THE CHARACTERIZATION OF IMMUNE RESPONSE IN PATIENTS WITH VIRAL HAPATITIS B INFECTION

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innate adaptive immunity (clinical outcomes) (multispecificity) envelope 171-180 HLA-Cw*08:01 53% HLA-Cw*08:01 cross-activity B C envelope 171-180 HLA-A (A*02, A*11, A*24) HLA-B (B*51) HLA-C HLA-A HLA-B HBeAg HBeAg Pegylated-interferon alpha Illumina Sentrix Humanref-8 v2 BeadChips microarray PBMC 24 (IFI16, MX1, MX2), (GBP3, IFI16), (APOBEC3F) and proteasome (PSMB8) (host factor)

Helper T cell 1 Helper T cell 2 22 Helper T cell 1 Helper T cell 2 IL-4 IL-10 IL-4 IL-10 Helper T cell 2 host factors clinical outcome

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PIMPAYAO SODSAI : THE CHARACTERIZATION OF IMMUNE RESPONSE IN PATIENTS WITH VIRAL HAPATITIS B INFECTION. ADVISOR : ASSOC.PROF. NATTIYA HIRANKARN, M.D.,Ph.D., CO-ADVISOR : ASSOC.PROF. TANAPAT PALAGA.,Ph.D., PROF. PISIT TANGKIJVANICH, M.D., 127 pp.

Hepatitis B infection is a major cause of liver diseases. Approximately 2 billion people worldwide and 400 million of them remain chronically infected. Defect or exhausted innate and adaptive immune responses lead to HBV persistence. It is believed that the vigorous and multispecific T-cell response are important to viral control in resolved HBV infection. Here, we firstly identified novel HLA-C-restricted CTL epitope of HBV antigen from resolved patient. The specific CTL response to amino acids 171-180 of envelope antigen restricted to HLA-Cw*08:01 molecule revealed responsiveness in 53% of tested patients with resolved HBV infection. The cross-activity of this CTL response was detected in HBV genotype B and C. The comparative specific CTL response against Env171-180 versus the known-HLA-A or -B-restricted epitopes indicated that the frequency and magnitude of HLA-Cw*08:01-restricted Env171-180 response are greater or at least comparable to known HLA-A*02, A*11, A*24 and B*51 restricted CTL responses. Moreover, we investigated whether gene expression patterns in peripheral blood mononuclear cells (PBMC) were different between sustained virological responder and non-responder to Pegylated-interferon alpha in chronic HBV infection with positive or negative HBeAg. The Illumina Sentrix Humanref-8 v2 BeadChips microarray was used for the analysis of global gene expression. We found that most of the significantly different genes were at higher level in the responder compared to the non-responder groups both at pretreatment and/or during treatment at week 24. Some interesting immune-related genes are previously reported to have anti-viral activity such as response to virus (IFI16, MX1, MX2), viral defense or viral genome sensor (GBP3, IFI16), regulation of viral reproduction (APOBEC3F) and proteasome (PSMB8). Another factor which influences the chronic status of HBV infection is host factor. Several polymorphisms of immune genes, such as cytokine genes, were previously reported to be individually associated with disease progression; however, the analysis of these polymorphisms together as the combination of Th1 and Th2 genotypes has never been investigated. In this study, twenty-two polymorphisms of cytokine and cytokine receptor genes were studied for their association with the risk of chronicity. Although the combined analysis of the role of Th1 and Th2 genotypes gave no positive association with chronic hepatitis B infection. Our genotype data showed that the patients with low IL-10 as well as low IL-4 producing allele has lower risk for chronic state suggesting a protective role of Th2. Taken together, host factors affect the distinct immune responses resulting in the different clinical outcomes of HBV infection. The more information in HBV pathogenesis and immune response is necessary for the further development of novel therapy for chronic HBV infection.

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HBV	Hepatitis B virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
PRR	Pattern Recognition Receptor
PAMP	Pathogen-Associated Molecular Pattern
CTL	Cytotoxic T Lymphocyte
Th1	T helper1 cell
TLR	Toll-Like Receptor
KC	Kupffer Cell
NK	Natural Killer Cell
APC	Antigen Presenting Cell
β_2m	β_2-microglobulin
TCR	T-Cell Receptor
ER	Endoplasmic Reticulum
pDC	Plasmacytoid Dendritic Cell
Treg	Regulatory T cell
PBMC	Peripheral Blood Mononuclear Cell
PD1	Programmed Death 1
IFN	Interferon
HLA	Human Leukocyte Antigen
GWAS	Genome-Wide Association Study
TNF-α	Tumor Necrosis Factor alpha
ISG	Interferon Stimulated Gene

PP2A	Protein Phosphatase 2A
PRMT1	Protein Arginine Methyltransferase 1
PegIFN	Pegylated Interferon
HBsAg	Hepatitis B Surface Antigen
PBS	Phosphate Buffered Saline
FBS	Fetal Bovine Serum
qRT-PCR	Quantitative Real-Time PCR
SVR	Sustained Virological Response
EDTA	Ethylene Diamine Tetra Acetic acid
IL	Interleukin
SSO	Sequence-Specific Oligonucleotide
HWE	Hardy–Weinberg Equilibrium
GBP3	Guanylate-Binding Protein 3
IFI16	Interferon-gamma-inducible protein 16
STING	Stimulator of Interferon Gene
HSV-1	Herpes Simplex Virus
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3
EBV	Epstein-Barr Virus

