside the nucleoplasm, the viral polymerase is used to repaired to complete the plus strand of rcDNA and then cellular enzymes is utilized to remove RNA-primers used for DNA-plus strand synthesis and viral polymerase including terminal protein on the 5'-end of minus strand. After this step, two complete strands are linked by covalent ligation and this molecule form super-coiled structure (beads-on-a string arrangement) called covalently closed circular DNA (cccDNA) with histone and non-histone protein. The minichromosomal cccDNA served as a template for viral replication and viral RNA synthesis transcribes into 4 RNA species; 3.5 kb pregenomic RNA (pgRNA) and 2.4 2.1 and 0.7 kb subgenomic RNA (sgRNA) by using host RNA polymerase II (2, 34). All viral transcripts are processed by using cellular transcriptional machinery including capping and polyadenylation at 5'- and 3'-end, respectively before exportation. Translation takes place in the cytoplasm where HBx protein is translated from 0.7 kb RNA and surface proteins are translated from 2.4 and 2.1 kb RNAs. Also that the pgRNA is translated into viral polymerase, HBcAg and HBeAg, it serves as a template for reverse transcription to synthesize negative-stranded DNA. HBV replication is initiated by binding of the terminal protein of viral polymerase to epsilon stem-loop of pgRNA. The RNA-Pol complex is then incorporated into the immature assembling nucleocapsid. This process is triggered by the reverse transcriptase domain in polymerase. Inside the RNA-containing capsid, pgRNA is reverse transcribed into minus-stranded DNA acted as a template for plus-stranded DNA synthesis and followed by degradation of pgRNA by RNaseH. The mature rcDNA-containing capsid is either re-imported into the nucleus to

maintain and amplify the cccDNA pool or transported into the endoplasmic reticulum (ER) to envelop and release to the bloodstream. It is estimated that cccDNA about 1-50 copies resided in an infected cell (32). In addition to an infectious particle or Dane particle, envelope proteins themselves can be budded into ER lumen, derived host lipid and secreted as non-infectious particles either filamentous or spherical forms. These particles are produced and exported in a 1,000 to 1,000,000-folds excess over infectious particles. HBV also produces and secretes HBeAg into blood circulation. Although it is not necessary for viral replication, it plays a major role in the contribution of chronic infection due to its immunomodulatory activities (2, 32, 34, 40).

### **Characteristics and early events in Hepatitis B virus infection**

The unique pattern of HBV infection is delayed viral replication after infection and absence of early clinical symptoms that why this infection is different from other viral infections (41, 42). The studies in humans and chimpanzees showed that following infection or inoculation, HBV does not rapidly amplify and spread because HBV-DNA and HBV antigens are undetectable in both serum and liver. Although chimpanzees were inoculated with high doses of HBV, this did not push HBV into a logarithmic phase of replication until 4-7 weeks after inoculation (43). In contrast to the study in acutely hepatitis C virus (HCV)-infected chimpanzees, HCV-RNA was detectable within one week following inoculation and the magnitude of viral titers rapidly increased after the primary manifestation. Several studies in animal models showed that the absence of HBV in the first few weeks of infection did not result from the innate immune response. Because the activation of many cytokines involving in HBV clearance (interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-2) and the accumulation of several inflammatory cells in hepatocytes were taken place after HBV started expansion phase. Furthermore, the results from global gene expression profiling from liver biopsies of HBV-infected chimpanzees demonstrated that no cellular genes were induced during an incubation period of HBV (43). These results confirmed that innate immunity does not influence the early phase of viral replication and spread (42, 44). However, the exact causes of the absence of HBV-DNA and their antigens have not well understood so far. It is possible that the other organs might be the initial site for infection as

observed in woodchuck hepatitis virus (WHV) whose bone marrow was the primary site for infection. Nevertheless, WHV is lymphotropic virus thus the missing of human HBV in the early event of HBV infection remains unclear (41). Although many studies showed that IFN- $\alpha$  and - $\beta$  could efficiently inhibit HBV replication, lack of type I IFN production is one of the outstanding characteristics of early HBV infection. The results from Wieland study confirmed this finding that no type I IFNs and Interferon-stimulating genes (ISGs) were induced in the initial event of HBV infection (43). This virus might develop strategies to evade immune sensors in innate immunity during the lag phase of infection. It has been suggested that 1.) cccDNA, the template for viral RNA synthesis, is located in cellular nucleus 2.) Due to replication inside nucleocapsid, either viral DNA or viral transcripts which are the potent inducers of type I IFN production are not detected by any sensing receptors (41, 42, 44-46).

#### **Immune response in HBV infection**

When HBV reach expansion period, most of the hepatocytes are infected and HBV-DNA can be detected in serum and liver as much as  $10^9$ - $10^{10}$  copies/ml (29). During this phase, HBV-infected chimpanzees showed acute hepatitis symptoms and many genes in the liver are induced responding to the infection (43). The large quantities of IFN- $\gamma$  and TNF- $\alpha$  which might be produced by natural killer (NK) cells and natural killer T (NKT) cells are also observed. There is evidence on humans and animal models showed that these cells were responsible for early inhibition of HBV replication. Despite stimulating NKT cells with either  $\alpha$ -galactoceramide or HBV antigens in HBV-transgenic mouse model, HBV replication could be inhibited by IFN- $\gamma$  produced by NKT cells (42, 44). The other study examining in resolved people demonstrated that the vast amount of HBV coincided with a large number of NK cells in the circulation of HBV-infected individuals followed by massive IFN- $\gamma$  production. About 2-4 weeks later from this, the plenty of HBV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells were recruited into the liver whereas HBV replication had declined (41, 45). The adaptive immune response to HBV is initiated by presenting viral antigen to CD4<sup>+</sup> and CD8<sup>+</sup> T cells by antigen presenting cells (APCs) including dendritic cells (DCs) and kupffer cells (KCs), macrophages in hepatocytes. In addition to the presentation, APCs can produce IL-12 and TNF- $\alpha$ . Both cytokines are

required for development and IFN- $\gamma$  production of CD8<sup>+</sup> T cells while naïve CD4<sup>+</sup> T cells need only IL-12 for their differentiation into T helper (Th)1 cells. After activation, naïve CD4<sup>+</sup> T cells will differentiate into Th1 and Th2 based on types of cytokine production while naïve CD8<sup>+</sup> T cells become cytotoxic T cells (CTLs). The Th1 cytokines including IFN- $\gamma$ , TNF- $\alpha$  and IL-2 are essential for the maturation and induction of CD8<sup>+</sup> T cells. Conversely, antibody-producing B cells require IL-4 and IL-10 which are produced by Th2 cells for inducing their production of antibody to HBV antigens such as HBsAg, HBcAg and HBeAg. These antibodies play a role in the neutralization of free virus; however, only HBsAb are life-long protective immunity to re-infection. Anti-HBs are synthesized since early infection but they are undetectable due to forming a complex with the excess HBsAg produced during viral replication. CTLs are also responsible for HBV clearance. Depletion of CD8<sup>+</sup> T cells in an acute phase of infection, HBV-infected chimpanzees could not control viral replication leading to chronicity. The other study in woodchucks also gives the consistent results. CTLs can control viral replication via cytolytic and noncytolytic mechanisms. The first one, following activation, CTLs can directly destruct the infected cells by stimulation of program cell death through perforin and Fas/Fas-ligand leading to release of the virus. Subsequently, viral particles will be neutralized by antibody from B cells. Another mechanism is the production and secretion of IFN- $\gamma$  and TNF- $\alpha$  to clear virus via inhibition of pgRNA synthesis, destabilization of viral capsid utilizing the NF- $\kappa$ B pathway, and degradation of viral protein through proteasome-dependent and kinase-dependent pathway and nitric oxide. Although it is found that several HBV components can be a target for CTLs, the core proteins, especially HBc 18-27, are immunodominant epitopes that induce efficient responses. It is widely accepted that CTLs are the principal effector cells for HBV clearance nowadays. However, the coordination of cellular including CD4<sup>+</sup> and CD8<sup>+</sup> T cells and humoral responses in adaptive immunity is necessary for efficient viral defense (41, 42, 44, 45).

### **Pathogenesis of hepatitis B**

HBV is a non-cytolytic virus. This fact is supported by several clinical studies which indicated that many HBV carriers do not show any symptoms and their livers

are minimal injury despite the highly viral replication in their hepatocytes (31). According to studies in chimpanzees and transgenic mice, the histological and biochemical changes in the liver were not observed until the occurrence of specific immunity. Therefore, it is believed that host immune responses to HBV not only result in viral clearance but also lead to liver pathology. The study in transgenic mice shows that liver pathology mainly results from CTLs; however, the non-specific inflammatory cells including neutrophils, NK cells and monocytes together with platelets contribute to severe liver injury (41, 44). In addition to the secretion of antiviral cytokines such as IFN- $\gamma$ , CTLs can directly destruct infected liver cells through programmed cell death. The high-mobility group box 1 (HMGB1) secreted by apoptotic cells induce neutrophils into the liver. These cells produce and secrete matrix metalloproteinase (MMP) which degrade the extracellular matrix of the liver resulting in increasing intrahepatic infiltration of inflammatory cells. The chemokines CXCL9 and CXCL10 produced by parenchymal and non-parenchymal in the liver in response to IFN- $\gamma$  also promote these migrations. Furthermore, platelets secrete some proteins such as P-selectin that interact with CD8+T cells to facilitate these cell into the liver through sinusoids. The more specific and non-specific inflammatory cells influx into the liver, the more severe pathogenesis occur (28, 29, 42, 46).

### **Chronic hepatitis B**

The severity of disease and clinical outcomes of HBV infection are various among people depending on either viral factors or host factors. The efficient and optimal immune response to eradicate virus leading to resolved and self-limiting infection can be observed in people acquired HBV at adolescence or adulthood while the individuals who have over-action of immune response resulting in severe hepatitis and fulminant hepatic failure (3). Conversely, inadequate and improper immunity cause failure of viral clearance and establish of persistence which mostly observed in people exposed to HBV at birth (90%) or early childhood (30%). The chronic hepatitis B (CHB) is defined as the state that HBsAg is still in the serum longer than 6 months because the immune responses are incapable of control and eliminate the virus. The mechanism of HBV chronicity is not well understood, and it might result from either

virus or host (31). HBV have evolved strategies to evade immune responses such as mutation of core proteins to escape CTL responses. The plausible factors from virus including HBx protein and HBeAg. The first one HBx protein, multi-functional protein, can alter several cellular pathways and this might influence on immune response and HBV antigen presentation (47). HBx protein can induce the expression of human leukocyte antigen (HLA) class I on hepatocyte surface that facilitate presentation leading to augment of CTLs; however, the increasing number of CD8+ T cells fail to control virus due to lacking effective activities. This phenomenon results in severe liver damage. Another viral factor HBeAg acts as immune tolerogen due to the similarity in structure to HBcAg which are the critical target of immunity. Thus, HBeAg leads to the reduction of response to HBV. In addition to depletion of HBV-specific T cells, HBeAg decreases TLR2 expression on monocytes leading to decrease of TNF- $\alpha$  production. The reduction of this cytokine results in the imbalance of Th1/Th2 responses and this effect causes anti-inflammatory cytokines production such as IL-4 and IL-10. These cytokines subsequently restrain the response of HBV-specific CD4+ and CD8+ T cells. However, the proportion of Th1/Th2 increase after emerging of HBeAb (HBeAg seroconversion) and this lead to effective CTL response as a result of production and secretion of IL-2 and IFN- $\gamma$ . Besides, the excessive production of non-infectious particle HBsAg might contribute to the low response of specific-T cells. Other than viral factors, host factors including host genetics and immune status may involve in chronic infection. The studies on the association of HLA-DRB and the ability of HBV clearance in HBV-infected people in Thailand, Gambia, Korea and several western countries give the consistent results that HLA-DRB1\*1301-2 are associated with immune response in the protection of CHB (31). In contrast to people with resolved infection, the HBV-specific CD4+ and CD8+ T cells in people with CHB are hypo-responsiveness. The reduction in the number of core-specific CD8+ T cells and their ability to produce IFN- $\gamma$  are observed especially in HBeAg-positive chronic carriers. HBV-specific CTLs are still detectable in the liver of these carriers but they are incapable of clearing virus due to their low responsiveness and they also cause liver damage. The possible mechanism that can be explained the defect of the immune response in CHB is HBV might inhibit



the function of DCs which play a role in T cell priming but this hypothesis is still controversial in the current. The over-expression of programmed cell death (PD)-1 on HBV-specific T cells may be the other mechanisms involving in viral persistence. Generally, PD-1 and its ligands including PD-L1 and PDL-2 are the mechanisms to reduce the inflammation in the liver but the increase in its expression leads to the exhaustion of HBV-specific CTL activity. The activity of CD8+ T cells may be restored by blocking the PD-1 molecule or its ligands. The last probable cause may be the function of regulatory T cells (Treg). These cells express CD4+CD25+ on their surface and they play a crucial role as a negative regulator by inhibition of proinflammatory response. Also, Treg cells can suppress CTL proliferation, inhibit CTL function and diminish the production of IFN- $\gamma$  and TNF- $\alpha$ . Some studies indicated that the number of Treg cells found in CHB carriers are higher than those of in normal or HBV-resolved individuals. However, depletion of these cells or inhibition of their functions lead to increase in cytokine production of CD8+ T cells (3, 42, 48).

### The progression of CHB

In the present, CHB is divided into 4 phases namely immune tolerance, immune clearance (HBeAg-positive chronic hepatitis), low replicative (inactive HBV carrier state) and re-activation phase (HBeAg-negative chronic hepatitis). The HBeAg status and its seroconversion of the first two phases are positive and negative, respectively while low replicative and re-activation phases are negative and positive to HBeAg status and its seroconversion, respectively. The virological, biochemical and histological evidence of each stage are shown in the Table 1.

*Table 1. Characteristics of each phase in CHB*

Phase	HBeAg	Anti-HBe	HBV DNA (IU/ml)	ALT*	Histological activity
Immune tolerance	Positive	Negative	Very high ( $>2 \times 10^7$ )	Normal	Normal/ minimal change
Immune clearance	Positive	Negative	High ( $>2 \times 10^6$ )	Elevated	Hepatic necroinflammation with variable fibrosis level
Low replicative	Negative	Positive	Low ( $<2 \times 10^3$ )	Normal	Inactive and minimal fibrosis
Re-activation	Negative	Positive	Moderate ( $>2 \times 10^3$ )	Elevated	Hepatic necroinflammation with variable fibrosis level

\*ALT referred to alanine aminotransferase, the marker for liver injury

The immune tolerance phase is the phase that takes long times more than 10 years especially in a neonate born from chronically infected mother or in people exposed to HBV in early childhood. However, lack or short periods of this phase are observed in the infected people acquired HBV through horizontal transmission. Despite high viral load in the blood circulation, the carriers do not show any clinical appearances of hepatitis because of HBeAg inducing unresponsive HBV core-specific CD4+ and CD8+ T cells. In general, it is not recommended to treat the patients in this phase with anti-viral drugs because the disease progression is not severe and the response to therapy is quite low with 5% of HBeAg seroconversion (anti-HBe) emergence each year. The mechanism of the transition from immune tolerance to immune clearance phase is still unknown. The period of immune clearance phase varies ranging from several months to several years. During this phase, HBV DNA levels are lower than those of in immune tolerance phase as a result of the immune response against HBV. This interaction destroys the infected hepatocytes through apoptosis leading hepatitis. The increase in ALT levels in this period reflects liver inflammation. Approximately 90% of patients achieved HBeAg seroconversion. Of these, 80-90% of patients enter low replication phase while 10-20% was remaining shifts to re-activation phase by skipping the third phase of progression. The yearly rate of HBeAg is 8-12% depending on the age of infection, route of transmission, serum ALT levels, immune status and viral genotype. Many studies reported that good prognosis was observed in the patients who got HBeAg seroconversion before the age of 40 or had a short period of immune clearance with rising ALT levels. In contrast, the patients taking long-time in this phase or getting hepatic flare several times without HBeAg seroconversion increase the risk of cirrhosis and HCC in the future. Low replicative phase takes place following the occurrence of HBeAg seroconversion. In addition to the reduction of HBV DNA and ALT levels, Anti-HBs can be observed in this phase. Although the most of patients have sustained remission, some patients either get HBeAg reversion and then return to the immune clearance phase again or shift to re-activation phase. Although the patients in HBeAg-negative chronic hepatitis phase have seropositive to anti-HBe, HBV DNA levels are higher than patients in low-replicative phase. Besides, the fluctuated level of ALT reflects that the immune response attempts to control the

virus but this reaction is inadequate to inhibit viral replication. The results from the molecular study of HBV from the blood of the patients in this phase show that there are double mutations in basic core promoter (BCP) resulting in the reduction of HBeAg production. This study also demonstrated that mutation in the pre-core gene causing stop codon leads to lack of HBeAg production (3, 31, 42, 46, 48).

### Current treatment in CHB

The currently anti-HBV drugs cannot completely eradicate virus due to the persistent form of cccDNA in hepatocytes. Therefore, the short-term purposes of treatment are to suppress viral replication and to achieve HBeAg seroconversion and/or HBsAg seroconversion while the long-term goal of therapy is to delay the development of cirrhosis and hepatocellular carcinoma. Before the beginning of treatment, the indicators should be considered including ALT levels, HBV DNA viral load and histological grade and stage as shown in the following Table 2 (2).

*Table 2. Indications of each antiviral treatment*

	Interferon (IFN)	Nucleotide analogues (NAs)
Age	Less than 60 and healthy	Any
Baseline HBV DNA level	Low	Any
Baseline ALT	Greater than 2-3 X ULN	Any
HBV genotype	A or B	Any
Cirrhosis	No	With or without decompensation

ULN = Upper limits of normal

For example, The American Association for the Study of Liver Diseases (AASLD) recommend that either HBeAg-positive or HBeAg-negative CHB patients with an HBV DNA greater than or equal to 20,000 IU/ml or 2-fold upper limits of normal of ALT level should be treated with anti-viral drugs. In current, there are seven drugs approved for CHB treatment and they are divided into 2 groups namely interferon and nucleos (t) ide analogue based on its actions as shown in the following Table 3 (2).

Table 3. Drugs for CHB therapy

Interferon-based therapy	NA-based therapy	
	Nucleoside analogue-based therapy	Nucleotide analogue-based therapy
IFN- $\alpha$ 2a and $\alpha$ 2b	Lamivudine	Adefovir
Pegylated IFN- $\alpha$ 2a and $\alpha$ 2b	Entecavir	Tenofovir
	Telbivudine	

IFN- $\alpha$  is one of the cytokines in immunity that has dual activities including anti-viral and immunomodulatory effects. The former action, IFN- $\alpha$  can inhibit viral replication in several steps of viral life cycle by induction of ISGs possessing anti-viral activities such as 2'-5'-oligoadenylate synthetase (2'-5'-OAS), myxovirus resistance-1 (Mx1), protein kinase R (PKR) and ISG15. This activation affects viral replication through blocking viral transcriptional and translational events and promoting viral RNA degradation (49). The latter action, IFN- $\alpha$  contribute the efficient immune response because it enhances the expression of HLA class I and co-stimulatory molecules on DCs, increase the activity of NK cells and CTLs and promotes the differentiation of naïve T cells to Th1 cells (50). Therefore, IFN- $\alpha$  is used either as anti-tumor drugs such as hairy cell leukemia, malignant melanoma and AIDS-related Kaposi's sarcoma or used as the anti-viral drug in several viral infections such as rhinovirus infection, chronic hepatitis C and chronic hepatitis B. The conventional IFN- $\alpha$  has been approved for treatment of CHB since 1991. The regimen of this drug are subcutaneous administration three times per week for at least 3 months. From the meta-analysis study, it showed that about 33% of HBeAg-positive CHB patients achieved successful responses defined as diminish ALT level, loss of HBeAg and develop HBeAg seroconversion (5). These responses were sustained in the long term despite off-treatment. Several reports indicated that IFN- $\alpha$  might prevent or delayed the development of cirrhosis and HCC. The inconvenience of frequent subcutaneous injection is the disadvantage for this therapy; however, pegIFN- $\alpha$  has been replaced. Pegylated IFN- $\alpha$  is modified interferon by attachment of polyethylene glycol molecule to interferon to improve its immunological, pharmacokinetic and pharmacodynamic properties. The large size of the peg molecule causes IFN- $\alpha$  to increase its half-life due to the reduction of

clearance at the kidney. In addition to an increase of biological activity of modified IFN- $\alpha$ , peg moieties may postpone the eradication of recombinant IFN- $\alpha$  by the immune system by reducing the immunogenicity of IFN- $\alpha$ . With these reasons, pegIFN- $\alpha$  allows being administered once weekly. There are 2 forms of pegIFN- $\alpha$  in current namely, pegIFN- $\alpha$ 2a (40 kDa of branching peg molecule) and pegIFN- $\alpha$ 2b (12 kDa linear peg molecule) (51). The recommended dose of pegIFN- $\alpha$ 2a and pegIFN- $\alpha$ 2b for both HBeAg-positive and HBeAg-negative chronic hepatitis patients are 180  $\mu$ g and 1.5  $\mu$ g/kg, respectively for 48 weeks. Following completion of pegIFN treatment, HBeAg seroconversion achieves in around 30-40% in patients with HBeAg-positive CHB. More than 80% of these patients have sustained response and loss of HBsAg. The responses including the amount of HBV DNA less than 20000 copies/ml and normalization of ALT level are observed in patients with HBeAg-negative CHB in around 36% after complete treatment. Furthermore, loss of HBsAg is also observed in 3% of these patients and the rate of HBsAg may increase to 10% after 4 years post-treatment (52). Although IFN therapy has benefits including 1) increase of HBeAg and HBsAg seroconversion 2) delay development of progressive disease 3) finite duration of treatment and 4) no drug resistance, the limitations of IFN treatment are following 1) subcutaneous injection 2) moderate HBV DNA suppression and 3) adverse effects such as influenza-like symptoms (fatigue and fever), weight loss, cytopenia, depression, anxiety, abnormal function of thyroid gland and suppression of bone marrow (5). In contrast to indirect action on viral replication of IFN- $\alpha$ , NA directly inhibits viral replication by acting as a competitive inhibitor of the HBV polymerase in several steps of HBV life cycle as shown in the Table 4.

Table 4. Action of NA

Structure	Drug	Polymerase function		
		Priming	Minus strand synthesis	Plus strand synthesis
L-nucleosides	Lamivudine (CA)		x	x
	Telbivudine (TA)			x
Deoxyguanosine analogue	Entecavir (GA)	x	x	x
Acyclic nucleotide phosphate's	Adefovir (AA)		x	x
	Tenofovir (AA)		x	x

CA = Cytidine analogue, TA = Thymidine analogue, GA = Guanosine analogue and AA = Adenosine analogue

These drugs can incorporate into viral genome during viral replication due to the similarity of their structure to natural nucleotide; however, the next nucleotide can't bind to NA because these drugs lack hydroxyl group in their structure. This lead to chain termination and eventual inhibition of viral replication (2). NA can restrain HBV polymerase activity by inhibition of priming in step of reverse transcription, inhibition of RNA dependent DNA polymerase or reverse transcriptase in step of elongation negative strand, and inhibition of DNA dependent DNA polymerase in step of plus strand synthesis. Although NA suppress viral replication resulting in the reduction of HBV DNA in liver and blood circulation, NA do not inhibit cccDNA synthesis in the liver but it is able to reduce the turn-over of virus into the nucleus of hepatocytes resulting in the reduction of cccDNA in the liver. In addition to inhibition of viral replication, NA can prevent and reduce the development of cirrhosis, liver failure and HCC (5). The 5 approved oral NA drugs for CHB treatment are lamivudine, adefovir, entecavir, telbivudine and tenofovir (2). Treatment with lamivudine results in reduction of HBV DNA, ALT normalization and achievement of HBeAg seroconversion in around 15-20% and 25-30% of patients with HBeAg positive CHB after 1-year and 2-year post-treatment, respectively. However, prolonged therapy with lamivudine cause development of viral resistance 14-24% and up to 76% of case after 1 and 5 years of treatment. This is the reason why lamivudine is limited in current. Due to high rate of viral resistance, lamivudine is not recommended to use as first-line therapy. Although the combination treatment between IFN and lamivudine are able to reduce the emergence of lamivudine-resistant HBV, the responses are not redundant or synergistic effects (4, 7, 53). The second one, adefovir dipivoxil is a guanosine nucleotide analogue.