CHAPTER I

INTRODUCTION

Chronic hepatitis B virus (HBV) infection remains a major worldwide public health problem. Globally, more than 400 million people are infected by HBV, and some of them may develop liver cirrhosis and hepatocellular carcinoma (HCC). Approximately, over two-thirds of all cases of liver cancers worldwide are also caused by HBV (1). HBV is a non-cytopathic virus which can induce inflammation and liver injury by stimulating immune responses (2). The liver diseases related to HBV have a wide spectrum including acute hepatitis, chronic hepatitis, cirrhosis and hepatocellular carcinoma. Disease severity varies among infected individuals depending on viral, host and environment. Some persons can control and eliminate the HBV without clinically evident liver disease or with acute inflammation of liver in short-term clinical sequelae; while, others fail to clear HBV and develop into chronic infection (3).

HBV activates a broad range of innate and adaptive immune responses that help to control and eliminate HBV in the acute phase of infection(4, 5). Subsequently, the cellular components of innate immunity can trigger adaptive immune response that has effective HBV clearance. Cytotoxic T lymphocytes (CTL) and T helper1 T (Th1) cells play a central role in the control of HBV infection as shown by several evidences. For example, the depletion of CTL following acute HBV infection lead to persistence of HBV infection (6). The patients with acute HBV infection develop large amount of HBV antigen-specific CTL response with HLA class I restricted to multiple epitopes in envelope, nucleocapsid and polymerase proteins (7-9) and this event still persists after recovery due to the continuation of long-lived memory HBV-specific CTLs (10). Several reports showed the lack or exhausted immune responses in chronic HBV patients. Although the HBV-specific CTLs could be induced, their low proliferation and exhaustion could lead to dysfunction of HBV-specific CTLs in chronic infection (11). The narrow antigen-specific T-cell response against HBV were also found in chronic infection (4).

This different level of CTL response between acute and chronic infection may reflect a crucial immunity to viral controlling and disease progression. Therefore, the knowledge of immunogenic regions and virus-specific CTL repertoires in hepatitis B infection are important to understand viral pathogenesis and the development of noveltherapy. The immunodominant epitope is responsible for effective specific CTL response with high magnitude and level of response frequency. At present the epitopes presented by the common HLA class I such as HLA-A2, HLA-A11 and HLA-A24 have been defined (8-10, 12, 13). However, the immunodominant CTL responses restricted by HLA-C have been recently characterized and defined in several infected diseases (14). HLA-Cw*03-restricted CD8+ T cells can induce escape mutations in HIV due to immune pressure, suggesting that HLA-C-restricted CTLs are involved in the control viral infections (15, 16). The characterization of new HLA-C-restricted HBV epitopes is needed to expand the basic biological knowledge and as a part to develop the new therapeutic technique for HBV patients especially the ones in chronic stage who have the defect in specific immune responses.

Besides the important role of CTL, immunomodulation therapy with PegIFN-α is the standard treatment currently and can control HBV effectively in the patient. There are several known antiviral actions of IFNα including (I) inducing interferon-stimulated genes to block viral replication (17), (II) recruiting and activating antigen presenting cells (APC) by upregulating MHC and subunit of proteasome; one of interferon-stimulated genes (ISG), to promote antigen processing (2, 18, 19), (III) cooperates with T cell receptor and co-stimulatory receptor signaling to drive clonal expansion and differentiation of specific CTLs (20). However, there are hundreds of cellular genes that were regulated by type I IFN that might contribute to the eradication of HBV. To further identify factors that contribute to sustain clearance of HBV, it is necessary to understanding the action of type I IFN more clearly. In this study, the difference of gene expression profile of chronic HBV infection with response and non-response to PegIFN-α treatment was observed.

HBV clearance is mediated by antiviral cytokines produced by immune cells of the innate and adaptive immune response, especially interferon (IFN)- /β/γ and tumor necrosis factor alpha (TNF-α) (21). HBV-specific T-helper 1 cells that produce Th1 cytokines are involved in HBV clearance in both acute and persistent infection (2). Both viral (viral load, genotype and genomic mutations) and host factors (age, sex and immune status) contribute to differential clinical outcomes. As a result, both the cytokine polymorphisms that dictate the functionality of cytokine and the immune response are associated with different outcomes of HBV infection (22). By investigating these factors, healthcare practitioners could predict the severity of the cases and thus suggest the most effective treatment options to the patients. Several HBV association studies of the human leukocyte antigen (HLA) genes, the single nucleotide polymorphisms (SNP) and the promoter region cytokine genes were reported (23-25). However, the cytokines work as a network *in vivo* and the association with several cytokines rather than with single cytokine lead to different outcomes. Thus lastly, we also reported the effects of 22 SNPs from 14 cytokine and cytokine receptor genes on the susceptibility to HBV-related chronicity among Thai patients with chronic HBV infection and healthy individuals.