Although the efficiency on suppression of HBV DNA is less than lamivudine, the occurrence of viral resistance is lower than first one with 2% and 29% of case following 1 and 5 years of therapy. Following 1 year of treatment with this drug, the patients with HBeAg-positive CHB achieve HBeAg seroconversion in around 12-18% of case and HBA DNA level in blood circulation is undetectable about 13-21% while HBV DNA level is undetectable approximately 51-63% in case of the patients with HBeAg-negative. Adefovir is suitable for patients with lamivudine-resistant HBV because two drugs are not cross-resistance. Due to the moderate suppression of HBV DNA, this drug is not suggested to treat HBeAg-positive CHB patients who have high HBV DNA level in their blood. Furthermore, it is not recommended to use as first drug for CHB treatment because of its nephrotoxicity. Thus, the use of adefovir is limited and it is mainly used in patients with lamivudine-resistant HBV (5, 7, 53). Entecavir is the first-line therapy for CHB that can efficiently reduce HBV DNA level both in patients with HBeAg-positive (67%) and HBeAg-negative (90%) CHB after 1 year of treatment and this effect still sustain following 5 years of continuous treatment with entecavir. Its potency in suppression of HBV DNA is higher than lamivudine and adefovir because it can inhibit HBV polymerase in 3 steps of HBV life cycle. However, the rate of occurrence in HBeAg seroconversion is not different from lamivudine and adefovir treatment, that is, the loss of HBeAg is achieved about 20% and 30% of patients with HBeAg-positive CHB after 1 and 2 years of entecavir treatment. The emergence of resistance is low with 1.2% after 5 years of treatment; however, the rate may increase up to 51% in patients with lamivudine-resistant HBV following 5 year treatment. The reasons why entecavir is low resistance are the effective viral suppression and the requirement of at least 3 sites of mutation for induction of entecavir resistance (5, 54-56). The reduction of HBV DNA levels by telbivudine treatment are 60% and 88% of patients with HBeAg-positive and HBeAg-negative CHB, respectively after 1 year of treatment. The occurrence of HBeAg seroconversion is 22.5% and 29.6% of case after 1 year and 2 year of treatment. The emergence of resistance is low with 5% and 25% of case after 1 and 2 years of treatment, respectively. The telbivudine-resistant HBV is cross-resistance to lamivudine and entecavir. The main limitation of this drug is undesired side effect including myalgia and peripheral neuropathy (55, 56). Another drug, tenofovir disoproxil fumarate

belongs to extremely HBV reduction and the magnitude of suppression is similar to entecavir and telbivudine. Moreover, tenofovir-resistance is not observed after 4 years of treatment and this drug is used to treat lamivudine-resistant HBV. The HBV DNA level is unquantifiable in around 76% of case and the emergence of HBeAg and HBsAg seroconversion are 21% and 2% of case after 1 year of treatment and 27% and 6% of case after 2 year of treatment in patients with HBeAg-positive CHB. For patients with HBeAg-negative CHB, the reduction of HBV DNA level is about 93% of case. With these reasons, tenofovir is suggested to use as a first-line therapy in naive both HBeAg-positive and HBeAg-negative CHB patients (5). Although the antiviral treatments for CHB in the present are efficient in the suppression of HBV DNA and achieve HBeAg seroconversion which are the aims of treatment, there are still some limitations which shown in the Table 5. Thus, the novel agents that overcome these restrictions are still required (5, 7, 53-57).

*Table 5. Comparison advantages and limitations of each treatment*

	<b>Interferon (IFN)</b>	<b>Nucleotide analogues (NAs)</b>
<b>Advantages</b>	No drug resistance	Oral administration
	Increased HBsAg seroconversion	More potent HBV DNA suppression
	More durable off-treatment	Minimal side effects
	Finite therapy duration	
<b>Limitations</b>	Subcutaneous injection	Risk of resistance on prolonged therapy
	Less potent HBV DNA suppression	No increase in HBsAg seroconversion
	Frequent side effects	Less durable off-treatment response
		Long-term therapy

### **Introduction to Interferon lambda (IFN- $\lambda$ )**

Interferons have been classified into 3 types including type I, II and III based on their structures, receptors and biological functions. Type III IFN or IFN- $\lambda$  is a novel cytokine of class II cytokine family additionally consisting of IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26), type I IFN (13 isoforms of IFN- $\alpha$  and 1 isoform of IFN- $\beta$ ,



IFN- $\epsilon$ , IFN- $\kappa$ , IFN- $\omega$  and IFN- $\tau$ ) and type II IFN (IFN- $\gamma$ ). This cytokine discovered in 2003 by two research groups independently contains 3 subtypes including IFN- $\lambda_1$ , IFN- $\lambda_2$  and IFN- $\lambda_3$ . These cytokines are also known as IL-29, IL-28A and IL-28B, respectively. In spite of the structural similarity to the IL-10 family, IFN- $\lambda$ s have biological functions like type I IFN. Type III IFNs exert their activities through their receptor complex containing IFN- $\lambda$ R1 (IL-28RA) and IL-10R2 (58, 59).

### Gene organization of IFN- $\lambda$ s

The IFN- $\lambda$  genes are encoded on chromosome 19 at 19q13.3 region. IFN- $\lambda_1$  gene contains 5 exons while IFN- $\lambda_2$  and IFN- $\lambda_3$  consist of 6 exons. In contrast to intron-lacking genes of type I IFN, The exon-intron organization are conserved in IFN- $\lambda$  genes which are similar to those of IL-10-like cytokines. During duplication, the fragment containing IFN- $\lambda_1$  and IFN- $\lambda_2$  genes was copied and subsequently integrated back into the genome in a mirror-inversion or head-to-head orientation with the IFN- $\lambda_1$  and IFN- $\lambda_2$  segment. This phenomenon results in the production of IFN- $\lambda_3$  and non-functional pseudogene IFN- $\lambda_4\psi$  on the negative strand while IFN- $\lambda_1$  and IFN- $\lambda_2$  genes lay on the positive strand. Thus, IFN- $\lambda_3$  gene is transcribed in the opposite direction of IFN- $\lambda_1$  and IFN- $\lambda_2$  genes. The coding region together with the upstream and downstream flanking sequences of IFN- $\lambda_3$  gene are nearly identical to those of IFN- $\lambda_2$  gene; therefore, IFN- $\lambda_2$  and IFN- $\lambda_3$  are more homologous each other than IFN- $\lambda_1$ . However, the promoter of IFN- $\lambda_2$  and IFN- $\lambda_3$  genes are similar to those of the IFN- $\lambda_1$  gene. The promoter of three IFN- $\lambda$  genes contains several binding sites for nuclear factor- $\kappa$ B (NF- $\kappa$ B), interferon regulatory factor (IRF) 3 and 7 which are similar to those of type I IFN. Thus, it is implied that the stimuli activating type I IFN expression may also induce type III expression (60-62).

### Induction of type III IFN expression

In response to viral infection, several cell types are capable of IFN- $\lambda$  production especially plasmacytoid dendritic cells (pDCs); nevertheless, macrophages which are the cells that massively generate IFN- $\alpha$  did not produce IFN- $\lambda$ . Like type I IFNs, IFN- $\lambda$ s are mainly induced by viral infection. The nucleic acids of virus which are one of the

pathogen-associated molecular patterns (PAMPs) are a potent inducer of IFN response. In the cellular cytoplasm, there are several sensing molecules called pattern recognition receptors (PRRs) including retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5) to detect the PAMPs while Toll-like receptors (TLRs) -3, -7/8 and -9 detect the PAMPs in the endosome. After recognition, signaling transduction is generated through adaptor molecules and kinases resulting in activation of IRF3, IRF7 and NF- $\kappa$ B, which in turn bind the promoter of type I and III IFN leading to IFN production. Both types of IFN are stimulated via these transcription factors; however, IFN- $\lambda$ 2/3 are mainly activated by IRF-7 like IFN- $\alpha$  while IRF3 primarily induce IFN- $\beta$  and IFN- $\lambda$ 1. For NF- $\kappa$ B pathway, it is the main activators of type III IFN expression because the inhibition of this pathway in DCs results in suppression of IFN- $\lambda$  production while type I production has a little effect (60, 63).

### Signaling pathway of IFN- $\lambda$

All 3 subtypes of IFN- $\lambda$  exert their biological activities by triggering signal to the heterodimeric receptor complex contained IFN-LR1 and IL-10R2 which are encoded on chromosome 1 (1p36.11 region) and chromosome 21 (21q22.11 region), respectively (58, 59). The former receptor is not only crucial for IFN- $\lambda$  signaling due to providing the binding energy but also unique for IFN- $\lambda$  and expresses in specific organs which are epithelial cell origin including pancreas, thyroid, skeletal muscle, heart, prostate testis and liver. While the latter receptor is ubiquitously expressed in many organs and this receptor is also utilized by IL-10 and IL-22. Also, IL-10R2 is required for proper signal transduction (58, 59, 64). After binding of IFN- $\lambda$  to its receptor complex, the Janus kinase 1 (Jak1) associated with IFN- $\lambda$ R1 and the Tyrosine kinase 2 (Tyk2) associated with IL-10R2 are subsequently activated resulting in cross-phosphorylation each other of two kinases. Following this activation, the two phosphorylated tyrosine (Tyr313 and Tyr517) on intracellular domain of IFN- $\lambda$ R1 are generated docking site for Src homology 2 (SH2) part of signal transducers and activators of transcription (STAT). This result in bringing this protein close to the activated kinases to phosphorylate the tyrosine residues to create a docking site at the C-terminal end of the STAT for the SH2 domain of the other STAT. In this case, the heterodimer of STAT-1 and STAT-2

recruit IRF9 to form the complex called IFN-stimulated gene factor 3 (ISGF3). This transcription factor subsequently translocates into the nucleus where it binds to the promoter of ISGs called IFN-stimulated response elements (ISRE) (60, 61, 63, 64). Consistent with these, Zhang et al. demonstrated that IL28B, IFN- $\lambda$ 3, induced STAT-1 and STAT-2 phosphorylation like IFN- $\alpha$  indicating that IL-28B signals through the JAK-STAT pathway. To confirm this finding, they treated JAK inhibitor 1 which is an inhibitor of Jak1 and Tyk2 into the liver cell line harboring full-length HCV RNA before treatment with IL28B to inhibit JAK-STAT pathway and they found that the expression of known ISG MxA and ISG15 was reduced and IL-28B did not suppress HCV core protein expression (65). From microarray study, it showed that the pattern of genes induced by IFN- $\lambda$  is nearly similar to those induced by IFN- $\alpha$  with different kinetics. The expression of IFN- $\alpha$ -induced genes was observed since 3 hours of post-treatment and they largely decreased their expression by 24 hours. Conversely, the expression of IFN- $\lambda$ -induced genes proceeded to increase their expression. Moreover, some genes were induced by IFN- $\lambda$  but not induced by IFN- $\alpha$ , confirming that IFN- $\lambda$  might induce these genes through signaling to other pathways (66).

### IFN- $\lambda$ subtypes

All 3 IFN- $\lambda$  subtypes belong to class II cytokine family. The outstanding characteristics of this family are structural feature and pattern of disulfide bonds. The crystal structure of IFN- $\lambda$ 3 revealed that IFN- $\lambda$ s are an  $\alpha$ -helical structure with 6 secondary elements which are similar to other class II cytokines. These cytokines have a unique pattern of disulfide bonds. IFN- $\lambda$ 1 has only 5 cysteines while the remaining have 7 cysteines. The study on the crystal structure of IFN- $\lambda$ 3 also showed that IFN- $\lambda$ 2/3 has 3 disulfide bonds in its structure. However, only 2 disulfide bonds are observed in IFN- $\lambda$ 1. In addition to the disulfide bond, the difference between IFN- $\lambda$ 1 and IFN- $\lambda$ 2/3 is glycosylation. The observation on the production of these proteins in mammalian cells found that both IFN- $\lambda$ 2 and IFN- $\lambda$ 3 were not glycosylated while IFN- $\lambda$ 1 have 1 potential N-linked and 6 potential-linked glycosylation sites. However, these glycosylations seem not to have any effect on its activity. Furthermore, the alignment

of amino acid showed that IFN- $\lambda$ 2 were highly identical to IFN- $\lambda$ 3 with 96% sequence identity whereas IFN- $\lambda$ 1 shared 81% sequence identity with IFN- $\lambda$ 2/3. To compare the sequence identity of type III IFN with other class II cytokines, for example, the sequence similarity between IFN- $\lambda$ 3 and IL-19 or IFN- $\lambda$ 3 and IFN- $\alpha$ 2 is 21% and 31%, respectively. With consideration of the similarity of amino acid sequence, type III IFNs are closely related to type I IFN more than IL-10-like cytokines (60-62). The potency of all subtypes was tested by regard to the ability of IFN- $\lambda$  subtypes to protect cell lysis by encephalomyocarditis virus (EMCV). The results determining by EC50 values showed that IFN- $\lambda$ 3 exhibited the highest potency among the others with 2-fold and 16-fold higher than IFN- $\lambda$ 1 and IFN- $\lambda$ 2, respectively. However, the potency of IFN- $\lambda$ 3 is less than that of IFN- $\alpha$  (10-fold) (10). In agreement with these results, Leiliang Zhang et al. demonstrated that all 3 types of IFN- $\lambda$  suppressed HCV replication and IL-28B seemed to be more potent than the others (IL28B > IL29 > IL28A). However, IFN- $\alpha$  appeared to be stronger to inhibit HCV replication about 15-folds than IL-28B (65). The results from Man-Qing Liu et al. study which determined the antiviral activities of IFN- $\lambda$ s on HIV replication also showed the correlated results (65). The weaker potency of IFN- $\lambda$  might result from the restricted expression of IFN- $\lambda$  receptor compared to the broad expression of IFNAR. The activities of IFN- $\lambda$ 2 and IFN- $\lambda$ 3 are different although two cytokines have the high identity and only differ by 6 amino acids. The cause of loss activity are still unclear nowadays; however, there is a hypothesis that the large molecule of Val95 in IFN- $\lambda$ 2 (Gly95 in IFN- $\lambda$ 3) located in helix D domain which is crucial for interaction with IL-10R2 might destabilize this domain resulting in less potency of IFN- $\lambda$ 2 (61).

### **Biological activities of IFN- $\lambda$**

The usage of receptors of IFN- $\lambda$  differs from those of IFN- $\alpha$ ; however, signal transduction of both cytokines trigger through the JAK-STAT pathway. Moreover, the patterns of gene expression induced by IFN- $\lambda$  are nearly identical to that induced by IFN- $\alpha$ . Therefore, IFN- $\lambda$ s possess the antiviral, antiproliferative and immunomodulatory activities like type I IFNs (58, 59, 64, 67). IFN- $\lambda$ s have shown its antiviral effects against

several viruses such as encephalomyocarditis virus (EMCV), vesicular stomatitis virus (VSV), influenza A virus (IAV), cytomegalovirus (CMV), human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus in several experimental models. In addition to the in vitro study, IFN- $\lambda$  also inhibits vaccinia virus (VV) and herpes simplex virus (HSV) in vivo. In agreement with antiviral function, IFN- $\lambda$ s are able to induce OAS1 and MxA expressions which are known as common features of the antiviral response (68). Besides, like antiviral activity, the antiproliferative effect of IFN- $\lambda$  is limited in some cell types especially in tissues from the epithelial origin which highly express IFN- $\lambda$ R1 (61, 69). One group studying the antitumor function of this cytokine demonstrated that the tumor size of tumor-introduced mice which were injected with the plasmid harboring murine IFN- $\lambda$  had smaller than that of untreated mice. They were supposed that this might result from increased NK activity and tumor cells apoptosis which are induced by IFN- $\lambda$ . Furthermore, IFN- $\lambda$ s contribute the adaptive immune response by up-regulating the expression of major histocompatibility complex (MHC) class I (61, 69).

### IFN- $\lambda$ and viral hepatitis

There are many studies about the effect of IFN- $\lambda$ s on HCV. All of these showed that HCV replication was suppressed in a dose-dependent manner after IFN- $\lambda$ s stimulation by determining the reduction of either HCV RNA or protein expression (65, 68, 70, 71). The combination treatment either between IL-29 and IFN- $\alpha$  or between IL-29 and IFN- $\gamma$  on hepatocyte cell line containing HCV RNA showed that the reduction of HCV RNA expression after treatment with combinations was higher than that treated with individual cytokines. The co-treatment of IL-29/IFN- $\alpha$  and IL-29/IFN- $\gamma$  reduced HCV RNA expression by 92 and 98%, respectively (68). Recently, the genome-wide association studies (GWAS) have indicated that a single nucleotide polymorphism (SNP), rs12979860, in the upstream of IL28B was associated with the response to pegIFN- $\alpha$  plus ribavirin (RBV) treatment and spontaneous clearance of HCV. Following standard therapy of pegIFN- $\alpha$  and RBV, the HCV-infected patients with CC genotypes have the rate of sustained virological response (SVR) higher than those with CT or TT genotypes (72-74). However, this SNP is not associated with clearance of HBV DNA, HBsAg and

HBeAg (75-77). Although the SNP in the IL28B gene is not linked to the clinical outcomes of HBV infection, IFN- $\lambda$ s have shown their antiviral activity against this virus. In 2005, Robek et al. determined the antiviral function of IFN- $\lambda$ 2 against HBV replication by using murine liver cell line harboring HBV (HBV-Met) as a model. The experimental result showed that 10 ng/ml of murine IFN- $\lambda$ 2 could inhibit HBV replication greater than 90% at 24 hours after post-stimulation and this result was also observed in HBV-Met cells treated with 200 U/ml of IFN- $\beta$  at the same time (71). Consistent with this study, the study of Seung-Ho Hong et al. also showed that IFN- $\lambda$ 1 suppressed HBV replication in human hepatocyte containing HBV cell line at 48 hours after post-treatment (78). As described above, the antiviral activity of IFN- $\lambda$  is weaker than that induced by IFN- $\alpha$ ; however, the restricted its receptor expression resulting in less of unfavorable side effects and the prolonged response of IFN- $\lambda$  may let this cytokine be the novel therapeutic agent. Recently, a clinical trial showed that the SVR of the patients with chronic hepatitis c treated with pegIFN- $\lambda$ 1 together with RBV for 24 weeks were similar to that of patients treated with standard regimen but the rapid reduction of HCV RNA level was observed in patients treated with pegIFN- $\lambda$ 1. Also, the patients treated with pegIFN- $\lambda$ 1 had fewer adverse effects and lesser elevated ALT than those treated with a standard course (79). For treatment with CHB, a clinical study revealed that pegIFN- $\lambda$ 1 reduced the amount of HBV DNA, HBsAg, and HBeAg greater than pegIFN- $\alpha$  in the early phase of on-treatment. At the end-of-treatment, the effects of pegIFN- $\lambda$ 1 on virologic, serologic, biochemical responses were comparable to pegIFN- $\alpha$ . However, no non-inferiority was observed in pegIFN- $\lambda$ 1 at week 24 post-treatment. The fewer adverse events were found in the patients treated with pegIFN- $\lambda$ 1 (9). In addition to reducing HBV DNA and HBsAg levels, another clinical study demonstrated that pegIFN- $\lambda$  showed immunomodulatory effects by the maintenance of HBV-specific CD8+ T cells and enhancement of natural killer cell polyfunctionality (80).

### **The tools used to study molecular mechanism**

So far, we have known that IFN suppresses HBV replication by preventing the association of viral RNA and immature nucleocapsids which take place in the

cytoplasm (81). Although several studies proposed the molecules involving in antiviral properties of IFN including MxA, apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) and myeloid differentiation primary response protein (MyD88), the picture of global effects responding to IFN is not complete (82-84). A microarray is a powerful tool providing the information about the biological process in mRNA level. Wei et al. used this tool to study the global effects of IFN- $\alpha$  and IFN- $\beta$  on HepG2 and HepG2.2.15 (HBV-transfected cell line) and showed that the functional categories of IFN- $\alpha$  responsive genes were cell cycle, apoptosis, extracellular matrix, signal transduction, interferon-inducible and proteasome components. From this result, the researchers were interested in MyD88 which are one of the components in signal transduction responding to IFN- $\alpha$  and they confirmed the role of this gene in antiviral activity by cloning this gene into the plasmid and then co-transfecting with HBV replicative cell line (85). MyD88 could inhibit HBV replication and reduce the synthesis of HBsAg and HBeAg. Although microarray is a large-scale quantitative analysis of mRNA expressions, mRNAs do not reflect the exact functional molecules while proteins do. Protein is vital components of an organism which play a role in physiological processes and complex biological networks are mediated by proteins. Furthermore, due to post-transcriptional processing and post-translational events, the poor correlation between mRNAs and proteins has been demonstrated. Therefore, high-throughput study at the proteome level, proteomics, should be carried out (86, 87). Wang et al. studied the global proteins of HBV-transfected cell responding to IFN- $\alpha$  using proteomics utilizing 2-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). They found that IFN- $\alpha$ -responsive proteins were involved in ATP binding, cell cycle, apoptosis, ATPase activity and electron transport. From this result, they were interested in prohibitin which its functions are cell cycle, apoptosis and proliferation. Prohibitin can bind and interact with p53 which possess antiviral and antitumor properties; however, p53 and its downstream apoptosis protein, caspase7, could not be observed in 2-DE. This result might be from the low amount of proteins and low sensitivity of gel staining. Nevertheless, the mRNA expression of prohibitin, p53 and caspase7 were detectable using RT-PCR. To confirm this result, annexin V-

FITC/PI assay revealed that IFN- $\alpha$  induced cell apoptosis. The researchers indicated that IFN- $\alpha$  induced prohibitin to bind to the p53 protein and allow infected cells to apoptosis (23). Although 2-DE is commonly used, there is some limitations including poor reproducibility (gel-to-gel variation), low sensitivity in identifying proteins with extreme pH (too low pH or too high pH), low sensitivity in identifying proteins which their molecular weights are too low or too high, labor-intensive technique, time-consuming as well as detection of low abundance or hydrophobic proteins (88-90). To overcome these limitations, gel-free proteomics has been developed. This technique, complex mixture of proteins are digested in solution and subsequently fractionated and analyzed by tandem mass spectrometry (88, 90, 91).