The understanding of the immune responses against HBV in various stages will lead to the development of the successful therapies in chronic HBV infection.

Hypothesis

Various mechanism of effective immune responses including the HBV-specific CTL, IFN type I and Th1 cytokines are important for the clearance of HBV infection. Our specific hypothesis includes:

1. HLA-C restricted CTL epitopes are immunodominant in HBV patients.
2. The upregulated genes in the PBMC from chronic hepatitis B patients who respond to PegIFN- compared to the nonresponder compose of important immunological genes responsible for viral clearance.
3. The combination of cytokine and cytokine receptor genes in the Th2 is a risk factor for chronic hepatitis B infection.

Objectives

1. To search new HBV-specific CTL response in resolved HBV infection.
2. To investigate the difference of gene expression profile between responder and non-responder to PegIFN- at pre-treatment and during treatment in CHB.
3. To study the association of cytokine and cytokine receptor gene polymorphism and risk of chronic HBV infection.

CHAPTER II

LITERATURE REVIEW

1. Natural History in Hepatitis B Infection

Hepatitis B virus is a member of the hepadnavirus, which has a hepatocyte as specific target for replication and persistent infection. It is a DNA virus having approximate 3,200 nucleotides that contain 4 open reading frames encoding envelope, nucleocapsid, polymerase and X proteins. The modes of HBV transmission are percutaneous, sexual, and perinatal in which bloodborne is a major route. The maternal-fetal transmission is a major cause of chronic infection in exposed infants whereas infection in adults typically are self-limited (3). Despite the safe and effective universal hepatitis B vaccination for more than 20 years, the HBV infection is still a global health problem. It is a major cause of chronic liver inflammation worldwide with approximately 400 million people having chronic HBV infection that is associated with the cirrhosis and hepatocellular carcinoma resulting in 1 million death a year. The prevalence is high ($\geq$8%) in Southeast Asia and Saharan Africa, intermediate (2-7%) in Mediterranean countries and low in Western Europe and USA (1). The HBV genotypes are relied on genome sequencing with the differences more than 8% divergence to distinguish each genotypes. Currently, HBV genotypes are classified into 8 genotypes (A-H) with distinct geographic distribution (26). Hepatitis B virus is a non-cytopathic virus, which can induce inflammation and liver injury by stimulating immune responses. The liver diseases related to HBV have a wide spectrum including acute hepatitis, chronic hepatitis, cirrhosis and hepatocellular carcinoma that varies between individuals depending on viral (genotype, mutation or dose of infection), host (age or immune response) and environmental (alcohol, co-infection with HCV or HIV) factors. Some persons can control and eliminate the HBV without any clinical symptoms or develop acute inflammation of liver with short-term clinical sequelae; while, others fail to clear HBV and develop chronic infection.

2. Immune Response in Hepatitis B Infection

2.1 Innate immunity to HBV infection

HBV activates a broad range of innate system and adaptive immune responses that help control and eliminate HBV in the acute phase of infection (2, 4). Unlike HCV, HBV-DNA and HBV antigens in the serum and liver are undetectable until the logarithmic phase at 4-7 weeks after infection (4). Interestingly, there are not much host responses in the liver of HBV infected chimpanzee model at lag phase (27). There are many possibilities of such the delayed detection of HBV antigens and HBV-DNA. The study in animal model suggested certain mechanisms e.g, 1) the early infection are only restricted to a few hepatocytes, 2) the HBV spreads with slow doubling time and 3) the initial site of infection is not in liver but in bone marrow of woodchuck hepatitis virus infection (28). However, the mechanism in human is not clear because it is difficult to exactly analyze the characteristic of initial antiviral defense mechanisms to HBV in acute asymptomatic HBV infection (first month after infection). Moreover, the real HBV-infected animal model is still lacking except in the chimpanzee which is not widely available. Even though HBV transgenic mice are used in several experiments but it is not a representative model because only they only express certain HBV antigens but not the whole HBV infected model. Once the viral antigens appear during log phase, they will be detected through PRRs such as Toll-like receptor (TLRs) leading to viral control by the intrinsic induction of cell death, activating the cellular immune genes or cytokine or chemokine production. These components can subsequently trigger the adaptive immune defense (29). Some important innate immunity factors are:

I. Kupffer cells

Kupffer cell (KC) is a macrophage living in the liver, representing 15-20% of total liver cell population. Macrophage is induced by TLRs leading to the alteration of its phenotype and function including 1) the production of type I IFN, pro-inflammatory cytokines, chemokines, nitric oxide and reactive oxygen species, 2) having phagocytic activity, 3) enhancing antigen presentation in response to IFN-γ. After the release of the

HBV particles, proteins and HBV DNA from infected hepatocytes, HBcAg is the main antigen that interacted with TLR2 on macrophage surface leading to IL-6, IL-12 and TNF-α secretion (30). The endosomal TLRs (TLR3, 8, 9) can detect phagocytosed HBV particles resulting in chemokine and cytokine production including type I interferon that trigger recruitment and activation of $CD8^+$ T cell, $CD4^+$ T cell, DCs and natural killer cells (NKs) (31-34). On the other hand, the inflammatory feature and liver injury in HBV infection is influenced by activated KCs expressing FasL resulting in apoptosis of infected and bystander hepatocytes (35, 36).