### **Quantitative proteomics**

MS is a powerful tool for protein identification; however, it does not provide the quantitative information from peptide intensities. Because of the efficiency of ionization, signals generated from MS are variable from peptide to peptide. Quantitation strategies using stable isotope labeling provide qualitative (protein identification) and quantitative information. Stable isotope labeling allows samples more than 2 different states to be analyzed in a single run (92). The chemical and physical properties of one isotope-labeled peptides are identical to the other isotope-labeled peptides but the difference in mass introduced by isotope labeling which can be distinguished by MS. The relative intensities of different isotopes generated by MS reflect the relative quantity of the peptides in various states of the samples (93). Each method of isotopic labeling can be introduced into different sample level either in viable cell in cell culture (stable isotope labeling by amino acids in cell culture, SILAC) or in protein level (isotope-coded affinity tag, ICAT) or in peptide level (isobaric peptide tags for relative and absolute quantification (iTRAQ), tandem mass tags (TMT), and dimethyl labeling) via metabolic or chemical labeling (94, 95). Stable isotope labeling by amino acids in cell culture (SILAC) is based on metabolic labeling in cultured cells. The isotopically labeled amino acids such as  $^{13}\text{C}_6$ -Arg are added to the culture medium; therefore, these isotopes incorporate into the cells during their growth. This labeling will complete when the cells grow and divide into several passages. After that,



the differentially labeled cells are combined, digested and analyzed by MS. The quantitative information is obtained by comparing the relative peak intensities generated from these differentially labeled peptides at the MS1 scan (96). Isobaric peptide tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT) label the samples at the peptide level by covalent labeling of primary amine both N-termini and side chain of lysine residues with isobaric tags. The isobaric tag contains 3 part namely, a reporter group, a mass balance group, and a peptide-reactive group. At the MS1 scan, all differentially labeled peptides have identical m/z due to the constant total mass of isobaric tags. Therefore, the relative quantification is obtained from the MS2 scan. Stable isotope dimethyl labeling is a simple, reliable and cost-effective quantitative proteomic method based on chemical labeling at the peptide level. The principle of this techniques is reductive amination. The formaldehyde interacts with the  $\epsilon$ -amino group of the side chain of a lysine residue or the N-terminus of a peptide (primary amine) to form Schiff base and these intermediates are subsequently reduced by sodium cyanoborohydride to form a secondary amine and convert to dimethyl amines as shown in the following Figure 1.

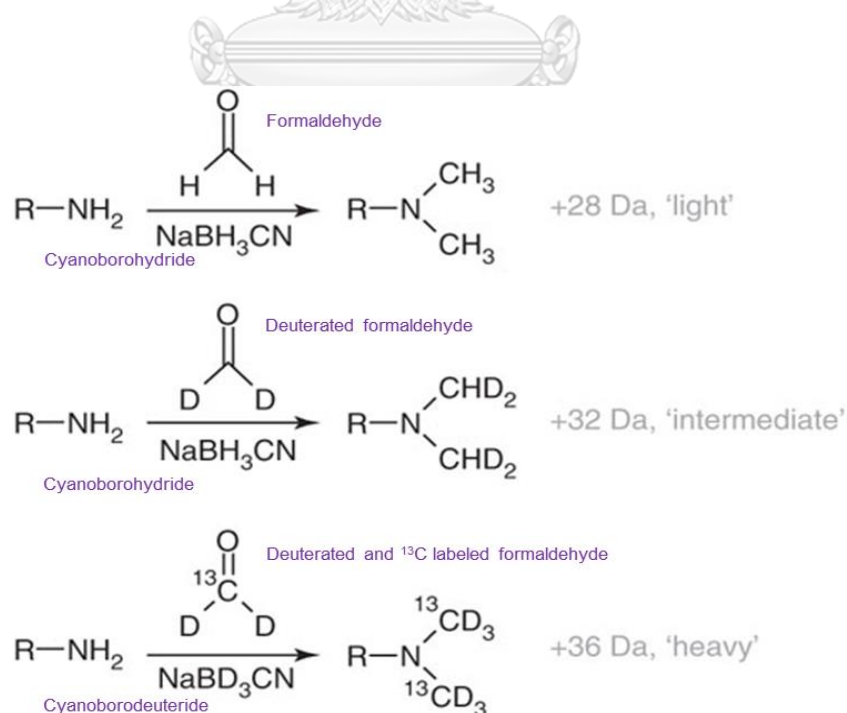


Figure 1. The reaction of dimethyl labeling.

Multiplex at least 3 labeling can be generated by different combinations of isotopomers of formaldehyde and cyanoborohydride:  $\text{CH}_2\text{O}/\text{NaBH}_3\text{CN}$  (light),  $\text{CD}_2\text{O}/\text{NaBH}_3\text{CN}$  (medium) and  $^{13}\text{CD}_2\text{O}/\text{NaBD}_3\text{CN}$  (heavy). The first combinations generate a mass increase of 28 Da while the medium and heavy labeling generate a mass increase of 32 Da and 36 Da, respectively. The labeled peptide differ in mass by only 4 Da between light and medium labeling and between medium and heavy labeling whereas light and heavy labeling have mass difference 8 Da. Like SILAC, the quantification of this method is based on MS1-scan (97, 98). The advantages and limitations of each labeling was shown in the Table 6.

*Table 6. Comparison of strengths and weaknesses of each labeling technique*

	Strengths	Limitations
SILAC	Introduction of isotope label at the cell or organism level No limits to amount of sample to be labeled	Not applicable to human samples Expensive to culture and reach full incorporation Labeled arginine might convert to proline
Dimethyl labeling	Cheap reagents Quick reaction Can be automated by performing the reaction online with LC-MS Applicable to any sample (animal/human tissue samples) Capable of labeling of sub-micrograms to milligrams of sample	Introduction of isotope label at peptide level Small isotope effect in LC separation
iTRAQ	Labeling of up to ten different samples No increased complexity at MS1 level Applicable to any sample (animal/human tissue samples)	Introduction of isotope label at peptide level Reagents are not very stable Expensive reagent Choice of the mass spectrometer is limited to those capable of measuring at low m/z

## CHAPTER III

### MATERIALS AND METHODS

#### **Experimental design and statistical rationale.**

Mixing of dimethyl-labeled samples corresponding to IFN- $\lambda$ 3, IFN- $\alpha$ 2a, and control (PBS) treatments were performed at  $n = 5$  vs. the typical  $n = 3$  in order to emphasize depth. Only biological replicates were performed. The normality of all proteomics, western-blotting and qPCR data was evaluated with the Shapiro-Wilk test. In all cases, the unpaired Student's t-test or one-way ANOVA was selected when the distribution of the data was normal; otherwise the Mann-Whitney U test was applied. For proteomic analysis, peak intensity  $\log_2$  ratios (L/M, L/H, M/H) were compared against a value of 0 (no change,  $\log_2(1)$ ). Significance was based on the following criterion:  $p$ -value  $< 0.05$  or cases where proteins were detected in only one condition making standard statistical analysis inapplicable, with the requirement that these proteins must be identified in at least 4 of 5 experiments. Proteins not fulfilling the above criteria as well as those appearing in fewer than 3 experiments, were excluded from downstream analysis. Regarding western-blotting and qPCR experiments,  $n=3$  was set. One-way ANOVA was performed for these experiments with  $p$ -value  $< 0.05$  considered significant. In the case of DAVID enrichment analysis, all proteins identified by mass spectrometry were input as background. When Fisher-based enrichment analysis was performed against an in-house database of proteomic/transcriptomic studies, resulting  $\log(p$ -values) were conservatively adjusted by subtracting the  $\log$  value corresponding to the total of all possible study/study combinations in the database.

#### **Chemicals and reagents.**

Culture media and all supplements used in this study were purchased from Gibco. Recombinant IFN- $\lambda$ 3 (5259-IL-025) was purchased from R&D Systems as a lyophilized form and dissolved to a concentration of 100  $\mu\text{g/ml}$  in sterile PBS containing 0.1% BSA. Aliquots of reconstituted IFN- $\lambda$ 3 were stored at  $-80^\circ\text{C}$  until use.

Recombinant IFN- $\alpha$ 2a (11100-1) was purchased from pbl assay science as a solution. The Lot No. of this product is 6336 which activity and specific activity are  $1.68 \times 10^8$  units/ml and  $7.3 \times 10^8$  units/mg, respectively. The concentration of IFN- $\alpha$ 2a was adjusted to 100  $\mu$ g/ml in PBS containing 0.1% BSA and kept at  $-80^\circ\text{C}$  until use. All reagents used in cDNA synthesis and qPCR were obtained from Applied Biosystems. Formaldehyde- $d_2$  and cyanoborodeuteride were purchased from Cambridge Isotope Laboratories (CIL). Triethylammonium bicarbonate (TEAB), 50% formic acid (FA), sodium deoxycholate (SDC), formaldehyde, deuterated  $^{13}\text{C}$ -labeled formaldehyde, sodium cyanoborohydride, and thiazolyl blue tetrazolium bromide (MTT) powder were purchased from Sigma. All reagents used in SDS-PAGE electrophoresis were purchased from Bio-Rad Laboratories. Anti-SAMHD1 (#12361), anti-STAT1 (#9175), and anti-GAPDH (#5174) antibodies were purchased from Cell Signaling Technology (CST). Anti-OAS3 (ab64163) was purchased from Abcam. Odyssey Blocking Buffer and IRDye 680RD secondary antibody were obtained from LICOR-Biosciences. Trifluoroacetic acid (TFA) and 25% ammonia solution were purchased from Merck. Dithiothreitol (DTT) and iodoacetamide (IA) were purchased from GE Healthcare. All other reagents not mentioned here or not specified elsewhere were obtained from Thermo.

#### **Cell culture and cell stimulation.**

HepG2.2.15 cells are stable HBV-transfected cells derived from a hepatoblastoma HepG2 cell line (20). These cells contain a plasmid which expresses the complete genome of HBV, which integrates into host cellular DNA (20, 99). Because HBV within HepG2.2.15 replicates and secretes HBsAg, HBeAg and HBV DNA into the culture media, HepG2.2.15 has been widely used as a model of chronic hepatitis B (22, 99). The HepG2.2.15 cell line was kindly provided from Professor Antonio Bertoletti (Singapore Institute for Clinical Sciences, A\*Star). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% MEM Non-Essential Amino Acids (MEM-NEAA), 1% Penicillin/Streptomycin and Geneticin (G418) at a final concentration of 150  $\mu$ g/ml. The cultured cells were grown in a humidified incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . One million HepG2.2.15 cells were seeded into 6-well plates with 1 ml media and maintained in

complete DMEM for 24 hours. For determining the effects of IFN- $\lambda$ 3 on HepG2.2.15, these cells were left untreated or treated with 1, 10, 100 or 1000 ng/ml of IFN- $\lambda$ 3 and incubated for another 24 hours. For proteomics analysis, HepG2.2.15 cells were plated at  $5 \times 10^6$  cells in T-75 flasks and grown in complete DMEM for 24 hours at 37°C. These cells were subsequently cultured in media with 100 ng/ml of IFN- $\lambda$ 3 or 100 ng/ml of IFN- $\alpha$ 2a or PBS (control) for another 24 hours. For further qPCR experiments with an optimized concentration of IFN- $\lambda$ 3, HepG2.2.15 cells were stimulated with or without 100 ng/ml of IFN- $\lambda$ 3 for 0, 8, 16, and 24 hours.

#### **RNA isolation, reverse transcription, and qPCR for gene expression.**

TRIzol Reagent was used to extract total RNA as specified in the accompanying manual. Briefly, the culture medium was discarded from culturing plates and 1 ml of TRIzol Reagent was directly added into each well followed by mixing the cells several times and incubating for 5 minutes at room temperature to ensure that the cells were completely lysed. Phase separation was generated by adding 100  $\mu$ l BCP (bromochloropropane, MRC), used instead of chloroform, with vigorous shaking. Following incubation at room temperature for 3 minutes, the mixtures were centrifuged at 12,000 x g for 15 minutes at 4°C. The colorless aqueous phase containing RNA was transferred into a new tube, precipitated by absolute isopropanol with incubation at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. Subsequently, RNA pellet was washed with 75% ethanol followed by centrifugation at 7,500 x g for 5 minutes at 4°C to remove any salts. The dried pellet RNA was re-suspended with RNase-free water and incubated in heat box at 60°C for 10 minutes. The concentrations of RNA were measured by spectrophotometer (Nanodrop, Thermo SCIENTIFIC) and their purities were determined by the ratio of absorbance at wavelength of 260 nm to 280 nm. RNA was stored at -80°C until further use. Complementary DNA (cDNA) synthesis was carried out using the Taqman Reverse transcription kit. Two hundred micrograms of RNA were served as template in the reaction. The master mix was prepared according to manufacturer's recommendation described in appendix. Conditions for reverse transcription were as specified in the manual that was 25°C for 10 minutes, followed by 48°C for 30 minutes and 95°C for 5

minutes. cDNA was kept at -20°C until further use. Relative gene expression was measured with the ABI Prism 7500 sequence detection system (Applied Biosystems). All primers and probes were designed with the “primer express 3” program (Applied Biosystems), and are shown in Supplementary Table. The master mix for all target genes composed of RNase-free water, 20 µM of each forward and reverse primers and Power SYBR Green PCR Master Mix. For housekeeping gene 18s rRNA, the components of master mix were similar to those of target genes but probe was utilized to monitor gene amplification instead of SYBR green dye. To monitor gene amplification, the intensity of fluorescence from the 18S housekeeping gene was monitored via *Taqman* probe while all target gene levels were monitored via SYBR green dye. To test the specificity of SYBR green dye, melting curve analysis was conducted for all target genes. The condition of amplification for both target and housekeeping genes was 1 cycle at 95°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The Melting curve analysis was performed only in the reactions using SYBR Green as a detector to ensure the specificity of primers and target genes. The intensity of fluorescent generated by SYBR Green or probe which was higher than threshold was measured as a Ct value. Relative gene expression was calculated with the  $2^{-ddCt}$  method. The Student's *t*-test and one-way ANOVA were used to compare the relative expressions of target genes in cells treated with various doses of IFN-λ3. A p-value less than 0.05 was considered significant.

#### **DNA extraction and absolute qPCR.**

After trypsinization and PBS-washing, cellular DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. In brief, cell pellets were re-suspended in PBS followed by adding QIAGEN proteinase and AL buffer. After vortex-mixing and incubation at 56°C for 10 minutes, absolute ethanol was added to the mixtures. These mixtures were applied into QIAamp Spin columns and centrifuged at 8000 x rpm for a minute. Buffer AW1 was added into the columns followed by centrifugation at 8,000 x rpm for 1 minute. Before DNA elution, Buffer AW2 was added into the columns and then centrifuged at 14,000 x rpm for 3 minutes. Distilled water was used as an eluent to elute DNA from columns and DNA was kept

at -20°C until use. Quantification of HBV viral load was performed by absolute quantitative real-time PCR using the ABI Prism 7500 sequence detection system. For ease of plasmid amplification, we used plasmids containing only the HBV gene *preS1* as a surrogate marker for HBV DNA (*preS1* was chosen because it is highly conserved across all HBV genotypes). First, *preS1* plasmids were extracted from *E. coli* transformants with the GeneJET Plasmid Miniprep Kit (Fermentas). The concentration of extracted plasmids was measured by spectrophotometer and copy/ $\mu$ l was determined by following formula.

$$\text{Copy}/\mu\text{l} = \frac{6.02 \times 10^{23} \text{ (copy/mol)} \times \text{DNA amount (g}/\mu\text{l)}}{\text{DNA length [Plasmid size + inserted gene]}(\text{bp}) \times 600}$$

The plasmid concentration was adjusted and diluted in a range of  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2$  copy/ $\mu$ l. These concentrations were used to construct a standard curve. Both standard and sample *pres1* were amplified at the same time using conditions described above. The fluorescent intensities were specified as  $C_t$  values. For standards,  $C_t$  values at the above concentrations were used to plot a standard curve. This curve and sample  $C_t$  values were used to calculate the amount of HBV DNA in the samples. The Student's *t*-test and one-way ANOVA were used to compare viral loads in cells treated with various levels of IFN- $\lambda$ 3, as well as untreated cells. A *p*-value less than 0.05 was considered significant.

#### **MTT assay.**

HepG2.2.15 cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells per well and incubated for 24 hours. The culture media was removed and replaced with fresh complete media in the absence or presence of IFN- $\lambda$ 3 (1, 10, 100 and 1000 ng/ml). The cells were further incubated for 24 hours followed by addition of 10  $\mu$ l 5 mg/ml MTT solution in each well with gentle shaking. Due to photosensitivity of MTT, this step was performed with light protection. After 4h incubation, the resulting purple formazan crystals were dissolved with dimethyl sulfoxide (DMSO, Riedel-deHaën) and subsequently measured with ELISA plate reader (Thermo) at wavelength 570 nm. The absorbance values of cells treated with each concentration of drug were compared to

that of control (untreated) cells and % cell viability was calculated. This experiment was performed in biological triplicate.

#### **Protein extraction and in-solution digestion.**

After trypsinization, the cells were lysed in 5% SDC in 25 mM TEAB containing 1X protease inhibitor cocktail (Thermo), followed by sonication. All cell debris was removed by centrifugation at 15,000 x g for 10 minutes and supernatant protein concentrations of each sample were measured via BCA Protein Assay (Thermo). Equal amounts of protein from treated and untreated HepG2.2.15 were reduced and alkylated by dithiothreitol (DTT) treatment for 30 minutes at 37°C and iodoacetamide (IA) treatment for 30 minutes at room temperature in the dark, respectively. These samples were further quenched with DTT at least 15 minutes at room temperature before incubating with trypsin (Promega) at a ratio of 1:50 at 37°C overnight. These mixtures were incubated with 0.5% trifluoroacetic acid (TFA) for 30 minutes and then centrifuged to remove SDC precipitate. The amount of tryptic peptides of each sample was determined with the Pierce Quantitative Fluorometric Peptide Assay (Thermo).

#### **Dimethyl labeling.**

Equal amount of peptides from untreated, IFN- $\alpha$ 2a treated, and IFN- $\lambda$ 3 treated HepG2.2.15 were reconstituted in 100 mM TEAB and labeled with light reagents, medium reagents, and heavy reagents, respectively, as shown in the Table 7 for an hour at room temperature. The 1% (vol/vol) of ammonia solution and FA were sequentially used to stop the reaction. These steps were performed on ice. Labeling efficiency was tested before combining these three samples. The mixed labeled-peptides were dried in a SpeedVac centrifuge at room temperature.



Table 7. Dimethyl labeling reagents

	Untreated	IFN- $\alpha$ 2a treated	IFN- $\lambda$ 3 treated
Dimethyl labeling	Light	Medium	Heavy
Formaldehyde isotope	4% CH <sub>2</sub> O	4% CD <sub>2</sub> O	4% <sup>13</sup> CD <sub>2</sub> O
Cyanoborohydride isotope	0.6 M NaBH <sub>3</sub> CN	0.6 M NaBH <sub>3</sub> CN	0.6 M NaBD <sub>3</sub> CN

### High-pH reversed phase fractionation.

The pooled peptides were reconstituted in 0.1% TFA and separated into 10 fractions to reduce complexity using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo) according to the instructions. Briefly, the high-pH step-elution solutions were prepared as following in Table 8. The resins in spin column provided in the kit were twice conditioned in acetonitrile followed by centrifugation at 5,000 x g for 2 min. Next, the resins were washed twice in 0.1% TFA followed by centrifugation at 5,000 x g for 2 min. The dissolved peptides were loaded onto the column and centrifuged at 3,000 x g for 2 min. The column was washed with LC-MS graded water followed by centrifugation at 3,000 x g for 2 min. The elution solution no. 1 was applied onto the column followed by centrifugation at 3,000 x g for 2 min and collected the eluate as fraction no.1. These steps were repeated using the remaining elution solutions. Eluates of each fraction were dried in a vacuum centrifugation before LC-MS/MS analysis.

*Table 8. The high-pH step-elution solutions preparation*

Fraction no.	% ACN	ACN ( $\mu$ l)	0.1% Triethylamine ( $\mu$ l)
1	2.5%	50	1950
2	4.0%	80	1920
3	5.5%	110	1890
4	7.0%	140	1860
5	8.5%	170	1830
6	10.0%	200	1800
7	12.5%	250	1750
8	17.5%	350	1650
9	25.0%	500	1500
10	50.00%	1000	1000

#### LC-MS/MS and analysis.

The fractionated samples were resuspended in 0.1% FA to a final volume of 15  $\mu$ l prior to MS injection. The peptides were then analyzed via an EASY-nLC1000 system (Thermo) coupled to a Q-Exactive Orbitrap Plus mass spectrometer (Thermo) equipped with a nano-electrospray ion source (Thermo). The peptides were eluted in 5-40% acetonitrile in 0.1% FA for 70 min followed by 40-95% acetonitrile in 0.1% FA for 20 min at a flow rate of 300 nL/min. The MS methods included a full MS scan at a resolution of 70,000 followed by 10 data-dependent MS2 scans at a resolution of 17,500. The normalized collision energy of HCD fragmentation was set at 32%. An MS scan range of 350 to 1400 m/z was selected and precursor ions with unassigned charge states, a charge state of +1, or a charge state of greater than +8 were excluded. A dynamic exclusion of 30 s was used. The peaklist-generating software used in this study was Proteome Discoverer™ Software 2.1 (Thermo). The SEQUEST-HT search engine was employed in data processing. MS raw data files were searched against the Human Swiss-Prot Database (20,219 proteins, June 2017) and the Hepatitis B Virus Swiss-Prot Database (225 proteins, June 2017), as well as a list of common protein contaminants ([www.thegpm.org/crap/](http://www.thegpm.org/crap/)). The following parameters were set for the search: (1)

digestion enzyme: trypsin; (2) maximum allowance for missed cleavages: 2; (3) maximum of modifications: 4; (4) fixed modifications: carbamidomethylation of cysteine (+57.02146 Da), as well as light, medium, and heavy dimethylation of N-termini and lysine (+28.031300, +32.056407 and +36.075670 Da); (5) variable modifications: oxidation of methionine (+15.99491 Da). The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.02 Da, respectively. Known contaminant ions were excluded. The Proteome Discoverer decoy database together with the Percolator algorithm were used to calculate the false positive discovery rate of the identified peptides based on *Q*-values which were set to 1%. The Precursor Ions Quantifier node in Proteome Discoverer™ Software was employed to quantify the relative MS signal intensities of dimethyl labeled-peptides. The control channels were used as denominators to generate abundance ratios of IFN- $\lambda$ 3/control and IFN- $\alpha$ 2a/control. Log<sub>2</sub> of the normalized ratio was used to calculate the mean and standard deviation of fold change across all five biological replicates. When these ratios were found in less than 3 experiments, the relevant proteins were excluded. Significantly differentially regulated proteins were determined by Mann–Whitney U test and unpaired t-tests with p-value < 0.05 considered significant.

### **Bioinformatics.**

We compiled a list of defense response to virus using a variety of resources as follows. The online resource Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8, <https://david.ncifcrf.gov/>) and Reactome (<https://reactome.org/>) were employed to classify the proteins regulated by IFN- $\lambda$ 3 into functional categories using all proteins identified by MS as background (for DAVID). We used terms such as ‘antiviral’, ‘antigen processing/presentation’ to help extract a custom list of broad antiviral proteins. Additionally, the list contains proteins involved in the HBV life-cycle that were derived from manual literature curation. Further analysis of up-/down-regulated proteins was performed against an in-house database currently under assembly. The goal is simply to expand on DAVID’s method by emphasizing datasets from individual MS and RNA-seq studies. Probabilities presented here are generated using Fisher’s exact test with a background proteome size of 10,000 and are

unadjusted. For consistency, lists of proteins with altered expression in our own HBV work are generated by filtering out all cases where differential expression is not accompanied by a p-value < 0.05 (i.e. fold-change is not a factor); the lists are then subjected to Fisher analysis.

#### **Western blotting for MS confirmation.**

The 15 mg of protein from IFN- $\lambda$ 3-treated and untreated HepG2.2.15 was subjected to SDS-PAGE electrophoresis. Both 10% separating and 4% stacking SDS-PAGE were prepared as describe in appendix. The electrophoresis was performed with a constant voltage 120V for 75 minutes. Proteins were transferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). The transferred membranes were stained with Ponceau S dye (AppliChem) to ensure that proteins were completely transferred onto the membranes followed by destaining with MilliQ. The membranes were blocked with Odyssey Blocking Buffer for an hour at room temperature with shaking, washed three times with TBST for 15 min and probed with anti-OAS3, anti-SAMHD1, anti-STAT1, or anti-GAPDH antibodies at 1:2,500 dilution at 4°C overnight. After washing three times with TBST, the probed membranes were incubated with IRDye 680RD secondary antibody at 1:10,000 dilution for 1 hour in the dark followed by three washes with TBST. The membranes were visualized using Odyssey CLx (LICOR-Biosciences).

#### **Data deposition.**

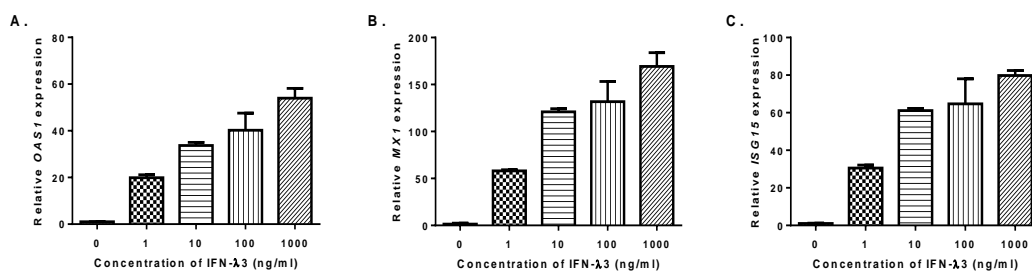
The mass spectrometry proteomics data, including annotated spectra for all modified peptides and proteins identified on the basis of a single peptide, have been deposited to: 1) the ProteomeXchange Consortium via the PRoteomics IDentifications (PRIDE) partner repository with the dataset identifier PXD007896 and 2) the MS-Viewer (<http://msviewer.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msviewer>) with the following keys: dhjzinh2g0, tjnx2fzkzu, 3zxxxfanwm, ac4wmxx0tv, and ao9nga6qqm.

## CHAPTER IV

### RESULTS

#### Validation of responses to IFN- $\lambda$ 3 treatment in HBV-transfected hepatoblastoma cell line model

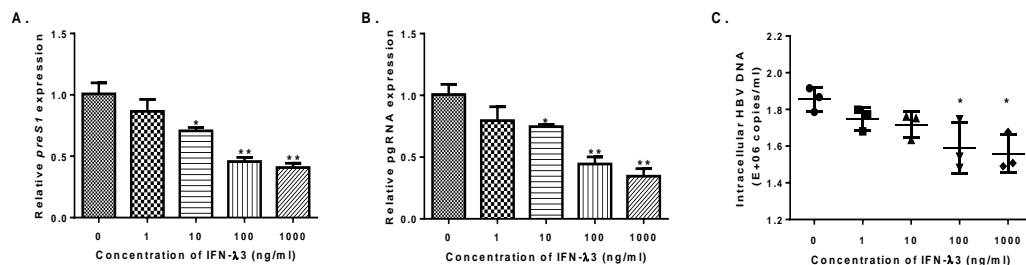
HepG2.2.15 is a hepatoblastoma cell line that contains a stable HBV expression plasmid that has been validated as an HBV infection model in previous reports (20, 99). The response to type III IFN treatment in HepG2.2.15 cells has not been reported. To determine whether HepG2.2.15 cells respond to IFN- $\lambda$ 3, we performed qPCR to investigate the expression of the classical interferon-stimulated genes (ISGs), namely *OAS1*, *MX1* and *ISG15* in HepG2.2.15 cells treated with various amounts of IFN- $\lambda$ 3 for 24h. Figure 2 shows that IFN- $\lambda$ 3 could significantly increase the expression of these 3 ISGs in a dose-dependent manner. These results indicated that HepG2.2.15 cells responded to IFN- $\lambda$ 3 stimulation.



**Figure 2. Relative quantification of ISG transcripts in HepG2.2.15 treated with IFN- $\lambda$ 3 for 24 h.**

The relative expression of *OAS1* (A), *Mx1* (B) and *ISG15* (C) genes in HepG2.2.15 cells 24h post-stimulation with different doses of IFN- $\lambda$ 3 is shown using Mean  $\pm$  SEM. As with type I IFNs, all 3 ISGs were significantly elevated by IFN- $\lambda$ 3 treatment ( $p < 0.001$ ) at all doses compared with a PBS control. These experiments were performed in triplicate.

For thoroughness, we investigated the anti-HBV effects of IFN- $\lambda$ 3 by determining the differential changes at 3 points in the viral life-cycle including the levels of *preS1* (typically used as a representative gene for HBV transcripts, given its high conservation across all genotypes), replicative intermediate pre-genomic RNA (pgRNA), and intracellular HBV DNA (both rcDNA and cccDNA). qPCR was performed on HepG2.2.15 RNA following treatment with various amounts of IFN- $\lambda$ 3 for 24 hours. As shown in Figure 3A and 3B, IFN- $\lambda$ 3 reduced both *preS1* and pgRNA expression compared with control in a dose-dependent manner. Measurement of intracellular HBV DNA showed that copy numbers of virus in IFN- $\lambda$ 3-treated HepG2.2.15 cells were diminished in a dose-dependent manner relative to control (Figure 3C). The reduction reached significant levels when the doses of IFN- $\lambda$ 3 were 100 ng/ml (p-value = 0.04) and 1,000 ng/ml (p-value = 0.0134). Collectively, these results indicated that IFN- $\lambda$ 3 inhibits HBV replication in HepG2.2.15 at the given time point.



**Figure 3. Effects of IFN- $\lambda$ 3 on HBV replication.**

HepG2.2.15 cells were incubated with IFN- $\lambda$ 3 (1, 10, 100 and 1,000 ng/ml) or treated with PBS for 24 hours. The relative transcript expression and the amount of HBV DNA are shown as Mean  $\pm$  SEM. IFN- $\lambda$ 3 significantly inhibited *preS1* and pgRNA expression at doses equal to or greater than 10 ng/ml (A and B). IFN- $\lambda$ 3 significantly suppressed viral propagation at doses equal to or greater than 100 ng/ml (C). These experiments were performed in triplicate. (\* represents a p-value less than 0.05 and \*\* represents a p-value less than 0.01).

Before we investigated the cellular response to IFN- $\lambda$ 3, the toxicity of this drug was considered. The MTT cytotoxicity assay was performed to determine HepG2.2.15 viability under distinct concentrations of IFN- $\lambda$ 3. The percentage of viable cells is illustrated in Figure 4. The increasing doses of IFN- $\lambda$ 3 significantly promoted cell death

only at the highest two doses in this experiment, where the viable HepG2.2.15 cells reduced to 94% and 84% in 100 ng/ml and 1000 ng/ml conditions, respectively. Thus, we settled on 100 ng/ml of IFN- $\lambda$ 3 for further experiments because this dose showed the ability to significantly inhibit HBV replication with minimal cytotoxicity on HepG2.2.15 cells.

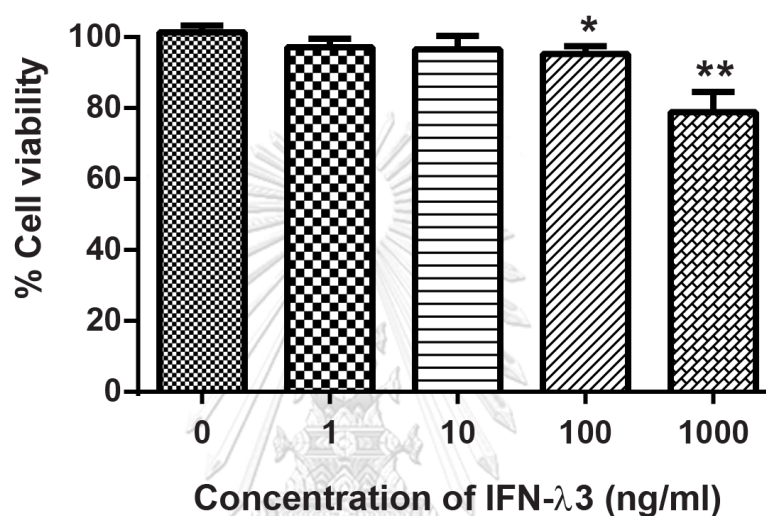


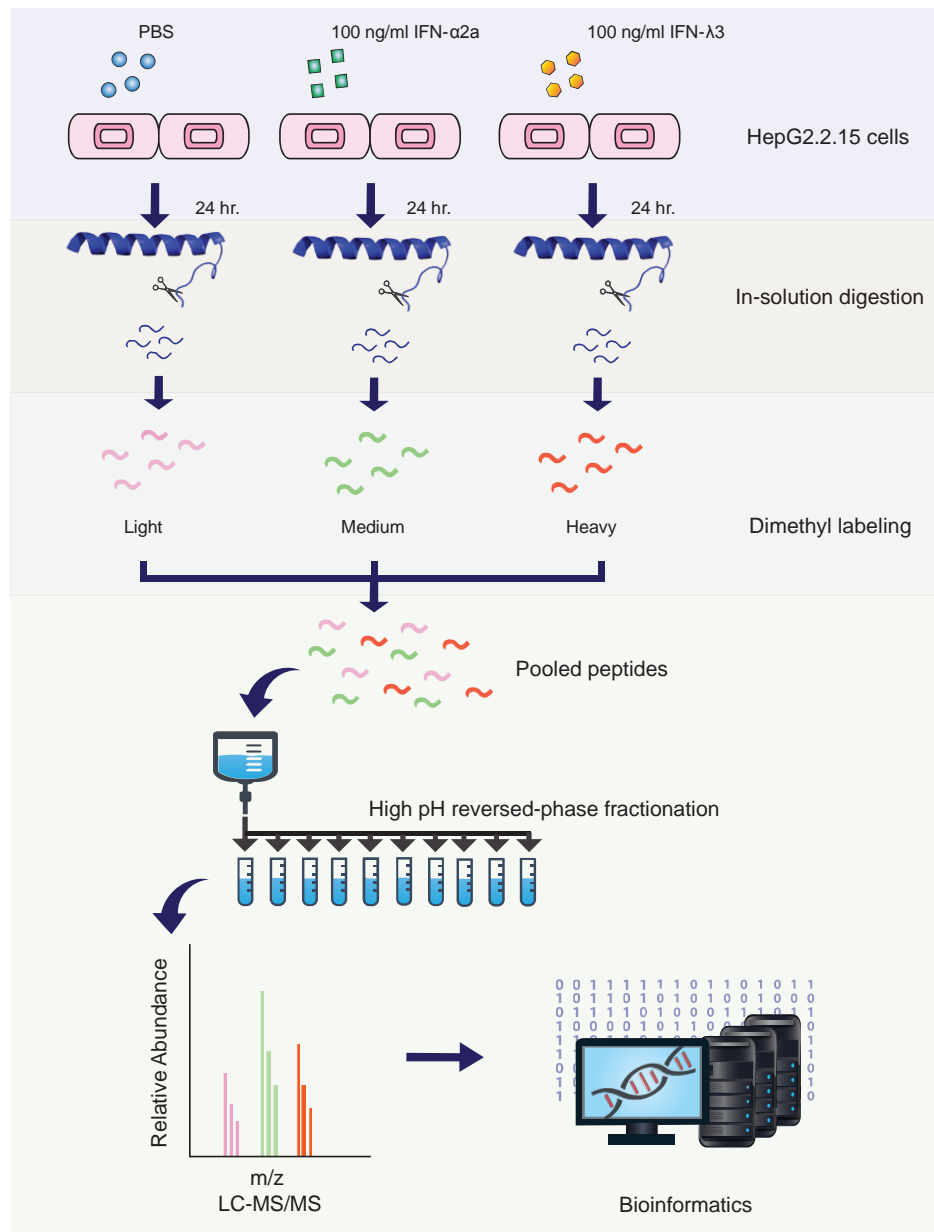
Figure 4. IFN- $\lambda$ 3 cytotoxicity assay.

The viability of HepG2.2.15 after IFN- $\lambda$ 3 treatment for 24 hours was determined by MTT assay. The percentage of cell viability is shown as Mean  $\pm$  SEM. IFN- $\lambda$ 3 showed minimal effect on cell viability when the doses were less than 1,000 ng/ml. These experiments were performed in triplicate. (\* represents a p-value less than 0.05 and \*\* represents a p-value less than 0.01).

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#### Quantitative proteomics analysis of IFN- $\lambda$ 3 responses in HepG2.2.15

Figure 5 shows the schematic workflow of this study. Briefly, untreated, IFN- $\alpha$ 2a-treated, and IFN- $\lambda$ 3-treated HepG2.2.15 cells were lysed and digested with trypsin. The tryptic peptides of these groups were labelled with light, medium, and heavy dimethyl reagents. Labeling efficiency was tested before combining these three samples and we found that greater than 99% of peptides were labeled as shown in Table 9. The pooled samples were fractionated for subsequent LC-MS/MS analysis.



**Figure 5. Quantitative proteomic workflow.**

HepG2.2.15 cells were treated with 100 ng/ml of IFN- $\lambda$ 3 and IFN- $\alpha$ 2a and PBS for 24 hours. Cell lysates of each group were digested and then labeled with different dimethyl reagents. After combining the 3 samples, these peptides were fractionated and then analyzed by LC-MS/MS.



Table 9. Dimethyl labeling efficiency

Labelling	Labeled peptides	Total peptides	% Efficiency
Light	5,775	5,786	99.81
Medium	5,232	5,255	99.56
Heavy	4,849	4,865	99.67

In total, 4,670 proteins were identified at a false discovery rate (FDR) of less than 1%, with 1,471 proteins identified on the basis of a single peptide. For the IFN- $\lambda$ 3 treatment condition, 2,904 proteins were identified in at least 3 of 5 replicates allowing evaluation of significance, shown in the corresponding volcano plot (Figure 6). The 737 proteins showed significant changes in abundance, with a slight bias towards down-regulated proteins in response to IFN- $\lambda$ 3 stimulation.

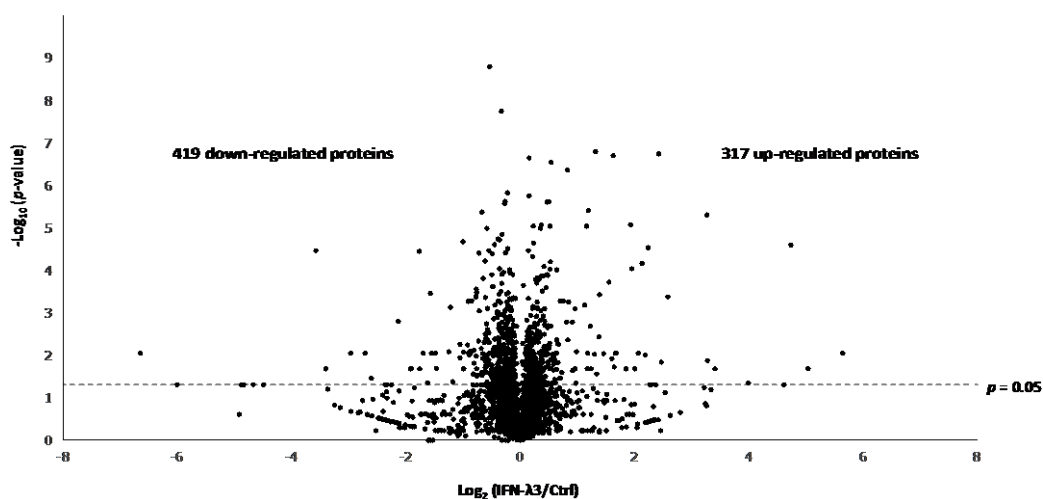


Figure 6. Volcano plot.

Volcano plot shows the distribution of identified proteins according to p-value and fold change, indicating significant level with a dashed line ( $p$ -value < 0.05).

Table 10 displays a list of significantly regulated proteins with  $\log_2(\text{IFN-}\lambda_3/\text{Ctrl})$  ratios of greater than |1|. We should point out that our primary intention in this work is to explore IFN- $\lambda_3$  effects on HepG2.2.15 cells; the effects of IFN- $\alpha_2a$  would be secondary, for the sake of comparison with IFN- $\lambda_3$  effects, and will be presented at the last part of the Results section.

Table 10. A list of significantly up-regulated (A) and down-regulated (B) proteins with  $\log_2(\text{IFN-}\lambda_3/\text{Control})$  ratios of greater than |1|.

A.

Accession number	Description	Gene ID	Average $\log_2$ ratios	Pathway
O14879	Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	4.74	Antiviral defense (Inhibit viral protein synthesis)
Q96AZ6	Interferon-stimulated gene 20 kDa protein	ISG20	3.28	Antiviral defense (Degrade viral RNA)
P05161	Ubiquitin-like protein ISG15	ISG15	3.27	Antiviral defense
Q15646	2'-5'-oligoadenylate synthase-like protein	OASL	2.58	RLR signaling pathway
O95786	Probable ATP-dependent RNA helicase DDX58	DDX58	2.47	RLR signaling pathway
Q9Y6K5	2'-5'-oligoadenylate synthase 3	OAS3	2.42	Antiviral defense (Degrade viral RNA)
Q29960	HLA class I histocompatibility antigen, Cw-16 alpha chain	HLA-C	2.24	Antigen processing and presentation
P09914	interferon-induced protein with tetratricopeptide repeats 1	IFIT1	2.19	Antiviral defense (Inhibit viral protein synthesis)
P42224	Signal transducer and activator of transcription 1-alpha/beta	STAT1	2.13	Type I and III IFN signaling
Q63HN8	Isoform 2 of E3 ubiquitin-protein ligase RNF213	RNF213	1.95	Ubiquitin proteasome pathway
Q01628	Interferon-induced transmembrane protein 3	IFITM3	1.93	Antiviral defense (Inhibit viral entry)
P52630	signal transducer and activator of transcription 2	STAT2	1.65	Type I and III IFN signaling
P29590	Protein PML	PML	1.63	Antiviral defense (Inhibit viral transcription)
P28838	cytosol aminopeptidase	LAP3	1.61	Antigen processing and presentation
Q10589	bone marrow stromal antigen 2	BST2	1.55	Antiviral defense (Inhibit viral egress)

Accession number	Description	Gene ID	Average Log <sub>2</sub> ratios	Pathway
Q6IA86	Isoform 6 of Elongator complex protein 2	ELP2	1.46	Regulation of transcription
Q08380	Galectin-3-binding protein	LGALS3BP	1.39	Cell adhesion
Q9H0P0	Cytosolic 5'-nucleotidase 3A	NT5C3A	1.38	Antiviral defense (Inhibit reverse transcription)
P35527	Keratin, type I cytoskeletal 9	KRT9	1.34	Intermediate filament organization
P41226	ubiquitin-like modifier-activating enzyme 7	UBA7	1.32	Ubiquitin proteasome pathway
Q9Y3Z3	deoxynucleoside triphosphate triphosphohydrolase SAMHD1	SAMHD1	1.19	Antiviral defense (Inhibit reverse transcription)
Q9BQE5	apolipoprotein L2	APOL2	1.22	Movement of lipids in the cytoplasm
P01892	HLA class I histocompatibility antigen, A-2 alpha chain	HLA-A	1.16	Antigen processing and presentation
Q9Y6A9	Signal peptidase complex subunit 1	SPCS1	1.13	Proteolysis

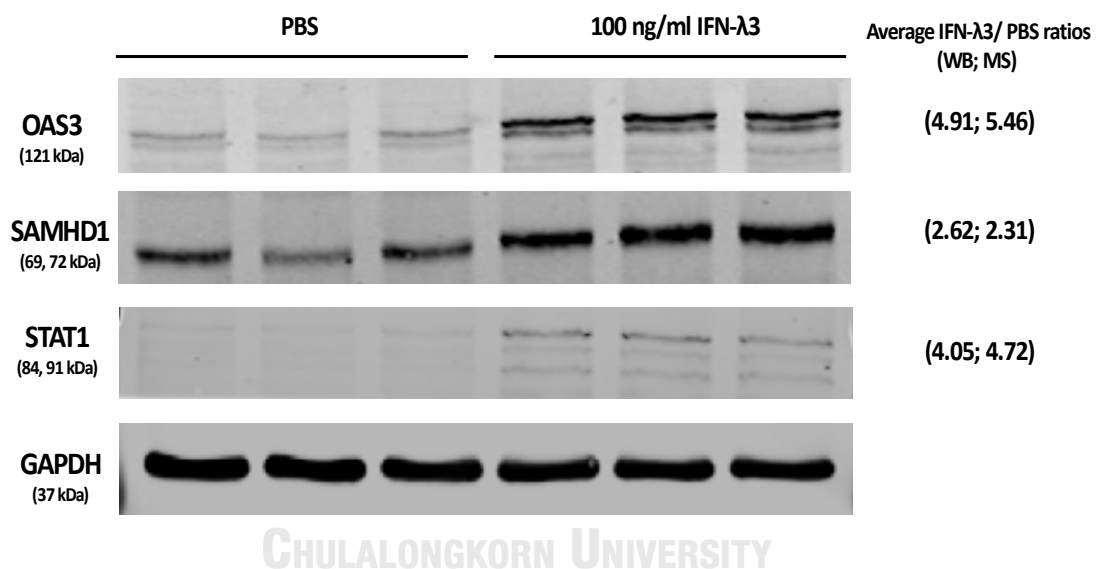
## B.

Accession number	Description	Gene ID	Average Log <sub>2</sub> ratios	Pathway
Q9BX93	Group XIIB secretory phospholipase A2-like protein	PLA2G12B	-1.05	Lipid catabolic process
Q15427	Splicing factor 3b subunit 4	SF3B4	-1.05	mRNA processing
Q9NPA8	Transcription and mRNA export factor ENY2	ENY2	-1.18	Regulation of transcription
P62158	Calmodulin	CALM3	-1.22	Regulation of synaptic vesicle exocytosis
O75410	Isoform 2 of Transforming acidic coiled-coil-containing protein 1	TACC1	-1.25	Cell proliferation
O75438	Isoform 2 of NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 1	NDUFB1	-1.47	Mitochondrial electron transport, NADH to ubiquinone
Q12929	Epidermal growth factor receptor kinase substrate 8	EPS8	-1.57	Actin polymerization-dependent cell motility
Q9NZD2	glycolipid transfer protein	GLTP	-1.62	Intermembrane lipid transfer
Q13126	Isoform 2 of S-methyl-5'-thioadenosine phosphorylase	MTAP	-1.76	Nucleobase-containing compound metabolic process
Q58FF7	Putative heat shock protein HSP 90-beta-3	HSP90AB3P	-3.58	Protein folding and response to stress

All HBV proteins (surface protein, core, polymerase, and HBx proteins) were identified in this study. However, calculation of significance could not be performed due to absence in multiple MS runs, with the exception of putative X-Core fused protein (which showed no significant change). Based on spectral counts, all of the HBV peptides were apparently expressed at very low levels relative to the entire HepG2.2.15 proteome (below 1 ppm, see in PRIDE partner repository as mentioned in Data Deposition). This finding is in agreement with a previous study that specifically examined intracellular HBV proteins in HepG2.2.15 cells, but found them to be

undetectable (100). Note that all HBV proteins, except HBV pol and HBx, are secreted (101-103), likely causing intracellular levels of these proteins to be scarce.

To confirm the results from MS analysis, three proteins known to be differentially regulated in response to HBV infection, namely 2'-5'-oligoadenylate synthase 3 (OAS3) (104), Sterile  $\alpha$  motif (SAM) and histidine/aspartate (HD)-containing protein 1 (SAMHD1) (105-107), and signal transducer and activator of transcription 1 (STAT1) (108, 109) were selected for validation by western blot analysis. Consistent with MS results, OAS3, SAMHD1 and STAT1 were up-regulated as a result of IFN- $\lambda$ 3 treatment (Figure 7).