II. NK and NK-T cells

NK cell is a member of lymphoid cell population which is one of the crucial immune cells of the innate immunity against HBV by direct cytotoxicity to infected cells and by the production of inflammatory cytokines (IFN-γ, TNF-α, GM-CSF). Intrahepatic NK cells can be induced by chemokines and cytokines produced by activating Kupffer cells (KCs) (32). The IL-12 and IL-18 from kupffer cells can also induce NK cells to produce IFN-γ. The NK-T cell is a T cell subset expressing the markers of NK and T cell, which can produce IFN-γ upon IL-12 and NK-cell stimulation (37). The cellular genes are activated such as IFN-γ (27, 38) and the decrease of HBV replication can be detected during IFN-γ production in the liver before the recruitment of T lymphocytes (39). This observation reflects the role of NK or NK-T cell function via IFN-γ to HBV controlling in experimentally infected chimpanzee during acute infection. A confirmatory experiment by activating NK-T cell with α-GalCer in the liver which subsequently produced IFN-γ and IFN-α/β to control HBV replication was done in the T cell-depleted mice (40). Moreover, non-classical NK-T cell can also be activated by HBV antigen upon adoptive transfer these cells into the liver of HBV transgenic mice (41). It is most likely that the NK and NK-T cells are activated by recognition of HBV antigens or stress-induced molecules on liver dendritic cells (42). Interestingly, the increase of NK cells in circulation was found during high level of HBV replication whereas HBV-specific CTLs were detected after decreasing of HBV replication in acute phase of naturally HBV-

infected patient (43). Low expression of MHC class I on surface of HBV infected cells (44) and increase of cellular stress molecules (45) can activate NK cells bias to NK activatory receptors for their effector function to recognize and kill infected cells (5). The activation of NK cells, which is one of innate immune cells producing IFN-γ is a crucial front line host defense mechanism and triggers the adaptive immunity for HBV clearance.

III. Type I Interferon

IFNs are a multigene family of inducible cytokines. IFNs affect a number of cellular processes including those regulating cell growth, differentiation, and apoptosis, as well as the modulation of the immune response (17). Both type I and II interferons (IFN-α/β and γ, respectively) are major molecules of defense against viral infections. IFNs block multiple steps of viral replication, including entry of the virus into the cells, transcription, translation, maturation, assembly and virion release (46). The diverse biological activities of the IFNs are mediated by a conserved signal transduction pathway. There are several known antiviral actions of IFNα including (I) inducing interferon-stimulated genes (OAS, RNAse L and PKR) to block viral replication (17), (II) recruiting and activating antigen presenting cells (APC) by upregulating MHC and subunit of proteasome; one of interferon-stimulated genes (ISG), to promote antigen processing (2, 18, 19), (III) cooperates with T cell receptor and co-stimulatory receptor signaling to drive clonal expansion and differentiation of specific CTLs (20).

However, there are hundreds of cellular genes that were regulated by type I IFN and might contribute to the eradication of HBV. To further identify factors that contribute to sustain clearance of HBV, it is necessary to understanding the action of type I IFN more clearly. Currently, several groups provided the information of IFN-differentially regulated genes in HBV infection using DNA microarray technique in HBV transfected hepatoblastoma cell line and HBV transfected mice model upon IFN-α/β induction. One study showed that 29 upregulated ISGs sharing between *in vivo* and *in vitro* model comprised of immunoproteasome, ubiquitin-like proteins, GTP-binding proteins,

chemokines and signaling molecules (47). Another study showed kinetics of IFN-α-induced expression profile either up-regulation or down-regulation in HBV transfected hepatoblastoma cell line including the genes involving in Interferon signal, kinase and protein degradation (48). One study reported that 2 ISGs (Diubiquitin and MyD88) could suppress HBsAg and HBeAg levels upon overexpressing these genes in HBV transfected hepatoblastoma cell line (49). From microarray studies above, some ISGs were indicated to have antiviral effect to HBV infection. For example, the proteasomes have an antiviral effect in HBV transfected hepatoblastoma cell line. It was found that proteasome inhibitor could block IFN-β-mediated inhibition of HBV replication with decline in HBV DNA levels (50). Moreover, another role of proteasome is the generation of peptides to be presented on MHC class I that supports adaptive immune response (51). The IFN can induce the LMP2 and LMP7 that are immunoproteasome catalytic subunit, and influence the pool of peptides of HBV polymerase and envelope proteins for processing and presentation by MHC class I to HBV-specific CTL (52). This observation suggested a role of IFN in regulating the adaptive immune response to HBV through alteration in HBV antigen processing by immunoproteasome activity.

It should be noted that the initial recognition of HBV infection in infected hepatocytes by toll-like receptors (TLRs) and the released IFN-α and IFN-β, type I interferon (4, 31) is quite different from other viral infection. While other viral infection lead to rapid and vigorous stimulation of IFN-α and IFN-β via the recognition of viral product within the infected cells, HBV escapes the detection of PRRs to single-stranded RNA replicative intermediate, by using transcriptional template (cccDNA) within the nucleus and newly transcribed viral genomes are generated within nucleocapsids (5, 29); thus, they werenotinteracted with PRRs. Therefore, the production of IFN type I in HBV infection is slower and with much lower level.