**Figure 7. Validation of altered proteins by immunoblotting assay.**

SDS-PAGE was performed on 24h-treated and untreated lysates, followed by membrane transfer and incubation with anti-OAS3, anti-SAMHD1, anti-STAT1, and anti-GAPDH overnight. The proteins of interest were visualized using the LI-COR Odyssey system. Consistent with proteomic results, the expression of OAS3, SAMHD1 and STAT1 increased after IFN- $\lambda$ 3 treatment. These experiments were performed in triplicate.

To further explore the possible roles of transcriptional regulation for significantly altered proteins, we selected a number of proteins involved in antiviral processes for qPCR analysis. Figure 8 shows that upregulation was seen at both protein

and RNA expression levels without exception. However, no clear pattern emerged when down-regulated proteins were examined for transcript levels.

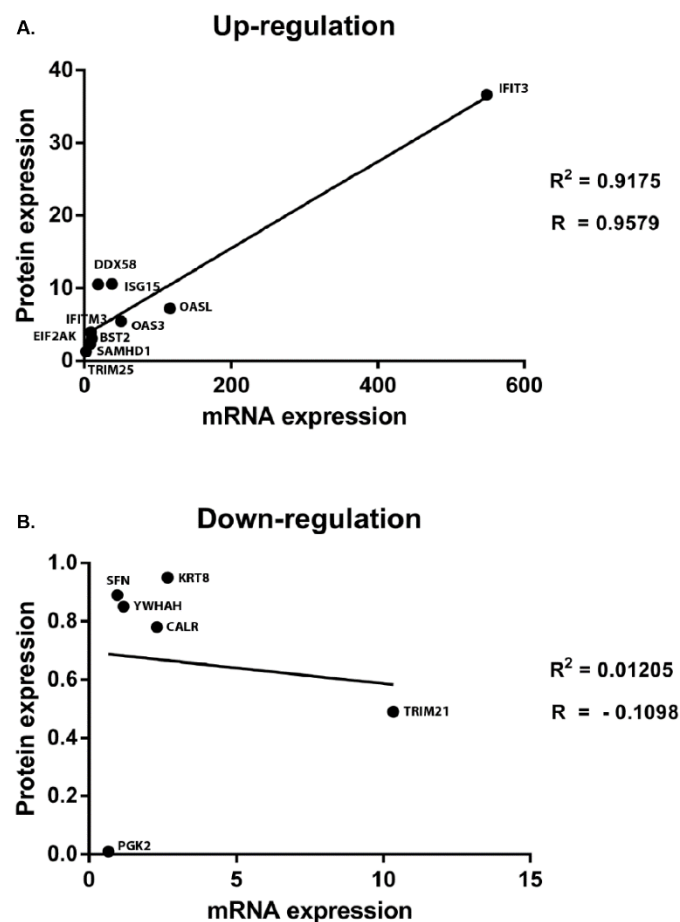


Figure 8. Correlation of mRNA and protein levels of up- and down-regulated proteins.

Various up- and down-regulated proteins were selected to investigate their transcript expressions by qPCR. Clear correlations between protein and mRNA expression were seen in up-regulated proteins (A) but not in down-regulated proteins (B).

### Bioinformatics analysis and anti-viral/immunomodulatory process mapping

The DAVID bioinformatics tool was used to classify and cluster significant functions of all up- and down-regulated proteins. Upon IFN- $\lambda$ 3 treatment, we found several biological processes expected to be canonically regulated on general IFN stimulation, such as immune response and viral infection defense. Additionally, however, several biological processes not previously emphasized upon interferon

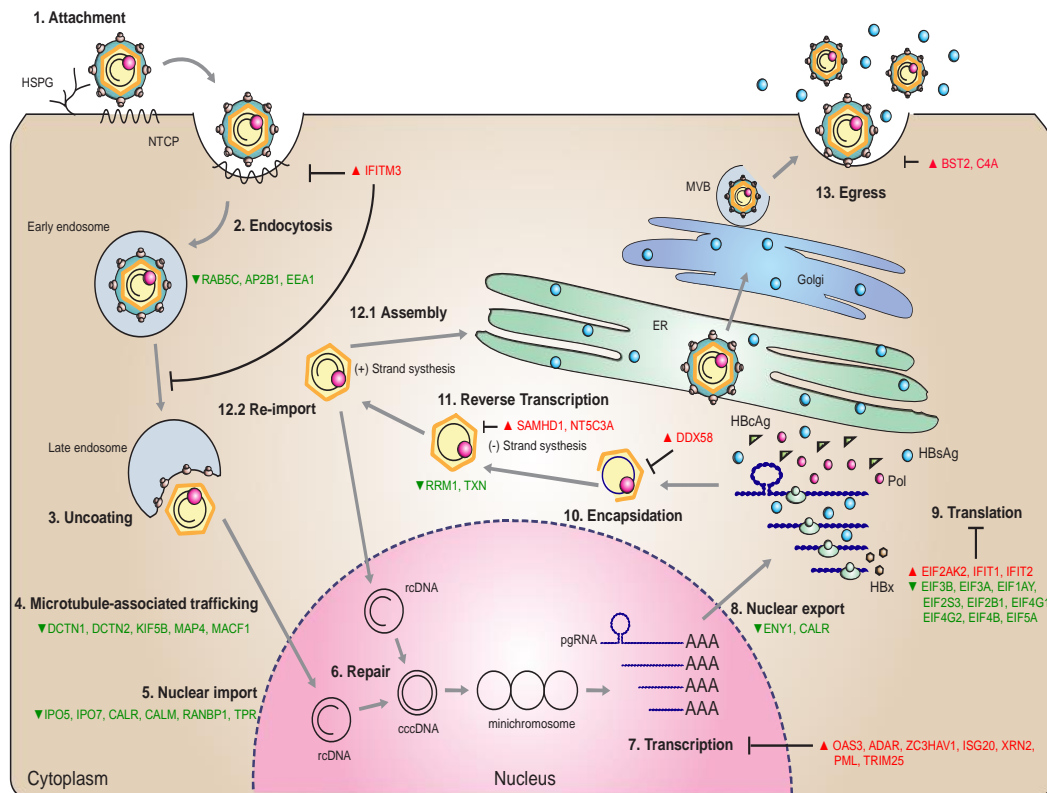
stimulation emerged from this work, including metabolism of RNA, major pathway of rRNA processing in the nucleolus and cytosol, selenoamino acid metabolism, peptide chain elongation, translation, prefoldin mediated transfer of substrate to CCT/TriC, transcription-coupled nucleotide excision repair (TC-NER), unfolded protein response (UPR), interleukin-23 signaling, and hypusine synthesis from eIF5A-lysine.

Following enrichment analysis, we conducted a protein-by-protein search for relevance to the viral processes. We manually searched the viral literature, as well as using DAVID's antiviral defense groups and antigen processing/presentation groups, to construct a map of points at which these proteins may partake in viral processes (Figure 9) (as described in Experimental Procedures). We also built a map displaying steps at which IFN- $\lambda$ 3 treatment could promote antigen processing and presentation of viral proteins. Finally, we constructed a map that highlights points at which IFN- $\lambda$ 3 stimulation may heighten expression of proteins in the RIG-I pathway that have been shown to be inhibited via HBV infection. Unless otherwise stated, we found no proteins that contradict the patterns that we report below.

Regarding the HBV life cycle, we identified many proteins which may be involved at numerous points (Figure 9). At the early steps of viral attachment, entry, and cytosolic release from endosomes (Figure 9, step 1 and 2), we detected an increased expression of IFITM3, known to alter intracellular cholesterol homeostasis, preventing viral fusion with endosomes in at least 4 out of 7 of the Baltimore viral groups (110). RAB5C and RAB7A, proteins previously implicated in viral trafficking (Figure 9, step 2), were down-regulated upon IFN- $\lambda$ 3 treatment (111), though RAB7A did not reach statistical significance. Two other trafficking proteins not mentioned in the viral literature, AP2B1 and EEA1, were significantly down-regulated. Regarding cytosolic-to-nuclear transport of the HBV capsid along microtubules (Figure 9, steps 4), we found that several molecules involved in microtubule assembly and function evinced decreased expression after IFN- $\lambda$ 3 treatment, consistent with the literature. These proteins were DCTN1, DCTN2, KIF5B, MAP4, and MACF1 (112, 113). For nuclear import (Figure 9, step 5), all identified proteins known to play roles herein (113, 114) were found to decrease as a result of IFN- $\lambda$ 3 stimulation (IPO5, IPO7, CALR, CALM, RANBP1,

and TPR). Within the nucleus (Figure 9, step 7), all identified ISG products shown previously to limit or degrade viral mRNA (49, 115, 116) were observed to be up-regulated: ADAR1, OAS3, ISG20, PML, XRN2, ZC3HAV1, and TRIM25. At the level of RNA export (Figure 9, step 8), ENY2 and CALR were down-regulated. Regarding translation (Figure 9, step 9), antiviral proteins (EIF2AK2, IFIT1, and IFIT2) involved in protein synthesis increased in treated cells, as shown in previous work with type I IFN treatment (49, 116, 117). In addition, almost all identified translation initiation factors (EIF1AY, EIF2S3, EIF2B1, EIF3B, EIF4G1, EIF4B, EIF4G2, and EIF5A) decreased in this study. Encapsidation follows translation of viral proteins (Figure 9, step 10). Interestingly, RIG-I was upregulated and has been shown to interfere with the interaction between pgRNA and HBV polymerase, required for encapsidation (118). Other effects of RIG-I upregulation are discussed below. Reverse transcription, which occurs within the capsid (Figure 9, step 11), has been demonstrated to be inhibited by SAMHD1 (HBV and HIV) (106, 119) and NT5C3A (IAV and CSFV) (120, 121); these proteins were upregulated in our study upon treatment. Proteins that might interfere with viral assembly (Figure 9, step 12.1) were not identified in this study. Finally at the egress step (Figure 9, step 13), two upregulated proteins thought to play a role in inhibiting HBV export, BST2 and C4A, were detected after IFN- $\lambda$ 3 stimulation.



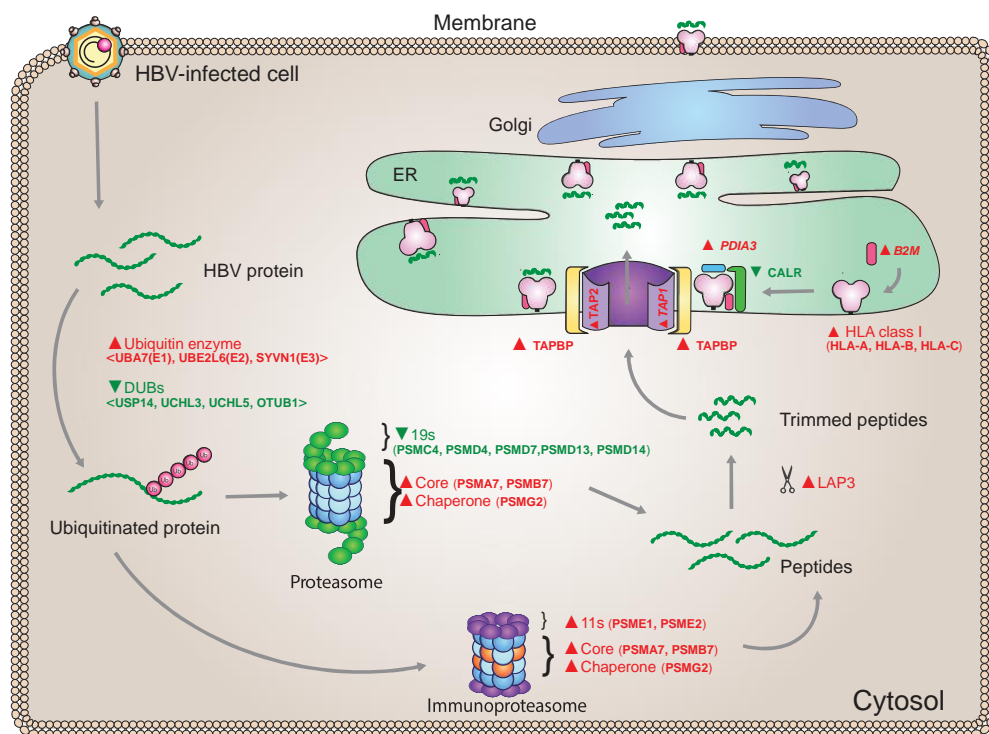


**Figure 9. Illustration of HBV life-cycle mapped to anti-viral proteins that were identified in this study.**

Antiviral proteins and host cellular factors of interest identified in this study were mapped into the HBV-life cycle. These proteins were identified at most steps of HBV replication. Proteins in red and green text represent up- and down-regulated proteins, respectively.

Regarding antigen processing and presentation, we identified 22 proteins that are involved in this process (Figure 10). Among proteins in the degradation-related ubiquitination cascade, we found only upregulation, specifically UBA7 (E1), UBE2L6 (E2), and SYVN1 (E3) after IFN- $\lambda$ 3 stimulation. Two ubiquitination enzymes were downregulated, but have not been reported to instigate degradation. All deubiquitinating enzymes (DUBs) identified in this study, i.e. USP14, UCHL3, UCHL5, and OTUB1, decreased as a result of IFN- $\lambda$ 3 treatment. Downstream of these processes, core proteasome components, PSMA7, PSMB7, and PSMG2, were up-regulated with no exceptions. Importantly, both cap components (PSME1 and PSME2) associated with the immunoproteasome were up-regulated, while all significantly altered cap components of the constitutive proteasome were down-regulated.

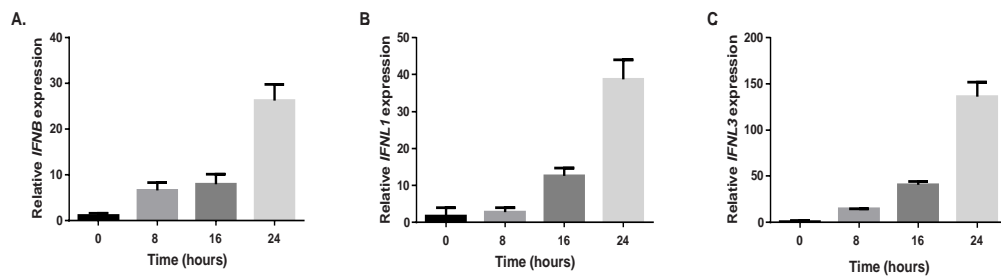
Evidence of possible post-proteasome processing was seen in the upregulation of a cytosol aminopeptidase, LAP3. Next, we saw upregulation of proteins involved in transport of peptides from the cytosol to the ER as well as assembly of peptide-HLA class I complexes; these proteins included TAP2, TAPBP, HLA-A, HLA-B, and HLA-C. Other proteins involved in the above step did not reach statistical significance but tended to increase expression following IFN- $\lambda$ 3 treatment; TAP1, PDIA3 (ERP57), and B2M. CALR, a player in this process, was actually down-regulated, the reasons for which will be discussed later.



**Figure 10. IFN- $\lambda$ 3 enhanced antigen processing/presentation.**

IFN- $\lambda$ 3 not only upregulated HLA class I expression but it also increased the expression of other effector molecules involved in antigen processing and antigen presentation. Ubiquitinating enzymes that promote protein degradation were found to be upregulated while deubiquitinating enzymes known to remove ubiquitin were found to decrease after IFN- $\lambda$ 3 treatment. Although all identified subunits of the constitutive proteasome cap were down-regulated, the 2 subunits of the immunoproteasome cap as well as some subunits of the proteasome core were elevated in expression. Several effector molecules involved in peptide loading on class I HLA as shown in red text were up-regulated with the exception of CALR, which was down-regulated as shown in green text. The italic red text represents proteins with increased expression levels that failed to reach statistical significance.

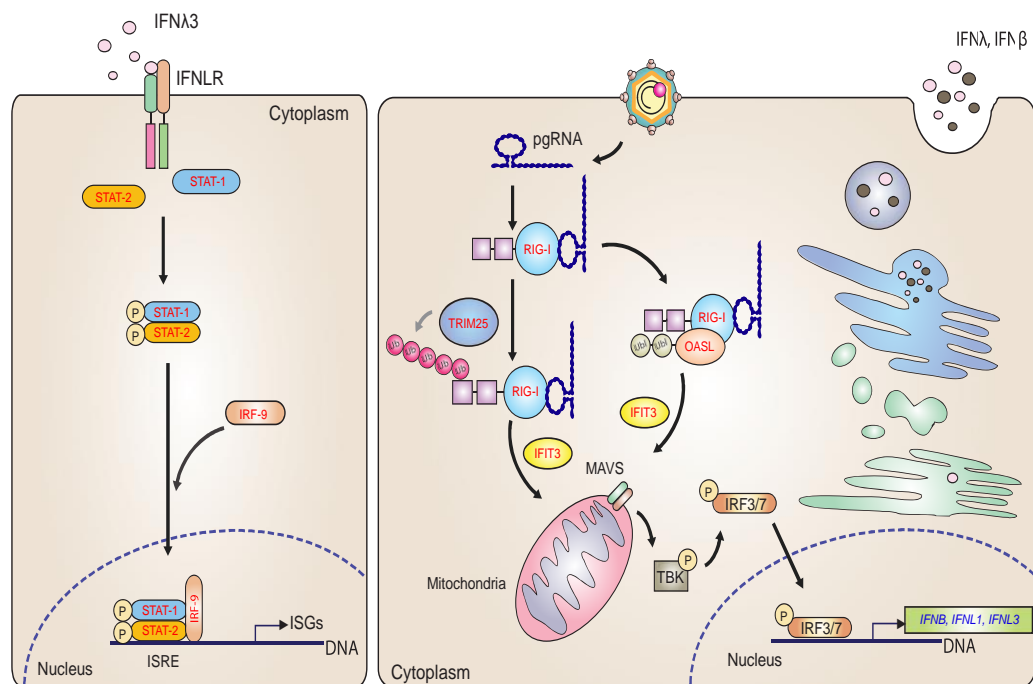
One of the important functions of the innate immune response against HBV is the sensing of viral RNA, leading to IFN activation, which includes components such as toll-like receptors, RIG-I, and MDA5. Here, we saw significant upregulation of RIG-I upon treatment, which could counteract the known suppression of RIG-I by HBV. Enhancers of RIG-I activity, such as OASL, TRIM25, and IFIT3 (RIG-G) were also significantly upregulated. Increased levels of type I and III IFNs, which are activated by RIG-I, would be expected, but were not observed via MS. However, qPCR work confirmed significant upregulation of these IFNs at the transcript level (Figure 11).



**Figure 11. qPCR analysis of IFN genes.**

Total RNA of HepG2.2.15 cells treated with IFN- $\lambda$ 3 for 8, 16, and 24 hours or left untreated was extracted and then converted to cDNA. The expression levels of IFNB, IFNL1, and IFNL3 (A, B, and C) were found to be upregulated in a time-dependent manner after IFN- $\lambda$ 3 treatment.

It is likely that the increase in IFN I and III gene expression due to RIG-I upregulation could lead to more production of IFNs at the protein level and in turn activate IFN receptors as positive feedback. All three canonical ISGF3 components (STAT1, STAT2, and IRF9) downstream from IFN receptor activation were upregulated, probably as a result of both initial IFN- $\lambda$ 3 treatment and further generation of type I and III IFNs (Figure 12).



**Figure 12.** *IFN- $\lambda$ 3 rescued the RIG-I signaling pathway.*

*IFN- $\lambda$ 3 upregulated the expression of RIG-I and IFIT3, which have been reported to be suppressed by HBV. IFN- $\lambda$ 3 also elevated the expression of OASL and TRIM25. These proteins promote type I and type III IFN production. These IFNs, in turn, activate the JAK-STAT pathway and induce the expression of ISGs. It is likely, then, that IFN- $\lambda$ 3 provides positive feedback to amplify ISG expression to control HBV replication. Proteins in red text represent upregulated proteins, while the blue text represents the up-regulated transcripts.*

We found a number of likely antiviral proteins that do not conveniently fit into the above viral processes. Firstly, three 14-3-3 proteins (YWHAZ, YWHAH, and SFN) were found to be down-regulated following IFN- $\lambda$ 3 treatment. Another down-regulated protein of interest was cyclophilin D (PPIF). Finally, CHID1 was actually the single most upregulated protein in our study on IFN- $\lambda$ 3 treatment. Though not prominent in the viral literature, CHID1 is known to bind LPS (122) and is seen to be down-regulated in several viral studies (123-126).

After removal of all anti-viral proteins, enrichment analysis of the remainder produced an interesting result. RNA-binding proteins, particularly spliceosome components, were both up- and down-regulated on IFN- $\lambda$ 3 treatment vs. control. For example, the splicing factor U2AF1 was significantly upregulated on treatment ( $p =$

0.00165). Searching through individual datasets from an in-house database containing a variety of studies and applying Fisher's exact test (see Experimental Procedures) showed a strong tendency toward both up-regulation and down-regulation of proteins that associate with the non-coding RNA NORAD (log  $p = -5$  and  $-13$ , respectively) (127). The same pattern applies to proteins shown to bind to the splicing factor U2AF2 (log  $p = -6$  and  $-9$ , respectively) (128).

Though our primary intent is to elaborate on IFN- $\lambda$ 3 potential, comparative studies against the best characterized interferon, IFN- $\alpha$ , are essential. Given the known parallelism in broad modes of action between the two IFNs, significant overlaps should be seen in sets of up-/down-regulated proteins upon treatment, offering a simple validation of IFN- $\lambda$ 3 activity. At the same time, and most intriguingly, the comparison would allow us to suggest possible differences in effect. To take advantage of the depth of our data, we constructed a tree depicting all 27 possible differential expression outcomes resulting from 3 pairs of treatment comparisons (i.e. IFN- $\lambda$ 3 vs. Ctrl, IFN- $\alpha$ 2a vs. Ctrl, IFN- $\lambda$ 3 vs. IFN- $\alpha$ 2a, see Figure 13).