In addition to the evasion of HBV in early phase of infection by the replication within the capsid, other strategies of HBV to inhibit function of IFN type I have been reported. For example, HBV polymerase can inhibit IFN-β production by interacting to DDX3, a transcriptional factor of IFN-β promoter, leading to blocking of IRF signaling of

TLR-3 and RIG-1 recognition (53, 54). HBV X protein was also reported to inhibit IFN-β production by interacting to IPS-1, PRRs adaptor, resulting in prohibiting of IRF signaling through RIG-1 recognition in HBV transfected cells (55, 56). HBV proteins not only inhibit production of type I IFN but also block its antiviral function. After type I IFN induction, JAK/STAT signaling is activated to drive the interferon stimulated genes (ISG). HBV polymerase protein could block IFN-α induced response by interfering with STAT-1 translocation to nucleus in transfected HBV hepatocyte (57). HBV protein blocked STAT-1 signaling by upregulating protein phosphatase 2A (PP2A) which can inhibited protein arginine methyltransferase 1 (PRMT1) of STAT-1 methylation. That results in the inhibition of IFN-α signaling in cells expressing HBV proteins and in liver biopsies of patients with CHB (58). Interestingly, HBV has the capacity to block both the production and antiviral activity of type I IFN.

2.2 Adaptive immunity to HBV infection

In adaptive immune response, cytotoxic T lymphocytes (CTLs) and T helper T cells play a central role in the control of virus infection. Antigen presenting cells (APCs) particularly DCs present and mediate maturation of HBV-specific CD4+ and CD8+ T-cells (2). T-helper cell type 1 (Th1) cells are required for effector CTL maturation while antibody production from B cells are helped by T-helper cell type 2 (Th2) cells which able to neutralize free HBV particles (4).

I. Role of T cell response

In acute HBV infection, HBV-CTLs or Th1 response to HBV is greatly effective in HBV clearance. After the HBV-DNA was detected in the exponential phase of HBV replication, HBV-specific CTLs are generated and recruited to liver by CXCL-10 to kill infected cells as well as to secrete cytokines (IFN-γ) (5). Depletion of CTL following acute HBV infection lead to persistence of HBV infection in chimpanzee and demonstrating the importance of both cytolytic and non-cytolytic activity of specific CTLs (6). Similarly in the hepatitis-infected woodchuck, the resolving woodchucks is associated with detection of vigorous and multispecific T cell and related to WHV

resolution whereas the infected woodchuck group having less virus-specific T cells was associated with the progression to chronic infection (59). The influence of multispecific CTL response in other viral studies was shown to effectively control virus. Several reports have revealed specific CTL responses to numerous HBV epitopes during and after acute HBV infection (8, 10, 60, 61). The heterozygosity of HLA molecules that can present simultaneously distinct epitopes to specific T cells refers indirectly the role of the multispecific CTL response (62, 63). Thus, the particular HLA class I profiles indirectly reflected the immunological events and outcome of infection (64). It is likely that the possible theory of efficient HBV controlling is a strong and multispecific T-cell response.

Specific T helper 1 (Th1) cells are also important in HBV immune response, particularly byproviding help to generate and retain the effector CTLs. The CD4 response was found in acute HBV patient and chronic HBV infection with HBeAg seroconversion. In addition to multispecific CTL response as described above, multispecificity of CD4 response was reported with several identified HBV epitopes restricted to HLA class II molecules (65, 66). Moreover, the influence of heterozygosity of HLA class II may be relevant to HBV control as well (66).

II. Immunodominance in HBV-specific T-cell Response

HBV-specific CTL response is thought to play a crucial role to control HBV infection. The T-cell recognition to HLA-HBV peptide complex on infected cells determines T-cell response. Therefore, the understanding the basic knowledge of endogenous antigen processing and presentation in infected cells or antigen presenting cell (APCs) is necessary. This review will focus on antigen processing of HLA class I molecule and the HBV immunodominant epitopes for HLA class I molecule that relates to our study.

A. Antigen processing

HLA class I molecules are composed of 2 noncovalently linked polymorphic chains and nonpolymorphic β_2-microglobulin (β_2m). The extracellular peptide-binding cleft is formed by folding of α1 and α2 segments of the α chain as parallel-paired α helices resting on antiparallel eight-stranded β-pleated sheet. This cleft has amino acid

variability that binds different peptides and distinct T-cell receptors (TCR) on T cells. The peptide-binding cleft can bind limited peptides of 8-11 amino acids. While α3 segment is responsible for binding to CD8, which is T-cell co-recoptor. HLA class I genes compose of HLA-A, -B, -C genes. Each individual carries 2 alleles of each these genes. While TCR is with fine specificity on T cell, HLA molecules have a broad specificity for peptide binding. One reasonable explanation is peptides sharing structural feature that can bind to the same HLA molecule. Once peptide loading to the cleft of HLA molecule, peptide contacts with amino acid residues of β-pleated sheet and α helices depending on binding of positively charged N terminus and negatively charged C terminus of peptide. β strands in the floor of the cleft contain pocket interacting to anchor residue of peptide. For example, hydrophobic amino acid at the C-terminal end of peptide (prefering to lysine and arginine for HLA class I molecule) and some side chains of such peptide fit into specific pocket. Each HLA-binding peptide contains only one or two anchor residues and that anchor residues show variability in other residues.

Most protein antigens for HLA class I molecule are intracellular pathogens that their protein are synthesized within the cells. Some antigens are internalized into phagosomes and then escape into cytocol. Proteolytic degradation of proteins by proteasome, which recognizes the ubiquitinated proteins, is a mechanism for generation of peptides. Next, synthesized peptides are transported from cytosol into endoplasmic reticulum (ER) and loaded onto newly synthesized HLA molecule. The peptide-HLA class I molecule complex can be transported to cell surface that is ready to be recognized by specific CTLs. T cell recognizes both specific antigenic peptide and polymorphic amino acid residue of α helices of HLA molecule (67-69).

B. Immunodominant epitopes in HBV-specific CTL Response

Because of the limitation of HBV propagation into cell culture, the characterization of HLA-restricted T cell response against HBV is analyzed using HBV synthetic peptides that could be mimicked to natural antigen processing and presenting by HLA molecules on surface of infected cells (12). The memory T cells specific for HBV

synthetic peptides can be detected directly *ex vivo* or by expanding *in vitro*. The patients with acute or resolved HBV infection develop amount of HBV antigen-specific CTL responses with HLA class I restricted to multiple epitopes in core, envelope, polymerase and X proteins (7-10, 12, 70) and these memory HBV-specific CTLs still persists after clinical recovery from acute and chronic infection (60, 71).

HLA-A or -B-restricted Epitopes

The majority of HBV epitopes are defined as HLA-A2 restriction such as Core18-27, Env183-191, Env335-343 and Pol455-463, which have strong specific-CD8 responses and high binding affinity. Core-specific CD8 T-cells is the most numerous in circulating CD8 T-cells up to 1.3% (4). The core18-27 restricted to HLA-A2 is a major immunodominant epitope in HLA-A2 patients and among other HLA-A2-restricted epitopes (evelope183-191, evelope335-343, Polymerase455-463) (61). In addition, some immunodominant peptides were identified as the overlapping binding epitope thatrestricted to both HLA-class I and class II molecules. This phenomenon is found in Core18-27 that is restricted with both HLA-A2 for HLA-class I and HLA-DP for HLA-class II. In addition, theoverlapping core19-27 was shown to be restricted to B51 allele (72). It is likely that epitope bound with multiple HLA molecules across HLA class I and HLA class II alleles have potential to induce both specific CD8 T cell and CD4 T cell. Moreover, some epitopes recognized by several HLA alleles within the same supertype are identified (61). All of these are valuable to generate the multiple-epitope-based therapeutic vaccine in diverse population. One limitation of classifying the HBV-specific CD8 response is the genetic variation of HBV-specific T-cell epitopes among HBV genotypes (13).

HLA-C-restricted Epitopes

Besides HLA-A and HLA-B restricted epitopes, HLA-C-restricted epitopes have not been previously defined in HBV. However, it is possible that some epitopes restricted to HLA-B might in fact be restricted to HLA-C due to their tight linkage disequilibrium (73). One of the reason for lower interest in HLA-C is resulted from the

fact that HLA-C alleles have low expression on the cell surface. There is only 10% of HLA-C expression comparing to HLA-A and HLA-B (74). However, the immunodominant CTL responses restricted by HLA-C have been recently characterized and defined in several infected diseases (14). HLA-Cw*03-restricted CD8+ T cells can induce escape mutations in HIV due to immune pressure, suggesting that HLA-C-restricted CTLs are involved in the control viral infections (15, 16). Moreover, HIV is one of the infected diseases that has been reported to be associated with HLA-C gene. Single nucleotide polymorphism (SNP) in HLA-C promoter at -35 was found to be associated to HIV control (75). The -35C allele is in strong linkage disequilibrium with several HLA-C alleles such as Cw*01:02, 02:02, 03:02, 06:02, 08:01, 12:03 and 14:02. In addition, this -35C allele was associated to higher cell surface expression of HLA-C which might lead to better control of viremia and progress slowly to AIDs (76).

Taken together, the characterization of new HLA-C-restricted HBV epitopes is needed to expand the basic biological knowledge and as a part to develop the new therapeutic technique for HBV patients especially the ones in chronic stage who have the defect in specific immune responses.

C. Immunodominance in HBV-specific CD4$^+$T-cell Response

In addition to HBV-specific CTLs, HBV-specific CD4 T-cells are detected in acute HBV patients during or after infection. The important HLA-class II-restricted epitopes are characterized in core, envelope and polymerase epitopes (13, 65, 72, 77). Several nucleocapsid epitopes and the mainly core epitopes within 50-69 amino acid region of HBV protein can be recognized by specific CD4 T-cells in most acute HBV patients (65). The increased core-specific CD4 responses was also detectable implicating for HBeAg seroconversion in acute exacerbations of chronic infection (78). Despite the lower envelope-specific CD4 T cells comparing to nucleocapsid-specific CD4 T cells in acute HBV patients, strong envelope-specific CD4 responses were shown in vaccinated subjects (79, 80). Moreover, not only core-specific CD4 T-cell response but also polymerase epitopes restricted to the most common HLA-DR alleles were identified (77).