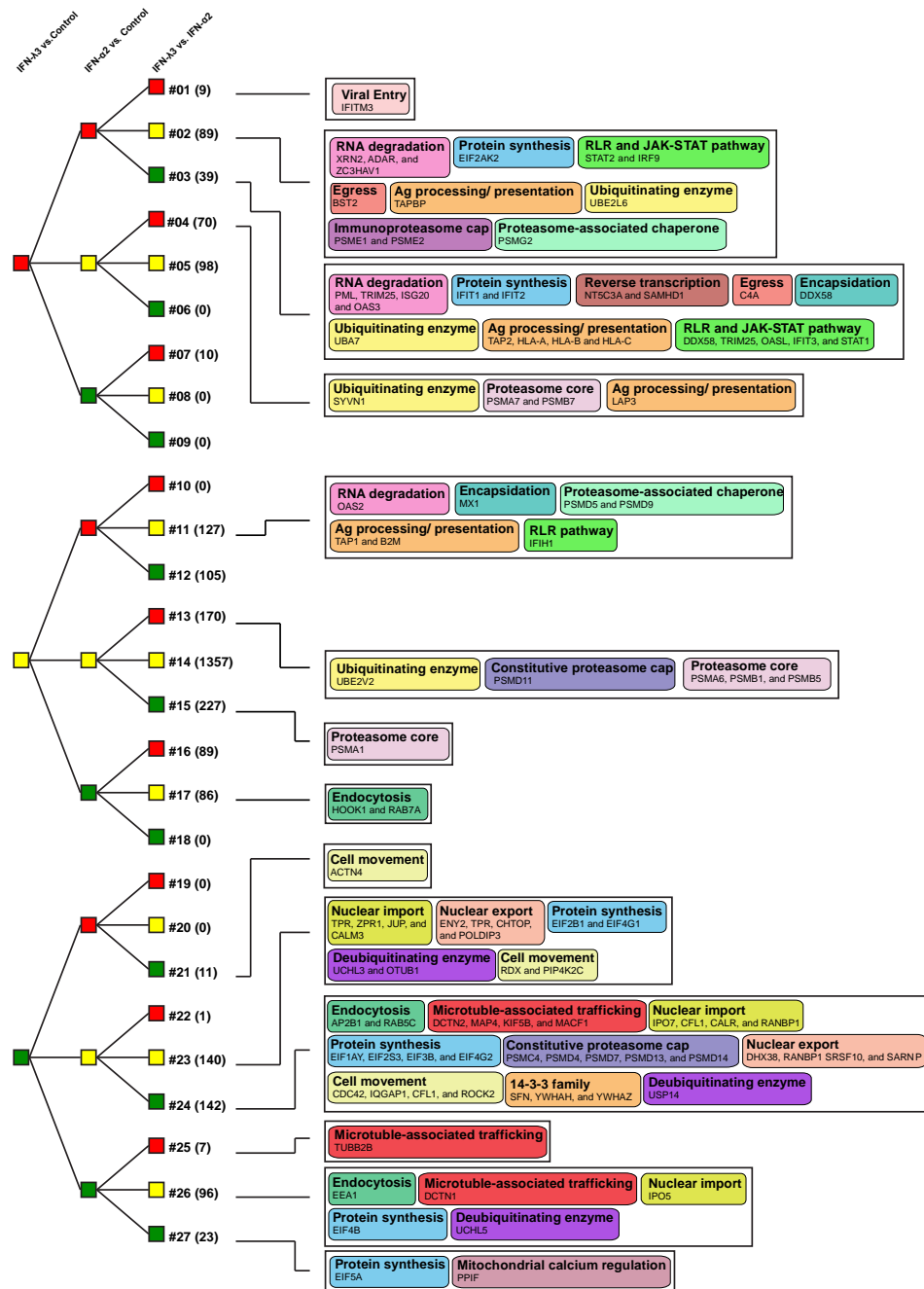
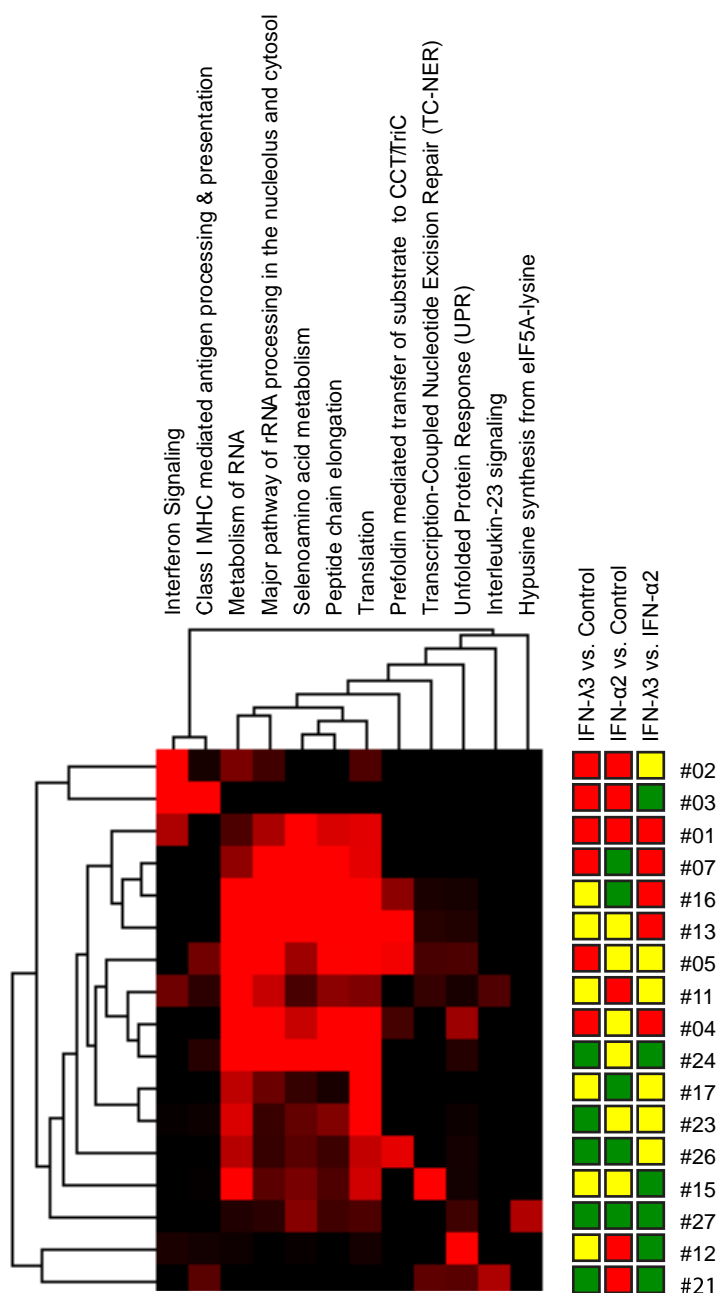


Figure 13. Outcome tree.

In order to compare the effects  $IFN-\lambda 3$  with  $IFN-\alpha 2a$ , we generated a tree representing the 27 possible outcomes. The boxes in red and green represent the group of proteins with significantly up and down-regulated expressions, respectively. Groups of proteins with insignificantly changed expressions are by yellow boxes. As an example, the topmost outcome represents proteins in which  $IFN-\lambda 3$  treatment causes significant upregulation versus control,  $IFN-\alpha 2a$  treatment causes significant upregulation versus control, and  $IFN-\lambda 3$  treatment causes significant upregulation versus  $IFN-\alpha 2a$ . The number in parentheses refers to the number of identified proteins in the group. The proteins in each group that might be involved in suppressing HBV replication are shown in colored boxes specifying various processes, particularly antiviral.

A simple comparison between IFN- $\lambda$ 3 vs. IFN- $\alpha$ 2a could lead to errors in interpretation. For example, the observation that IFN- $\lambda$ 3 treatment results in up-regulation or down-regulation of a protein vs. IFN- $\alpha$ 2a renders an uninteresting result if IFN- $\lambda$ 3 does not have any significant effect vs. Ctrl. This scenario can be seen in outcomes #12 and #16 in Figure 13. Another problem in interpretation arises when both IFNs have the same directional effect on cells but one effect is significantly more potent than the other; this would not be noticed upon mere IFN- $\lambda$ 3 vs. IFN- $\alpha$ 2 comparison. This scenario can be seen in outcomes #3 and #25 in Figure 13. The tree shows the number of MS-derived proteins at their appropriate outcome branch points and shows proteins associated with various biological processes (as described in Experimental Procedures) for each outcome. As one simple example of the power of this approach, note that IFITM3 was significantly upregulated upon treatment with both IFNs, however this effect was significantly stronger upon IFN- $\lambda$ 3 treatment (outcome #1). Another example was in outcome #4 where IFN- $\lambda$ 3 treatment caused upregulation of particular proteins involved in antigen processing and presentation (SYVN1, LAP3, PSMA7, and PSMB7), while IFN- $\alpha$ 2a did not produce any significant effects. Interestingly, the mirror image of this branch, outcome #24 where IFN- $\lambda$ 3 caused down-regulation while IFN- $\alpha$ 2a did not, was also populated with a subset of proteins involved in antigen processing and presentation, specifically those associated with the constitutive proteasome cap (PSMC4, PSMD4, PSMD7, PSMD13, and PSMD14). A priori, one would expect the most interesting outcomes to be found in cases in which IFN- $\alpha$ 2a and IFN- $\lambda$ 3 have clearly opposite effects (outcomes #7 and #21). We did not identify proteins clearly related to antiviral processes in these two outcome groups. This reason pointed to broader unbiased analysis. Hence, we performed “Reactome” enrichment analysis against all outcome groups for complete enrichment analysis beyond the aforementioned antiviral processes. The resulting Reactome enrichment groups were subjected to 2-D cluster analysis (Figure 14).



**Figure 14. Enrichment heatmap.**

Reactome enrichment analysis was performed on the tree outcomes that contained at least 9 members, excluding outcome #14 in which no significant alterations were seen on any treatment. Probabilities (Reactome's hypergeometric test,  $-\log(p\text{-value})$ ) were generated for every intersection of these tree outcomes with the specified Reactome groups. This matrix of probabilities was then submitted for two-dimensional cluster analysis using Cluster 3.0 and Java Treeview. The result is displayed as a heatmap. Red color intensity indicates the level of significance.



This analysis offers new insights. Firstly, we note the aforementioned cases in which IFN- $\lambda$ 3 and IFN- $\alpha$ 2a showed opposing effects. In outcome #7, where IFN- $\lambda$ 3 exclusively caused upregulation, the ‘peptide chain elongation’, ‘metabolism of RNA’, ‘major pathway of rRNA processing in the nucleolus and cytosol’, ‘selenoamino acid metabolism’, and ‘translation’ terms are highlighted, while in outcome #21, where IFN- $\lambda$ 3 exclusively caused downregulation, the ‘interleukin-23 signaling’ term is emphasized. Next, we observe that in most cases where proteins were upregulated upon IFN- $\alpha$ 2a treatment (outcomes#1, #2, #3, #11, and #21), ‘interferon signaling’ and/or ‘class I MHC mediated antigen processing & presentation’ terms were apparent, reinforcing expectations regarding IFN- $\alpha$ ’s potency on these pathways. Interestingly, specific terms related to ‘translation’ and ‘metabolism of RNA’ were significantly associated with 13 out of 17 different outcome groups. To our best knowledge, these biological processes have been not mentioned as consequences of general IFN activation. Finally, enrichment terms that are largely unexplored with regard to IFN treatment include ‘transcription-coupled nucleotide excision repair (TC-NER)’ (1 significant occurrence), ‘interleukin-23 signaling’ (1), ‘unfolded protein response (UPR) (2)’, ‘prefoldin mediated transfer of substrate to CCT/TriC (4)’, and ‘hypusine synthesis from eIF5A-lysine (1)’. Interestingly, the final enrichment term was only associated with a single case, that of proteins downregulated in all treatment pairs (outcome #27). These results could help point toward differential effects of IFN- $\lambda$ 3 vs. IFN- $\alpha$ 2a.

## CHAPTER V

### DISCUSSION

HBV infection continues to be a major global health concerns affecting more than 4 billion people worldwide. CHB can develop to liver cirrhosis and hepatocellular carcinoma causing 887,000 deaths each year. Although IFN- $\alpha$  and NAs are used as the first line of treatment for CHB, each drug has some limitations such as undesirable side effects of IFN- $\alpha$  and occurrence of drug-resistant HBV following NAs treatment. The new agents, which overcome these restrictions, are still required in the present. The novel cytokine IFN- $\lambda$  (type III IFN) is being explored as the new therapeutic drug in several diseases such as viral infections and cancers because it possesses biological activities-like type I IFN including anti-viral, anti-proliferative and immune-modulating effects. Type III IFN has been shown to exert antiviral effects on several viruses including EMCV, IAV, HSV, VSV, HIV, HCV and HBV (17, 60, 66, 67, 70, 78, 116, 129). In clinical trials, peg IFN- $\lambda$  treatment showed reduced HBV DNA, HBsAg and HBeAg in CHB patients at levels greater than or comparable to those treated with peg IFN- $\alpha$  during on-treatment and at end-of-treatment (9). Although the virological, serological, and biochemical responses of peg IFN- $\lambda$  at week 24 after treatment were not superior to peg-IFN- $\alpha$ , adverse events associated with drug treatment were mostly seen in patients treated with peg-IFN- $\alpha$ . Numerous HCV studies also illustrate this tendency toward diminished side-effects (13, 14). Another clinical trial demonstrated that patients treated with peg IFN- $\lambda$  had improved anti-HBV immunity through an increase in poly-functional NK cells and maintenance of both HBV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells, important arms of immunity for viral elimination (8). The mechanisms involved in these immunomodulatory effects remain to be elucidated. At the level of cell culture, only two studies (71, 78) have focused on the effects of IFN- $\lambda$  treatment of HBV-transfected cells, one of which offers a comparison against a type I IFN, IFN- $\beta$ . Here, a comparison against IFN- $\beta$  showed that IFN- $\beta$  exhibited greater potency in viral elimination at the 6 hour time-point, but potency was equivalent at 24 hours.

In this study, we selected HepG2.2.15 cells as CHB model in vitro because this cell line supports HBV replication and it also secretes HBsAg, HBeAg as well as HBV DNA into the culturing media like natural HBV life cycle. Here, in addition to up-regulation of OAS1, MxA and ISG15 expressions, we first demonstrated that IFN- $\lambda$ 3 exerted its antiviral activities by reducing the expression of HBV RNA transcript (pres1) and replicative intermediate (pgRNA) as well as reducing amount of intracellular HBV DNA in a dose-dependent manner. Our findings were in accord with the results from Robek et al. (71) and Seung-Ho Hong et al. (78), these suggested that all 3 IFN- $\lambda$  subtypes could suppress HBV replication in vitro. Although the antiviral effects of IFN are dose-dependent manner, IFN itself has toxicity to cells. IFN- $\lambda$ 3 at 100 ng/ml, used to treat the cells in subsequent experiments, was optimal concentration based on its inhibitory effects on HBV replication with minimal cytotoxicity. In addition, the previous studies showed that this concentration of IFN- $\lambda$ 3 could inhibit HCV (65) and HIV (129) replication in vitro. For time of treatment, 24 hours were chosen from the previous time-course study using microarray approach because it was the time that the expressions of most genes and the maximal magnitude of responses were induced in HCV-infected cells treated with IFN- $\lambda$ 1 (17). This was also the same time that Leiliang Zhang used to investigate the efficiency of IFN- $\lambda$ 3 to inhibit HCV replication in OR6 and JFH1 cells which is the hepatoblastoma harboring HCV RNA (65).

Surprisingly, the effects of IFN- $\lambda$  treatment have not been studied with high-resolution mass spectrometry to date, possibly because it is assumed that type I and type III IFNs share the same signaling pathways. In fact, modern, deep proteomic studies of the effects of IFN treatment in general are lacking, perhaps because of an underlying assumption that RNA-seq results should parallel those generated via MS. Here, we used a modern high-resolution LC-MS/MS system with upstream high-pH reversed phase fractionation to yield a total of 127,989 peptides (25,181 unique peptides) representing 4,670 proteins. The peptides were labeled with dimethyl isotopes, allowing accurate quantification under different treatments. This study provides the most comprehensive examination of changes in protein abundance under IFN treatments to date.

In order to provide objective measures for study-study comparisons, in the discussion below we perform numerous comparisons between our studies and others with Fisher's exact test (see Experimental Procedures). As expected, canonical IFN-stimulated biological processes were prominent in IFN- $\lambda$ 3 treatment. In particular, proteins significantly upregulated upon IFN- $\lambda$ 3 treatment vs. Ctrl (hereafter  $\lambda$ 3/c) overlapped with the GO "antiviral defense" term at p-value =  $10^{-14}$  (with the  $\alpha$ /c condition giving p-value =  $10^{-12}$ ). Similarly, the  $\lambda$ 3/c list significantly overlapped (p-value =  $10^{-13}$ ) transcripts whose up/down regulation patterns (i.e. "co-expression") mirror those of RIG-I transcripts reported in the ARCHS4 resource (130); the  $\alpha$ /c list gave p-value =  $10^{-18}$ , perhaps illustrating the expected difference in potency. Comparing proteins significantly upregulated in  $\lambda$ 3/c against those upregulated in  $\alpha$ /c, we derive p =  $10^{-138}$ , a clear demonstration of similarities in effect vs. control. Stepping outside the bounds of our own work, Bolen's transcriptomic comparison of the effects of 5 interferons, including IFN- $\lambda$ 3, on Huh7 cells can be examined against our proteomic results (16). Here, a list of highly upregulated transcripts derived from Bolen's 6, 12, and 24 hour  $\lambda$ 3/c time-points significantly intersected with our own  $\lambda$ 3/c results (p-value =  $10^{-25}$ ). Of interest, the degree of overlap (p-value =  $10^{-5}$ ) was less significant when performing the same exercise with down-regulated transcripts vs. proteins, paralleling the tendency of lower correlation seen in our own qPCR results for a number of down-regulated proteins (Figure 8). Other comparisons illustrating the expected canonical effects would be an  $\alpha$ /c result in Huh7 (131) (a  $\lambda$ 3/c experiment was not performed) where upregulated transcripts align with our own  $\lambda$ 3/c results (p-value =  $10^{-30}$ ), and a list of genes likely to be activated by STAT2 based on curated STAT2 chip-seq results that significantly overlaps with our  $\lambda$ 3/c results (p-value =  $10^{-25}$ ) (132).

In addition to acting on viral replication directly, we propose that the proteolytic pathway may be induced by IFN- $\lambda$ 3 to inhibit HBV replication. The proteasome plays a crucial role in degradation of misfolded, unfolded or damaged proteins which were occurred in the natural state and responding to the oxidative stress. The abnormal proteins were cleaved by protease enzymes into small peptides

with 7-8 amino acid in length and further hydrolyzed into shorter fragments to newly synthesize protein or to be chaperoned by HSPs to facilitate antigen presentation in case of viral infection. Like other viral infections, HBV produces and secretes its proteins into the host cells during its propagation and these proteins facilitate the efficiency of HBV replication.

Robek et al. demonstrated that small molecule-based inhibition of proteasome activity could restrain the anti-HBV activity of type I IFN *in vitro*, suggesting that antiviral effects of IFN might relate to the proteasome (133). Yao et al. demonstrated that suppression of HBV replication was accompanied by an increase of 5 proteasome subunits in HepG2.2.15 cells in response to interleukin-4 (IL-4) treatment (134). This implied that IL-4 exerted one of its anti-HBV effects through proteasome pathway to degrade the proteins produced during HBV propagation. Here, we found that some core subunits of proteasome (PSMA7 and PSMB7) were increased their expressions as a result of IFN- $\lambda$ 3 treatment. This suggested that antiviral effects of IFN- $\lambda$ 3, in part, might depend on proteasome by degradation of the essential proteins either viral or host cellular proteins which were required for HBV replication. In our study, we also note up-regulation of immunoproteasome members (cap components PSME1 and PSME2) on IFN- $\lambda$ 3 and IFN- $\alpha$ 2a treatment. The immunoproteasome is distinct from the constitutive proteasome in the composition of its catalytic subunits, its cap, and its peptide processing capacity (135). Interestingly, all significantly altered cap components of the constitutive proteasome were downregulated only upon IFN- $\lambda$ 3 treatment, suggesting coordinated modulation towards the antigen processing form of the proteasome on IFN- $\lambda$ 3 treatment. To our knowledge, this is the first report suggesting a shift from proteasome constitutive degradation mode to antigen processing/presentation mode in response to type III IFN treatment.

HBV has been termed a “stealth” virus because innate immune responses in chimpanzees are not observed shortly after infection (43). The synthesis of type I IFNs is induced by the activation of RIG-I-like receptor (RLR) signaling pathways in response to viral infection. Previous studies have shown that HBV polymerase and HBx proteins interfere with the interaction of RIG-I and downstream signaling molecules, resulting in

diminished type I IFN production (136-138). HBV has also been shown to alter RIG-I expression by inducing up-regulation of miR146a, which may directly down-regulate RIG-I and RIG-G transcripts (139). IFN treatment has not previously been shown to up-regulate RIG-I pathway components in HBV infected cells. We found, for the first time, that treating HepG2.2.15 with IFN- $\lambda$ 3 increased the expression of RIG-I. In addition, upon IFN- $\lambda$ 3 treatment, we observed elevated expression of RIG-G, known to enhance the activity of RIG-I, as well as TRIM25 and OASL, which promote the interaction between RIG-I and IPS1 on mitochondria or peroxisomes, leading to increased IFN production (140, 141). We propose that IFN- $\lambda$ 3 restores RIG-I and associated downstream signaling to produce type I IFN in HBV infected cells and likely other viruses. Although we did not find any IFNs in our proteomic work, possibly due to low abundances, qPCR verified increases in type I IFN (IFN- $\beta$ ) and type III IFN (IFN- $\lambda$ 1 and IFN- $\lambda$ 3) upon IFN- $\lambda$ 3 stimulation.

Several biological processes not mentioned above emerged from this work, including the involvement of 14-3-3 proteins, cyclophilin D, calreticulin, and cell motility proteins. These subjects are explored in detail below. In our study, the 14-3-3 proteins YWHAZ, YWHAH, and SFN were found to be down-regulated following IFN- $\lambda$ 3 treatment. Several studies have shown that 14-3-3 proteins may facilitate the replication of viruses (142, 143). The 14-3-3 proteins are highly conserved regulatory molecules having multiple functions, including involvement in signal transduction via kinases and phosphatases, the cell cycle, and apoptosis. These proteins have been shown to play important roles in viral infection. For example, complex formation of cdc25 with the HIV accessory protein Vpr, an event that alters the host cell life cycle, is facilitated by 14-3-3 proteins (142). Also, HCV core protein interacts with 14-3-3 proteins resulting in enhancement of Raf-1 kinase activity together with control of hepatocyte growth (143). Interestingly, sequence analysis revealed a 14-3-3 binding domain in HBx protein (144), suggesting that 14-3-3 proteins could directly interact with HBV. Notably, we could not observe significant alterations in 14-3-3 proteins upon IFN- $\alpha$ 2a treatment; the differential effects of IFN- $\lambda$  vs. IFN- $\alpha$  on 14-3-3 levels would be worthy of further investigation.

The downregulation of cyclophilin D upon IFN- $\lambda$ 3 treatment observed in our work suggests that this molecule may play a role in inhibition of HBV replication. One of the pathways affected by HBx is calcium signaling, as  $\text{Ca}^{2+}$  is necessary for viral replication and core assembly (145, 146). Calcium ions are the second messenger with various functions in the cell biology. These ions are stored in the mitochondria which acts as the regulator of intracellular calcium signaling. In addition to interference with RIG-I signaling, the HBx protein can interact with and modulate the mitochondrial permeability transition pore (MPTP) leading to the release of mitochondrial calcium into the cytoplasm (145). The opening of the MPTP anion channel is regulated by cyclophilin D (147), thus downregulation of cyclophilin D would serve to counteract  $\text{Ca}^{2+}$ -assisted viral replication and core assembly. Increasing levels of cytosolic calcium causes the stimulation of PYK2 kinase, in turn activating Src kinase signal transduction to promote HBV reverse transcription, replication, and core assembly (146, 148). In several studies, cyclosporine A (CsA) was used as an MPTP specific blocker, thus reducing core assembly and inhibiting HBV replication (101, 147, 148). Consistent with CsA treatment, the calcium ion chelating agent BAPTA-AM also suppressed HBV propagation (148).

Calreticulin (CALR), down-regulated upon IFN- $\lambda$ 3 treatment in our study, is an ER calcium-binding chaperone involved in the regulation of calcium homeostasis, the folding of newly synthesized proteins, nuclear import, and peptide loading on MHC class I (149-151). In HBV-transfected cells, Yue et al. demonstrated that the inhibition of IRF-7 translocation into the nucleus was induced by CALR, resulting in suppression of type I IFN production (152). Moreover, this group showed that CALR inhibited JAK-STAT pathway induction by IFN- $\alpha$  by inhibiting STAT1 phosphorylation, diminishing expression of PKR and OAS. Thus, the observation of downregulation of CALR would be expected to have an anti-viral effect. However, the downregulation of CALR would also seem to interfere with antigen processing/presentation. We note that most antigen processing/presentation molecules were upregulated upon treatment in our work, possibly compensating for this effect. Overall, the observed IFN- $\lambda$ 3-induced downregulation of CALR in our study may have an inhibitory effect on HBV replication.