3. The different immunological and virological events between self-limited infection and chronic infection

At the moment, the exact mechanism that leads to chronic HBV infection is not clear. Both viral factors (viral load, genotype and genomic mutations) and host factors (age, sex and immune status) contribute to the persistent or chronic infection. Unlike chronic infection, self-limited infection has efficient adaptive immune response which is triggered and activated by powerful innate immunity. Interestingly, the recovered HBV patients who have detectable HBsAg in the serum can detect the low level of HBV replication in the liver. It is believed thatthis event helps maintain long-lasting memory T cells against specific HBV epitopes (60, 81-83). In this case, HBV reactivation might occur in immunosuppressive stage such as patient under chemotherapy.

3.1 Defect of innate immunity in HBV chronicity

Although not fully understand at the moment, the fate of HBV clearance or chronic progression is likely determined by the efficient adaptive immune function which is directly influenced by innate immunological action at early phase of HBV infection. After the primary HBV infections, the immune system fails to eliminate the virus after 6 months of infection, and allows virally persistent infection in approximately 5% of adults and 95% of neonates (84). Chronic HBV infection is associated with defective innate immune response indicated in the lack of massive IFN-γ production mainly from NK cells. That seems to be a factor to activating effective adaptive immune response and influence the outcome of HBV infection (85).

The decrease of TLR2 expression on hepatocytes, kupffer cells and peripheral monocytes is influenced by HBV interference in HBeAg-positive chronic patients and HepG2 cell line expressing HBeAg (86) as well as the reduction of TLR9 expression in plasmacytoid dendritic cells (pDCs) (87). Thus it leads to the decrease activity of DCs and decrease IFN type I production (86, 88, 89).

3.2 Defect of adaptive immunity in HBV chronicity

Despite the induction of HBV-specific CTLs , their low proliferation and exhaustion might explain the dysfunction of HBV-specific CTLs in chronic infection (11). Resolved HBV infection is strongly related to vigorous and multispecific T-cell response. Conversely, weak or undetectable and narrow antigen-specific T-cell responses against HBV were found in chronic HBV infection (8-10, 29, 90, 91). The frequency and magnitude of specific CTL response to different HBV epitopes are quantitatively different in distinct phase of HBV infection that is related to HBV clearance or persistence. Interestingly, the core18-27-specific CTL response which is often immunodominant in acute or self-limited infection was less or undetectable in chronic HBV infection with high HBV-DNA level ($>10^7$ copies/ml) (71). It is likely that the core18-27-specific CTL response related to HBV control whereas envelope- or polymerase-specific CTL were related to high level of HBV replication in chronicity (92).

Persistence of HBV infection relates not only to the impairment of the number and function of HBV-specific CTLs but also reducing activity of Th1 CD4+ T cells (93-95). In one example of HBV/HCV coinfection, although the multispecific CTL response to HBV epitopes could be detected but most immunodominant epitope-specific CTL and CD4 T-cell response (helper T cell) were absent or exhausted in patients with chronic progression (96). It is suggested that coordination of CD4 T-cell and CTLs is important. Recently, the genetic association analysis reveals that *HLA-DRA1* and *HLA-DPB1* are strongly associated with chronic HBV infection (24).

Regulatory T cells (Treg) can modulate the virally immunological response through direct cell-cell contact and their suppressive cytokines. There are two different roles of Treg. One is to protect the immunopathological liver damage while another one is to support the viral persistence by inhibiting the proliferation and function of HBV-specific T cells as well as B-cell antibody production in chronic phase of infection. Frequency of Treg in the peripheral blood and in the liver of chronic HBV infection was higher than that in resolved infection or in severe patients. Moreover, increased Treg number was related to high level of HBeAg and failure of control HBV (97, 98). Depleted

Treg from peripheral blood mononuclear cells (PBMC) leaded to the increased proliferation of core-specific T cells of chronic HBV patients (99). However, this relationship of Treg and HBV clearance is still controversy (100, 101). Thus, Treg is likely important to protect liver damage from harmful immune responses while the association of Treg with chronic progression needs more information to further clarify its controversy.

Recently, the role of programmed death (PD)-1/PD-1L pathway was raised to explain the T-cell responsiveness in chronic infection. PD-1 is expressed on activated T and B cells including exhausted CTL cells while its ligands are expressed on several cell types including hepatocytes and dendritic cells (102). PD-1 was firstly described in chronic viral infection with LCMV that resulted in exhaustion of LCMV-specific CTL and simultaneously increase of PD-1 expression (103). These cells could be restored their function of proliferation, cytotoxic activity and cytokine production by PD-1/PD-1L blockade. For chronic HBV infection, blocking PD-1/PD-1L pathway increased IFN-γ producing CTL in liver of transgenic mice (104). Similarly, PD-1 expression was increased together with the impaired proliferation and cytokine production of HBV-specific CTLs from PBMCs expanded with synthetic peptide panel covering all HBV proteins in chronic HBV patients but not in resolved subjects. Their functions were recovered by blocking PD-1/PD-1L signaling (105). This concept was also confirmed in HBV-specific intrahepatic CTLs (106).