Enrichment analysis (DAVID) showed that the most significantly down-regulated proteins following IFN- $\lambda$ 3 treatment were involved in cell motility and cell adhesion. We believe these processes could be relevant to the effects of IFN- $\lambda$ 3 treatment on liver cancer. Tan et al. demonstrated that HBV-transfected cells showed morphological changes due to formation of filopodia and lamellipodia, leading to cell migration. Of interest, CHB patients have 5-15 fold increased risk of HCC, a characteristic of which is increased cell motility (153, 154). CDC42, down-regulated on IFN- $\lambda$ 3 treatment, has been reported to control cell proliferation, adhesion, and metastases. High expression of this protein was observed in several types of cancers including HBV-related HCC. Inhibition of CDC42 by CRISPR/Cas9 knockout and treatment with a specific CDC42 inhibitor in HBx-Huh7 cells (155) reduced cell proliferation and promoted apoptosis in these cells. In addition to CDC42 downregulation, we also found that its downstream effectors, actin and IQGAP1, showed decreased expression following IFN- $\lambda$ 3 treatment. Also, proteins involved in cell movement and focal adhesion such as ACTN1, ACTN4, RDX, CFL1, PIP4K2C, and ROCK2 were down-regulated.

We have shown that IFN- $\lambda$ 3's effects were largely in accord with those expected of canonical IFN activity. Nevertheless, our tree and cluster analyses (Figure 13 and 14) highlight a number of differential effects between IFN- $\lambda$ 3 and IFN- $\alpha$ 2a worthy of further investigation. Most obviously, despite the division of treatment outcomes into 27 non-intersecting protein groups, unbiased Reactome enrichment analysis repeatedly elicited an RNA-metabolism/translation theme. This broad theme can be divided into Reactome sub-groups (e.g. metabolism of RNA, major pathway of rRNA processing in the nucleolus and cytosol, translation, transcription-coupled nucleotide excision repair (TC-NER), and peptide chain elongation). However, we could not specifically find IFN- $\lambda$ 3- or IFN- $\alpha$ 2a dominant patterns in these sub-groups, suggesting that investigation of this broad theme might best be undertaken at the level of individual proteins. Perhaps the strongest general observation would be a tendency for involvement of ER-associated proteins (GO "establishment of protein localization to endoplasmic reticulum", which is primarily composed of ribosomal proteins) in cases where IFN- $\lambda$ 3 treatment results in significantly greater protein abundance vs. IFN-



$\alpha 2a$ ; in the 5 outcomes where such an effect would be discernable,  $-\log(p) = 4.7, 3.2, 4.8, 8.2,$  and  $5.3$ . On the other hand, such significance ( $-\log(p) = 4.1$ ) is seen only once out of six outcomes where IFN- $\alpha 2a$  treatment results in significantly more abundant protein expression. This result could imply differential regulation of these ER targeting proteins on IFN- $\lambda 3$  vs. IFN- $\alpha 2a$  treatment. Altered regulation of these ER proteins could interfere with viral replication (156, 157). It is interesting to note that some viruses utilize the ER as a replication compartment, whereas others do not. Thus, our work suggests that in addition to IFN's role in regulating ER-targeting, one IFN treatment might be more appropriate than another for particular infections. Of other Reactome groups that emerged during clustering, we find the "hypusine synthesis from eIF5A-lysine" case especially interesting, as it is only associated with a single outcome group, that in which  $\lambda 3 < c, \alpha 2a < c, \lambda 3 < \alpha 2a$  (i.e. the two IFNs cause downregulation, but IFN- $\lambda 3$ 's effect is most potent). Hypusine, a rare amino acid, is apparently found only once in the human proteome, as a modification of eukaryotic initiation factor 5 (eIF5A), with proviral implications for HIV and Ebola (158). Thus, IFN- $\lambda 3$ 's antiviral effect may be especially potent in down-regulating hypusination. The case of hypusination also illustrates how IFN- $\lambda 3$ -induced alterations to the RNA-metabolism/translation machinery could target particular viruses.

At the level of individual studies, we note that, in addition to the aforementioned  $\lambda 3/\alpha 2a$  comparison, Bolen's data (16) also allows a  $\lambda 3/\lambda 2$  comparison; here, upregulated transcripts aligned strongly with our own  $\lambda 3/c$  upregulated proteins ( $p\text{-value} = 10^{-27}$ ), suggesting possible differential effects even within the IFN- $\lambda$  subgroup. As a side note, IFITM3, strongly upregulated in our own  $\lambda 3/\alpha 2a$  comparison (outcome #1), was the single most significantly upregulated transcript in the  $\lambda 3/\lambda 2$  comparison ( $p\text{-value} = 10^{-10}$ ). Having noted possible IFN- $\lambda 3$  vs. IFN- $\alpha 2a$  differences, we should reiterate the points that: 1) our experimental design did not allow for discrimination of kinetic effects (e.g. those caused over time by differing receptor/ligand affinities and half-lives) vs. substantial alterations at the level of pathways and 2) the effects of IFN- $\lambda 3$  and IFN- $\alpha 2a$  were indeed substantially similar, especially with regard to canonical IFN effects such as regulation of anti-viral proteins,

antigen processing/presentation, and the RIG-I signaling pathway. A recent work does make a strong case for a differential  $\lambda 3/\alpha 2a$  effect on murine intestinal epithelial cells (159), but the effect is pronounced only upon polarization of cells, and the underlying microarray data does not correlate with our proteomic data. Thorough studies focused on possible differential effects on PTMs may help resolve these questions; the immediate result of IFN-receptor/ligand interaction is, after all, a phosphorylation event. Comprehensive identification of phosphorylation events and other kinds of PTMs will help us to elucidate possible differential effects in different IFN treatment.

In conclusion, our study found that IFN- $\lambda 3$  exhibited anti-HBV activities by significant inhibition of HBV replication and expression of HBV RNA. We utilized high-throughput quantitative proteomics to obtain a comprehensive understanding of molecular events upon treatment of HepG2.2.15 with IFN- $\lambda 3$ . To our knowledge, in fact, this study is the most comprehensive proteomics-based analysis of IFN treatment to date. For the first time, we reported significant upregulation of immunoproteasome components, restoration of HBV-inhibited RIG-I pathway proteins, as well as a number of proteins not previously associated with IFN- $\lambda 3$  treatment. Further study of these proteins is required. Clearly, IFN- $\lambda 3$  exhibited both antiviral and immunomodulatory effects to inhibit HBV replication; therefore, IFN- $\lambda 3$  is an attractive novel candidate for CHB treatment and the altered proteins might be new therapeutic targets in CHB infection.

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## APPENDIX

### The previous thesis proposal and preliminary results

B cell, one of the immune cells, plays critical roles in adaptive immune responses. Once B cell binds to antigen through B cell receptor (BCR), it undergoes proliferation and differentiation into either memory B cells or plasma cells, also known as antibody-secreting cells. Antibodies can lead to opsonization and phagocytosis of microbes, neutralization of microbes and toxins, antibody-dependent cellular cytotoxicity (ADCC), and activation of the complement system. Besides, B cells also act as antigen-presenting cells by presenting antigen to helper T cells via MHC class II molecules. Another function of the B cell is cytokine production. B cells can produce and secrete several cytokines including IL-2, TNF- $\alpha$ , IL-6, IL-12, MIF, and interferon- $\gamma$  which are in turn to activate a large number of immune cells such as T cells (including Th17 cells), macrophages, and natural killer (NK) cells (160). All of these functions and many physiological processes in B cells are regulated by BCR signaling (161).

Although the picture of BCR signaling has been elucidated showing the incorporation of many signaling molecules, all of this knowledge came from either individual molecules in specific pathways by immunoblotting assay or high-throughput, system-wide microarray analysis (162-164). It is widely accepted that transcriptional changes show the poor correlation with protein expression and posttranslational protein modifications especially phosphorylation cannot be predicted in transcript levels (164, 165). Besides, the previous studies in BCR signaling have been limited only in the cancer cell, not primary cells. Therefore, I would like to investigate the global picture of BCR signaling by showing the comprehensive, dynamic profile and direct protein-protein interaction from mouse splenic B cells through phosphoproteomics approach.

Firstly, the splenic B cells were isolated from 2-mo C57BL/6 (WT) mice using anti-CD43 antibody-conjugated microbeads. With this negative selection procedure, mature resting B cells were purified from other splenocytes. The cell viability and purity of isolated B cells were determined by flow cytometry. The result, as shown in

Figure I, indicated that B cell viability and purity were greater than 99% and 95%, respectively.

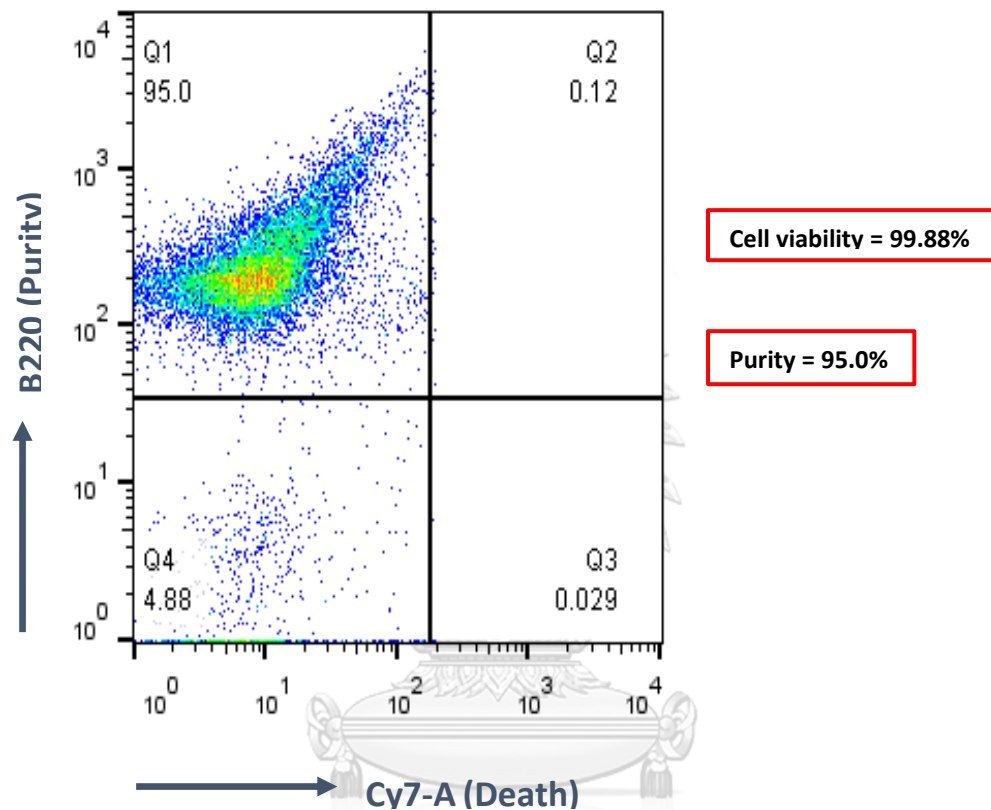
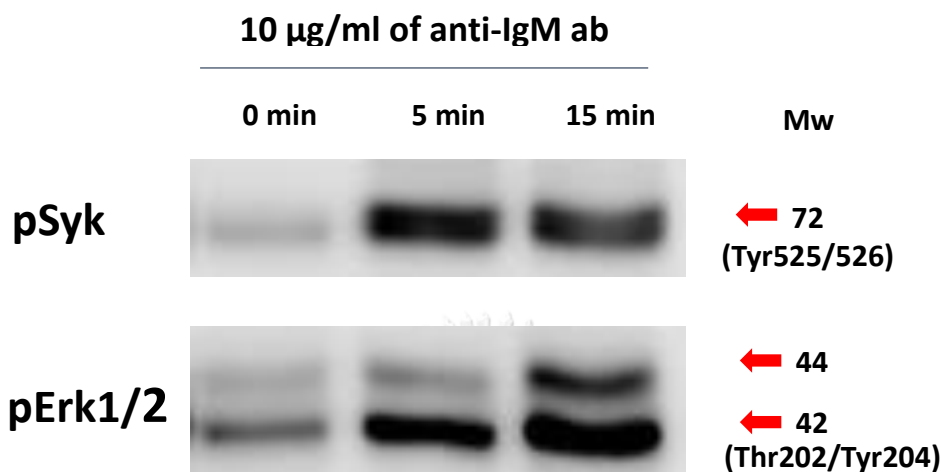


Figure I. Cell viability and purity of isolated B cells. Mouse B cells were isolated from splenocytes, stained with PE/Cy7 anti-B220 antibodies, and analyzed by flow cytometry. Cell viability and purity of isolated B cells were 99.88% and 95.0%, respectively.

Next, B cells were stimulated with 10  $\mu\text{g/ml}$  of intact anti-IgM antibodies for 0, 5, and 15 minutes. The representative downstream molecules in BCR signaling including pSyk and pErk1/2 were monitored by the immunoblotting assay. As shown in Figure II, the results revealed that elevated expressions of both proteins were correlated with increasing times of stimulation. This result indicated that the conditions used to stimulate B cells were optimal. Unfortunately, the amount of proteins extracted from splenic B cells were quite low although 45-50% of mouse splenocytes

are B cells. Therefore, it is necessary to pool splenic B cells from at least 5 mice together for phosphoproteomic analysis.



**Figure II. The expression of selected downstream molecules of BCR signaling.** Splenic B cells were stimulated with 10 µg/ml of anti-IgM antibodies for 0, 5, and 15 min. The immunoblotting assay was performed to determine the expression of 2 surrogate markers. The phosphorylated Syk and Erk1/2 were up-regulated their expressions following anti-IgM treatment.

In addition to investigating the global BCR signaling in normal condition, I also study BCR signaling in *Fcgr2b*<sup>-/-</sup> lupus mouse model. Since activation and inhibition of BCR signaling are tightly regulated in balance to provide proper functions of B cells. Dysregulation in BCR signaling may result in diseases such as malignancies including chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL) and autoimmune diseases (166, 167). For example, Lyn-deficient mice showed aberrant functions of B cells including enhanced activation of downstream effectors, high calcium ion flux, B cell hyperactivity and autoantibodies production (168, 169). Mice with X-linked immunodeficiency (Xid) which had a point mutation in PH-domain of Btk demonstrated defective BCR signaling and impaired B cell responsiveness and maturation (170).

Systemic lupus erythematosus (SLE) also called lupus is a chronic inflammatory autoimmune disease affecting multiple organ systems. The exact etiology of lupus remains to be defined; however, many studies have proposed that interplay between genetic and environmental factors leads to breaking immunological tolerance including

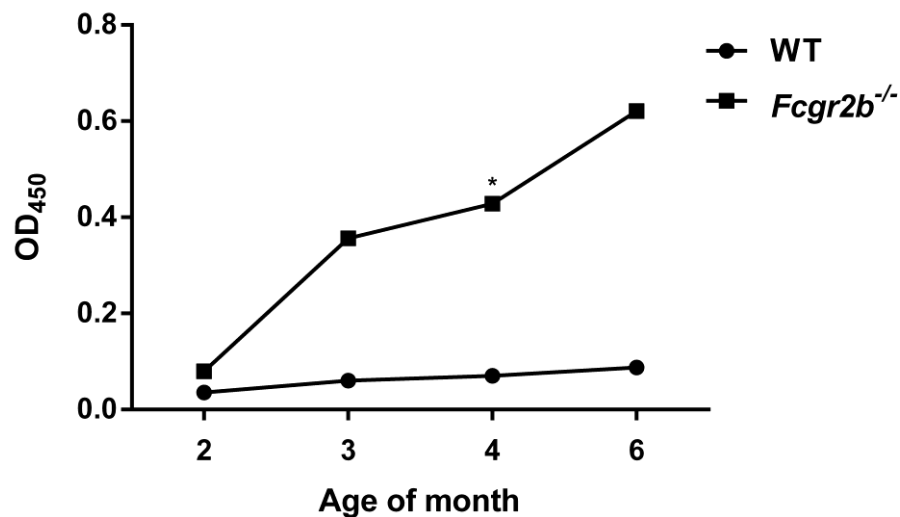
loss of tolerance to the nuclear antigen, immune complex (IC) formation, autoantibody production and loss of T- and B-cell regulation. The lupus pathogenesis has been reported that, in part, is mediated by autoantibodies (171, 172). Several studies reported that B cells from lupus patients have functional abnormalities (173, 174). These might result from aberrations in BCR signaling. The first observation of abnormal BCR signaling in lupus patients was conducted by Liossis and colleagues. This study showed that stimulation of circulating B cells from lupus patients with anti-IgM produced significantly higher fluxes of free intracytoplasmic Ca ions when compared to normal B cells. Also, this study revealed that the cellular proteins with molecular sizes between 36 and 64 kDa were significantly hyperphosphorylated at tyrosine in anti-IgM treated lupus B cells compared to those of B cells from normal controls (175). Therefore, it is essential to understand the defects in the BCR signaling pathway in SLE clearly.

In the present, several spontaneous, induced, or engineered lupus mice are available. The most common spontaneous lupus mouse models are (NZBxNZW)F1, MRL/*lpr*, and BxSB (176, 177). Although these three models show manifestations of the lupus-like disease including autoantibody production and glomerulonephritis; they do not develop a full panel of lupus symptoms and thus do not adequately represent SLE features in human. While complex genetics can accelerate the pathogenesis of the spontaneous lupus models as mentioned above, environmental factors can also trigger lupus-like disease such as exhibited in the pristane-induced lupus model. The latter mouse model develops lupus manifestations including immune complex glomerulonephritis and many lupus-associated autoantibodies after 4-month injection of pristane; however, arthritis, cerebritis, and skin involvement are absent (178). Due to the aforementioned limitations, attempts have been made to precisely engineer relevant molecules to create lupus mouse model that closely mimics human lupus. One of the engineered models is *Fcgr2b*<sup>-/-</sup> mouse model (179). *Fcgr2b* is a single chain, low-affinity receptor for the Fc portion of IgG that plays crucial roles in immune regulation by preventing excessive immune reactions and autoimmunity (180). Interestingly, the polymorphism (I232T) in *Fcgr2b* which attenuates inhibitory effects of this receptor is associated with susceptibility to SLE in Asians including Thai, Chinese, and Japanese (181). The *Fcgr2b*<sup>-/-</sup> lupus mice exhibit strong expression of IFN signature,



anti-dsDNA, ANA, lupus nephritis, and anemia like SLE patients. Therefore, this *Fcgr2b*<sup>-/-</sup> lupus model might be the best representative of human SLE.

Previous study reported that 6-mo *Fcgr2b*<sup>-/-</sup> lupus mice showed the full lupus manifestations (179). It might be possible that the abnormality in BCR signaling resulted from not only intrinsic defects in B cells but also other factors. Therefore, the early ages of *Fcgr2b*<sup>-/-</sup> lupus mice before disease onset should be considered. The disease progressions of *Fcgr2b*<sup>-/-</sup> lupus mice were monitored by determining the levels of serum anti-dsDNA which is one of the specific markers characterized in SLE (182) from lupus mice at different ages. The results as shown in Figure III demonstrated that the rising levels of this autoantibody were associated with increasing age. At the age of 4 months, the levels of anti-dsDNA were significantly higher than those of 2 months.



**Figure III. Levels of anti-dsDNA antibodies.** Serum of WT and *Fcgr2b*<sup>-/-</sup> lupus mice were collected at 2, 3, 4, and 6 age of months. The levels of anti-dsDNA were determined by ELISA. The levels of anti-dsDNA in lupus mice were higher than WT mice at the same age and reached the statistical significance at the age of 4 months.

However, Figure IV showed that anti-nuclear antigen (ANA), the first detectable autoantibody in human SLE, were initially positive in mice at the age of 3 months (+1) while the levels of ANA in the first two months mice were comparable to WT mice (negative). This result implied that 3-mo mice were getting the development of lupus disease. Thus, the mice at the age of 2 months was selected as a model for this study because the immune cells may be immature in younger 1-month mice. Other lupus

phenotypes were also determined as shown in Figure V and Figure VI. These results suggested that, like the WT mice, lupus manifestations were not observed in 2-mo *Fcgr2b*<sup>-/-</sup> lupus mice compared to 6-mo *Fcgr2b*<sup>-/-</sup> lupus mice.

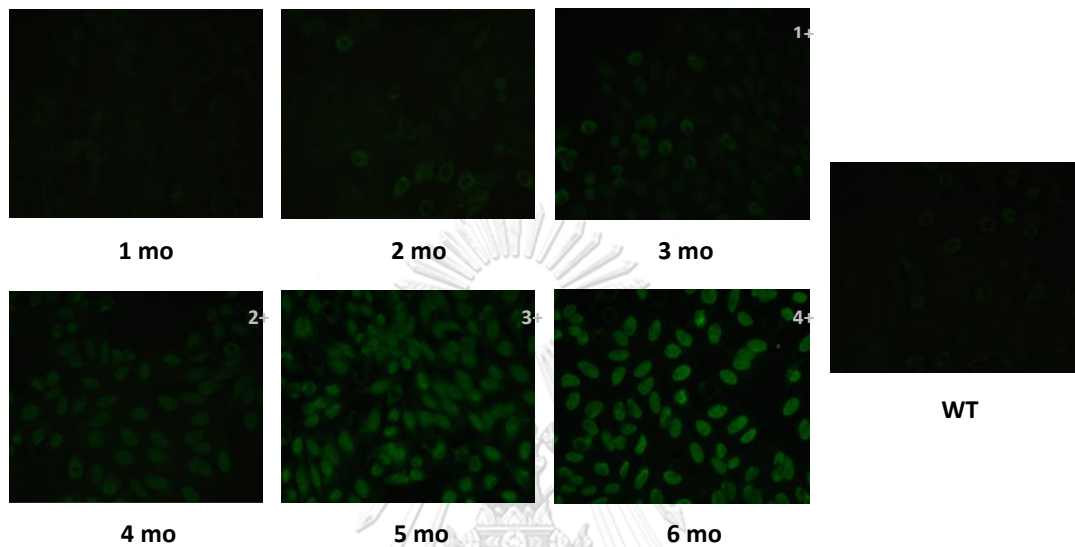
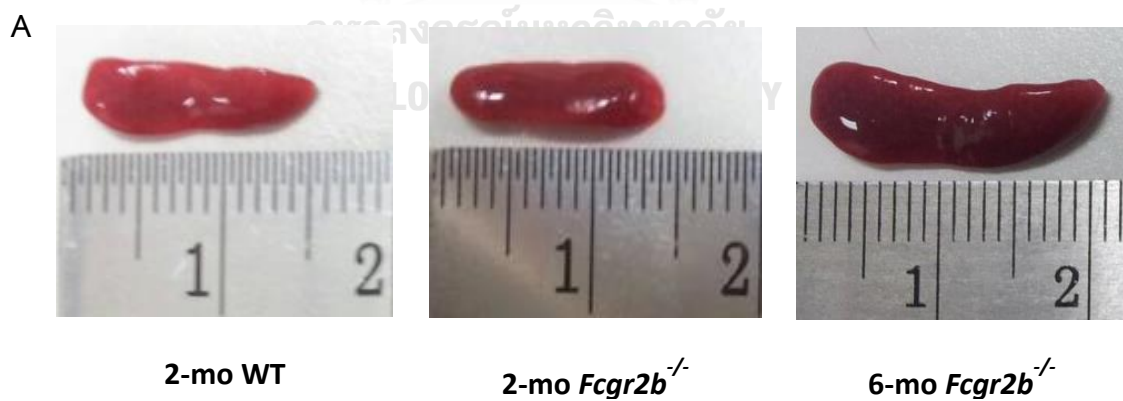


Figure IV. Anti-nuclear antibodies (ANA). Serum ANA of the different age of *Fcgr2b* deficient mice was determined. Compared to WT mice, ANA was initially detectable in the lupus mice at 3 months.



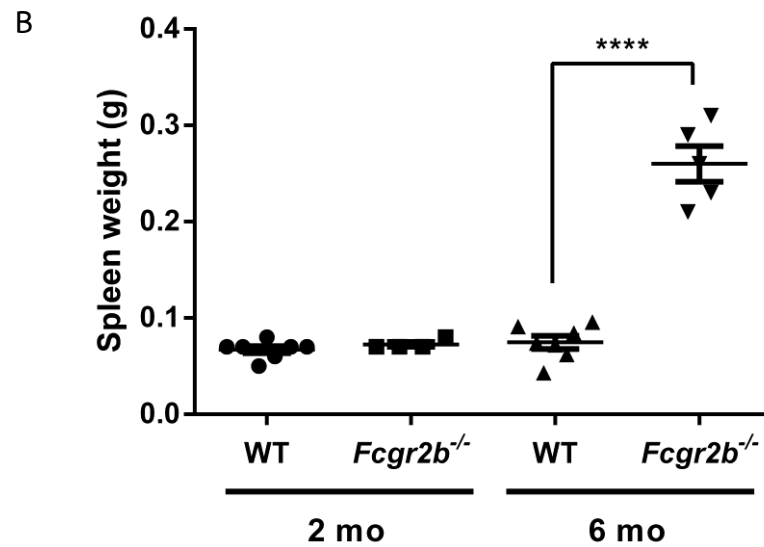


Figure V. Spleen size and weight. There were no differences in the size (A) and weight (B) of spleen from both WT and *Fcgr2b*<sup>-/-</sup> mice at the age of 2 months. Spleen from 6-mo *Fcgr2b*-deficient mice was used as a control

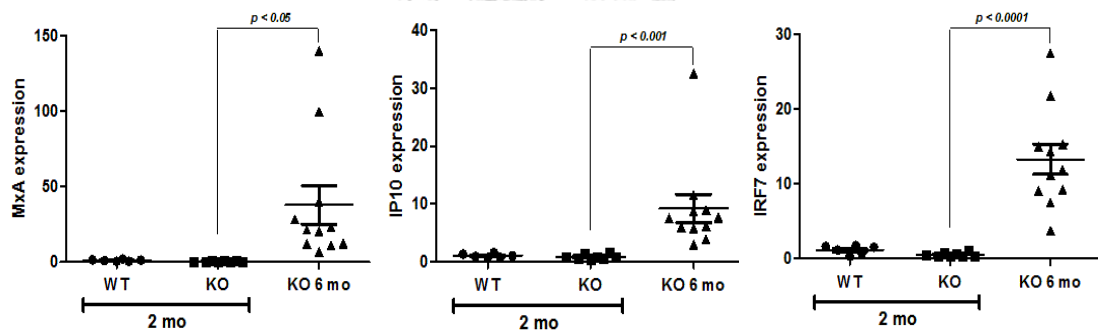


Figure VI. IFN signature in kidney. The kidney from WT and *Fcgr2b*<sup>-/-</sup> lupus mice at indicated were taken and extracted for RNA. The expressions of selected IFN signature including *Mx1*, *Ip10*, and *Irf7* were investigated by qPCR. The results demonstrated that IFN signatures in both WT and *Fcgr2b*<sup>-/-</sup> lupus mice at the age of 2 months were not different compared to those of 6-mo *Fcgr2b*<sup>-/-</sup> lupus mice used as the control. The p-value less than 0.05 was considered significant.

Next, splenic B cells were isolated from WT and *Fcgr2b*<sup>-/-</sup> lupus mice at the age of 2 months and stimulated with 10  $\mu$ g/ml anti-IgM antibodies for 5 minutes. The expressions of pSyk and pErk1/2 were determined by immunoblotting assay. As shown in Figure VII, the expressions of pSyk and pErk1/2 in stimulated lupus B cells were greater than those of stimulated normal B cells. These indicated that there were some defects in BCR signaling in lupus mice. Like the previous work, I had to collect the samples from several mice for phosphoproteomics.

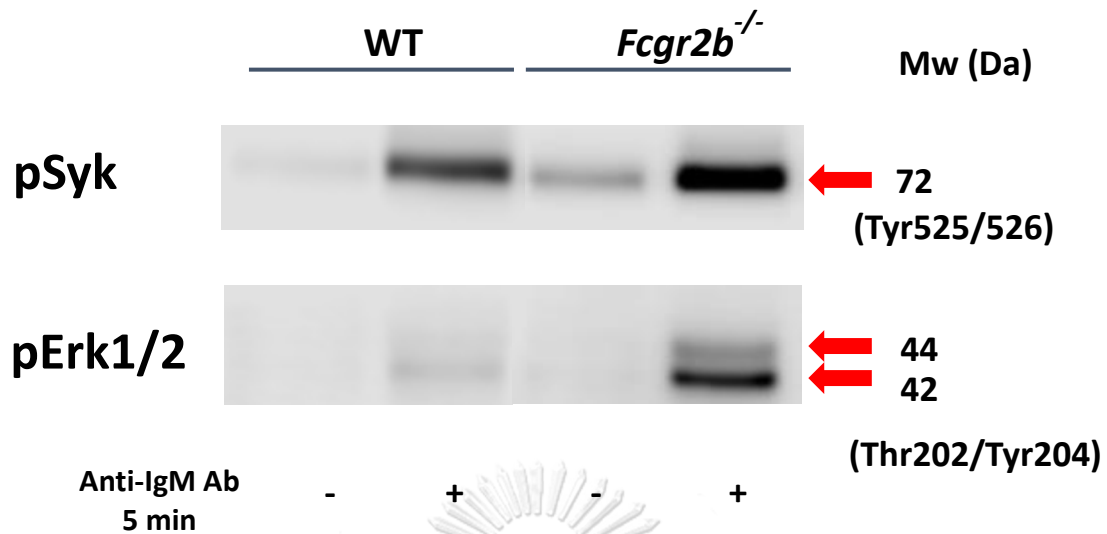


Figure VII. The expression of pSyk and pErk1/2 in 2-mo WT and *Fcgr2b*<sup>-/-</sup> lupus mice. The immunoblotting assay was performed to determine the expressions of pSyk and pErk1/2 in WT and *Fcgr2b*<sup>-/-</sup> lupus mice. After stimulation, the expressions of 2 surrogate markers in *Fcgr2b*<sup>-/-</sup> lupus mice were stronger than WT mice.

Another challenge in the study of cell signaling is that phosphorylation is a substoichiometric event and phosphoproteins are generally low-abundance. Phosphorylation is one of the most important post-translational modifications (PTMs) responsible for regulating several biological processes including signal transduction, metabolism, protein synthesis, transcriptional and translational regulation, proliferation, differentiation, and apoptosis. Most of the mechanisms in signal transduction involve in this PTM; therefore, the study of phosphoproteins may gain knowledge more than those studies on protein expression (183). Because phosphopeptides present in a small proportion together with lower ionization efficiency of phosphopeptides, these need to be enriched before MS analysis (184). Metal oxide affinity chromatography, especially TiO<sub>2</sub>, is an alternative method to enrich phosphopeptides based on electrostatic interaction with high specificity and highly robust toward salts/detergents (185).

As I aforementioned that proteins extracted from B cells were quite low; therefore, I used peritoneal macrophages challenged with *C. neoformans* to set up phosphopeptide enrichment condition instead. Since I did the other project, in parallel, which aimed to explore the severity of cryptococcosis in both pristine-induced and

*Fcgr2b*<sup>-/-</sup> lupus mice. This project also determined the phagocytosis and macrophage killing activity which are the main mechanisms controlling the organisms in cryptococcosis (186). Macrophages used in this study were isolated from the mouse's peritoneal cavity and the purity was examined by flow cytometry. With this isolation procedure, I could purify macrophage up to  $1 \times 10^8$  cells/mouse. Besides, the amount of proteins extracted from macrophages were more than those extracted from B cells because of the bigger size. Hence, I took these advantages using the macrophages as a model for optimizing phosphoenrichment condition. Due to the fact that the expressions of phosphoproteins in resting state are relatively low, the peritoneal macrophages were challenged with *C. neoformans* to stimulate the signaling in the cells before protein extraction. The proteins from lupus macrophages were digested into the peptides and phosphopeptides were enriched by TiO<sub>2</sub>. The enriched peptides were subjected to LC-MS/MS. The MS results indicated that the purity of phosphopeptides were 90% (Data not shown).

It was taken about 6-8 months to collect the samples which the amount of proteins were sufficient for phosphoproteomics. The expressions of pSyk and pErk1/2 were determined by immunoblotting assay prior to performing in-solution digestion. Unfortunately, as shown in Figure VIII, the expression of pErk1/2 was obviously decreased in stimulated B cells compared to the previous results. It might be possible that lysis buffer containing 5% sodium deoxycholate (SDC) and 1X phosphatase inhibitor cocktail in 50 mM TEAB did not preserve the phosphoproteins. Although SDC is an MS compatible agents, it is not a strong detergent like sodium dodecyl sulfate (SDS).

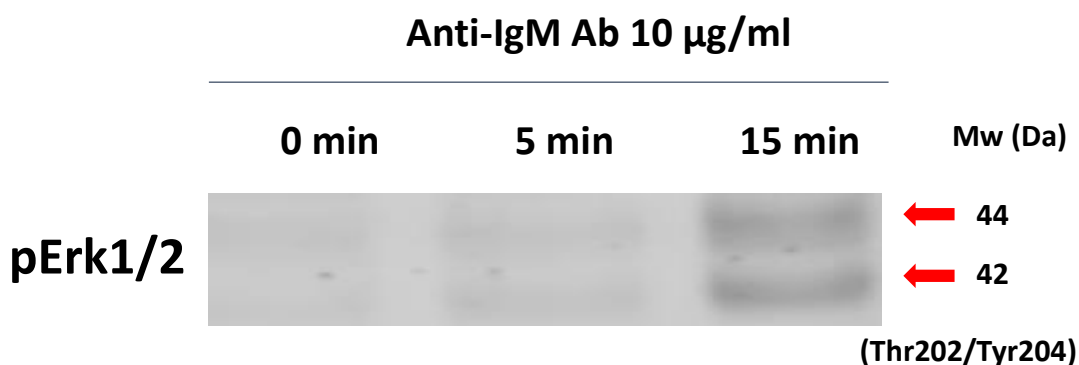


Figure VIII. Degraded pErk1/2 proteins in samples stored in long-term. After stimulation, proteins were extracted from B cells, lysed in 5% SDC containing 1X protease and phosphatase inhibitor and stored at  $-80^{\circ}\text{C}$ . Once the proteins were collected enough for phosphoproteomics, these proteins were subjected to SDS-PAGE and probed with anti-pErk1/2. The result showed that the expression of pErk1/2 after stimulation were obviously decreased compared with the previous results.

With this reason, I had to change the project from “Quantitative phosphoproteomic analysis of BCR signaling networks” to “Comprehensive proteomics identification IFN- $\lambda$ 3-regulated antiviral proteins in HBV-transfected cells” which is the study of protein abundance responding to certain stimuli in cell line instead.

Supplementary Table: Sequences of primers and probe

Primer	Sequence
<i>preS1</i>	F: 5'-GGGTCACCATATTCTTGGAAC-3' R: 5'-CCTGAGCCTGAGGGCTCCAC-3'
pgRNA	F: 5'-CTCAATCTCGGGAACCTCAATGT-3' R: 5'-TGGATAAAACCTAGCAGGCATAAT-3'
<i>OAS1</i>	F: 5'-AGCCTCATCCGCCTAGTCAA-3' R: 5'-CCTCGCTCCCAAGCATAGAC-3'
<i>MX1</i>	F: 5'-TGGTGTGACATACCGGAAGA-3' R: 5'-CCTTGCATGAGAGCAGTGATG-3'
<i>ISG15</i>	F: 5'-CGCAGATCACCCAGAAGATT-3' R: 5'-GCCCTTGTTATTCTCACCA-3'
<i>TBK1</i>	F: 5'-AGCGGCAGAGTTAGGTGAAA-3' R: 5'-CCAGTGATCCACCTGGAGAT-3'
<i>DDX58</i>	F: 5'-GCCATTACACTGTGCTTGGAGA-3' R: 5'-CCAGTTGCAATATCCTCCACCA-3'
<i>IFNB</i>	F: 5'-TGGGAGGCTTGAATACTGCCTCAA-3' R: 5'-TCCTTGGCCTTCAGGTAATGCAGA-3'
<i>IFNL1</i>	F: 5'-CGCCTTGAAGAGTCACTCA-3' R: 5'-GAAGCCTCAGGTCCCAATTC-3'
<i>IFNL3</i>	F: 5'-AGTTCCGGGCCTGTATCCAG-3' R: 5'-GAGCCGGTACAGCCAATGGT-3'
<i>SFN</i>	F: 5'-AAGAAGCGCATCATTGACTCAGCC-3' R: 5'-TGTTGGCGATCTCGTAGTGGGAAGA-3'
<i>YWHAH</i>	F: 5'-TGCAGAACTGGATACGCTGAGTGA-3' R: 5'-TCACCCTGCATGTCTGAAGTCCAT-3'
<i>CRT</i>	F: 5'-AAGTTCTACGGTGACGAGGAG-3' R: 5'-GTCGATGTTCTGCTCATGTTTC-3'

Supplementary Table (Continued): Sequences of primers and probe

Primer	Sequence
<i>Bst2</i>	F: 5'-CCGTCCTGCTCGGCTTT-3' R: 5'-CCGCTCAGAACTGATGAGATCA-3'
<i>IFIT3</i>	F: 5'-GAACATGCTGACCAAGCAGA-3' R: 5'-CAGTTGTGTCCACCCTTCCT-3'
<i>IFITM3</i>	F: 5'-ATGTCGTCTGGTCCCTGTTC-3' R: 5'-GTCATGAGGATGCCCAGAAT-3'
<i>GAPDH</i>	F: 5'-AGATCCCTCCAAAATCAAGTGG-3' R: 5'-GGCAGAGATGATGACCCTTTT-3'
<i>KRT8</i>	F: 5'-TCTGGGATGCAGAACATGAG-3' R: 5'-CTCCTGTTCCCAGTGCTACC-3'
<i>PGK2</i>	F: 5'-AAGTCAGCCATGTCAGCACTG-3' R: 5'-GCCTGCTGCTTGTCCATTACA-3'
<i>TRIM21</i>	F: 5'-AAGCTCCAGGTGGCATTAGG-3' R: 5'-ACTGTTTTCTTCCAGTCTGCTCT-3'
<i>SAMHD1</i>	F: 5'-TCACAGGCGCATTACTGCC-3' R: 5'-GGATTTGAACCAATCGCTGGA-3'
<i>TRIM25</i>	F: 5'-AAAGCCACCAGCTCACATCCGA-3' R: 5'-CGGTGTTGTAGTCCAGGATGA-3'
<i>EIFAK2</i>	F: 5'-ATGATGGAAAGCGAACAAGG-3' R: 5'-TTCTCTGGGCTTTTCTTCCA-3'
<i>OAS3</i>	F: 5'-CCCTGGTCTGAGACTCACGTTT-3' R: 5'-GACTTGTGGCTTGGGTTTGAC-3'
<i>OASL</i>	F: 5'-CGTGAAACATCGGCCAACTAAG-3' R: 5'-GTACCCATTTCCCAGGCATAGA-3'



## Reagents

Culturing media and reagents involving in cell stimulation and viability assay

### 1.) Complete DMEM (100 ml) (Store at 4°C)

Incomplete DMEM	90	ml
FBS	10	ml
5000 U/ml Penicillin/Streptomycin	1	ml
100X MEM-NEAA	1	ml

### 2.) 1X PBS pH 7.4

PBS	1	pouch
Type I water	1	liter

PBS powder was dissolved in distilled water, sterilized this solution by autoclaving at 121°C for 15 minutes and stored at room temperature.

### 3.) 0.1% BSA (Store at 4°C)

BSA	0.01	g
Sterile PBS	10	ml

### 4.) 5 mg/ml MTT solution

MTT	50	mg
Sterile PBS	10	ml

MTT was dissolved in sterile PBS and filtrated by 0.2  $\mu$ M acrodisc syringe filter. This solution was kept at 4°C with light protection.

Mastermixes for reverse transcription and quantitative real-time PCR

### 1.) Mastermix for cDNA synthesis (1 reaction)

RNase free water	5.5	$\mu$ l
10X RT buffer	3	$\mu$ l

25 mM MgCl <sub>2</sub>	6.6	μl
10 mM dNTPs	2	μl
50 M Random hexamer	0.5	μl
20 U/μl RNase inhibitor	0.6	μl
50 U/μl Multiscribe	0.25	μl
RNA 200 ng/μl	11.5	μl

### 2.) Mastermix for qPCR using SYBR green (1 reaction)

Power SYBR Green PCR Master Mix	10	μl
RNase-free water	7	μl
20 μM forward primer	0.5	μl
20 μM reverse primer	0.5	μl
50 ng/μl of cDNA or DNA	2	μl

### 3.) Mastermix for qPCR using probe (1 reaction)

TaqMan Universal PCR Master Mix	10	μl
RNase-free water	6.8	μl
20 μM forward primer	0.5	μl
20 μM reverse primer	0.5	μl
20 μM probe	0.2	μl
50 ng/μl of cDNA	2	μl

Reagents in SDS-PAGE preparation

### 1.) 1.5 M Tris-HCl pH 8.8

Tris base	181.7	g
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This agent was dissolved in 750 ml of type I water and then adjusted pH to 8.8 by HCl. The final volume of this solution was adjusted to 1,000 ml with type I water. This solution was kept at 4°C.

### 2.) 1 M Tris-HCl pH 6.8

Tris base	121.1	g
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This chemical was combined with 750 ml of type I water followed by pH adjustment to pH 6.8 using HCl. The volume was adjusted to 1,000 ml by type I water and this solution was stored at 4°C.

### 3.) 10% SDS

SDS	10	g
Milli Q	100	ml

### 4.) 10% APS

Ammonium persulfate	0.1	g
Milli Q	1	ml

This solution should be fresh preparation.

Buffer for running SDS-PAGE

### 1.) 10X SDS-PAGE running buffer

Glycine	144	g
Tris base	30.2	g
SDS	10	g

All chemicals were dissolved in Milli Q and adjusted to final volume of 1,000 ml with MilliQ. This buffer was kept at room temperature.

### 2.) 1X SDS-PAGE running buffer

10X SDS-PAGE running buffer	100	ml
Milli Q	900	ml

## Reagents and buffers in western blotting assay

**1.) Laemmli buffer (2X)**

10% (w/v) SDS	4	ml
Glycerol	2	ml
1 M Tris-HCl (pH 6.8)	1.2	ml
Milli Q	2.8	ml

After combination of all agents, bromophenol blue was added to the solution with final concentration 0.02% (w/v). This buffer was stored at  $-20^{\circ}\text{C}$  until use. 30 mg of DTT was added to 1 ml of this buffer prior to use.

**2.) 12.5% SDS-PAGE (1 gel)**

Milli Q	4,175	$\mu\text{l}$
40% Acrylamide gel	3,125	$\mu\text{l}$
1.5 M Tris-HCl pH 8.8	2.5	ml
10% SDS	100	$\mu\text{l}$
10% APS	100	$\mu\text{l}$
TEMED	5	$\mu\text{l}$

**3.) 4% Stacking gel (1 gel)**

Milli Q	3,650	$\mu\text{l}$
40% Acrylamide gel	625	$\mu\text{l}$
1 M Tris-HCl pH 6.8	625	$\mu\text{l}$
10% SDS	50	$\mu\text{l}$
10% APS	50	$\mu\text{l}$
TEMED	6	$\mu\text{l}$

**4.) 1X Transfer buffer**

Trans-Blot Turbo 5X Transfer buffer	20	ml
Ethanol	20	ml
Type I water	60	ml

**5.) 10X TBS**

Tris base	60.5	g
NaCl	88.7	g

Both Tris base and NaCl were dissolve in 1,000 ml of type I water and this buffer was kept at room temperature.

**6.) 1X TBST**

10X TBS	100	ml
Tween 20	1	ml

Both solutions were mixed and adjusted volume to 1,000 ml with Milli Q. This buffer was kept at room temperature.

Reagents for in-solution digestion

**1.) Lysis buffer (Store at -20°C)**

Sodium deoxycholate	13.6	g
1 M triethylammonium bicarbonate (TEAB)	0.5	g
Distilled water	25	ml
100X Protease inhibitor cocktail		

**2.) 25 mM TEAB**

1 M TEAB	100	mg
Type I water	10	ml

**3.) 100 mM DTT**

DTT	10	ml
25 mM TEAB	0.05	g

**4.) 100 mM IA**

Iodoacetamide	10	ml
25 mM TEAB	72.07	g

**5.) Trypsin solution**

Trypsin  
Suspension buffer

Reagents for in-solution dimethyl labeling

**1.) 100 mM TEAB**

1 M TEAB	1	ml
Type I water	9	ml

**2.) 4% CH<sub>2</sub>O**

37% CH <sub>2</sub> O	10.81	μl
Type I water	89.19	μl

**3.) 4% CD<sub>2</sub>O**

20% CD <sub>2</sub> O	20	μl
Type I water	80	μl

**4.) 4% <sup>13</sup>CD<sub>2</sub>O**

20% <sup>13</sup> CD <sub>2</sub> O	20	μl
Type I water	80	μl

**5.) 0.6 M NaBH<sub>3</sub>CN**

NaBH <sub>3</sub> CN	7.54	mg
Type I water	200	μl
<b>6.) 0.6 M NaBD<sub>3</sub>CN</b>		
NaBD <sub>3</sub> CN	3.95	mg
Type I water	100	μl
<b>7.) 1% Ammonia solution</b>		
25% Ammonia solution	20	μl
Type I water	480	μl
Reagents for LC-MS/MS		
<b>1.) Buffer A (0.1% in 5% ACN)</b>		
Acetonitrile	1	ml
50% Formic acid	40	μl
LC-MS grade water	19	ml
<b>2.) Buffer B (0.1% FA in 100% ACN)</b>		
Acetonitrile	20	ml
50% Formic acid	40	μl
<b>3.) 25 fmol BSA standard</b>		
1 pmol of digested BSA	5	μl
0.1% FA in LC-MS grade water	195	μl
<b>4.) Blank</b>		
0.1% FA in LC-MS grade water	200	ul

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PUBLICATION	1.) Surawut, S., Panpetch, W., Makjaroen, J., Tangtanatakul, P., Thim-Uam, A., Wongphoom, J., Tumwasorn, S., and Leelahavanichkul, A. (2018) Helicobacter pylori Infection Increased Anti-dsDNA and Enhanced Lupus Severity in Symptomatic FcγRIIb-Deficient Lupus Mice. <i>Frontiers in Microbiology</i> 9, 1488 2.) Makjaroen, J., Somparn, P., Hodge, K., Poomipak, W., Hirankarn, N., and Pisitkun, T. (2018) Comprehensive proteomics identification of IFN-lambda3-regulated anti-viral proteins in HBV-transfected cells. <i>Molecular and Cellular Proteomics</i> , In press. 3.) Surawut, S., Makjaroen, J., Thim-Uam, A., Wongphoom, J., Palaga, T., Pisitkun, P., Chindamporn, A., and Leelahavanichkul, A. (2018) Increased susceptibility against <i>Cryptococcus neoformans</i> of lupus mouse models (pristane induction and FcγRIIb deficiency) is associated with activated macrophage, regardless of genetic background. <i>Journal of Microbiology</i> , In press.
AWARD RECEIVED	First Runner-up from The 34th Annual Meeting The Allergy Asthma and Immunology Association of Thailand