3.3 Host genetic factor on HBV chronic infection

I. Effect of HLA molecules

The susceptibility to infectious diseases including chronic HBV infection is determined by host genetic factors (107). HLA gene profile in individual patients is one of the important susceptible genes to infection due to its role in antigen presentation to induce antigen-specific T-cell repertoire for effective antiviral function. Different HLA alleles were related to the fate of infection (108). According to HLA restriction to peptides as described previously, the particular HLA which is restricted to specific immunodominant epitope such as core18-27-restricted to HLA-A2, the infected patients

having such HLA may have a better chance to control virus. Thus the influence of HLA alleles and their subtypes are associated with viral persistence or resolution and risk or protective to viral infection.

Self-limited HBV infection is associated with strong polyclonal and multispecific CD4 T-cell responses while lacking number or having dysfunction of helper T cell (109). Polymorphisms of HLA class II may influent the resolution of HBV infection. Several studies investigated the association of HLA polymorphisms with outcome of HBV infection. Failure to develop anti-HBV immunity following HBV vaccination in non-responders was shown to associated with HLADRB1*03:01 and HLA DRB1*04:01 (110-112). In addition, the associations between the *HLA-DP* variants and the protective effects against persistent HBV infection and with clearance of HBV have been reported based on Genome-Wide Association Study (GWAS) and candidate gene approach in Asian, African and Caucasian populations (113-116).

II. Effect of single nucleotide polymorphism (SNP) of cytokine genes

HBV clearance is mediated by antiviral cytokines produced by immune cells of the innate and adaptive immune response, especially IFN-$\alpha/\beta/\gamma$ and tumor necrosis factor alpha (TNF-α) (21). In addition, Th1 cytokines produced from HBV-specific T-helper 1 cells and other immune cells are important for the development of successful CTL and NK-cell response resulting in resolution of both acute and persistent infection (2). On the other hand, secretion of Th2 cytokines such as IL-4, IL-10 and regulatory cytokines such as TGF-β leads to a weaker T-cell response. The polymorphisms of cytokine and cytokine receptor genes influencing their functionality through gene expression, mRNA stability or protein structure have been reported to associated with the susceptibility to various chronic diseases (117). Polymorphisms for pro-inflammatory cytokines associated with HBV persistence were investigated at the position -308 and -238 of *TNF-*α (118, 119), position -511 of *IL1*β (120) and position -174 of *IL6* (118). Moreover, the association of polymorphisms of Th1 cytokine genes with HBV chronicity was also reported in *IL2* (121, 122), *IFN-*γ (121, 123) and *IL18* (124) as well as the

association of polymorphisms of Th2 cytokine genes with HBV chronicity such as *IL4* -590 (121, 122) and *IL10* with single SNP and haplotype (125). However, the cytokines work as a network *in vivo* and the association with several cytokines rather than with single cytokine may lead to different outcomes.

3.4 Effect of viral factor on chronic HBV infection

Interestingly, the numbers and antiviral function of HBV-specific CTL in circulation and liver were negatively associated with the level of HBV-DNA (90, 92). The core18-27-specific CTL response which is often immunodominance in acute or self-limited infection was less or undetectable in chronic HBV infection with high HBV-DNA level (>10^7 copies/ml) (71). That indicates the effect of the level of viral load on regulating the protective HBV-specific T-cell response in chronic HBV. In addition, the tolerogenic effect of HBeAg was suggested due to the low level of core-specific CTL in chronic HBV patients with positive HBeAg. In addition, HBeAg was able to cross placenta to make tolerogenic effect and associated to viral persistence in mice model (126). In addition, HBeAg could bias helper T-cell response from HBV-specific Th1 to HBV-specific Th2 (127). Moreover, a numerous of soluble surface antigen were secreted during HBV replication that might result in T-cell defect or T-cell deletion (4). These observation above might explain how the treatment with antiviral drugs could restore protective T cell responses in chronic HBV infection (127, 128).

Antiviral pressure from CTL is an important factor to increase the HBV mutation in chronic HBV infection leading to the reduction of HBV-specific T-cell response, especially in the chronic HBV patients who express a narrow repertoire of anti-HBV CTL responses (91). Interestingly, the mutation usually occurs in the core region rather than in the envelope and polymerase epitopes suggesting that the CTL against core region is a protective immune response (92, 129).

4. Treatment of chronic HBV infection

The goals of therapy in chronic hepatitis B is 1) to suppress HBV replication, 2) to reduce liver cell inflammation and 3) to prevent the progression of fibrosis before the

development of cirrhosis and HCC. Currently, two treatment strategies are available which affect different steps in the life cycle of the HBV, 1) antiviral therapy with nucleos(t)ide analogues such as lamivudine, adefovir and entecavir and 2) immunomodulatory therapy mainly consisting of (Pegylated) Interferon-α (1).

4.1 Antiviral therapy

Licensed drugs in United States for the treatment of HBV infection are composed of lamivudin, adefovir, entecavir, telbivudine and tenofovir. The expected outcomes of antiviral drugs are on serologic (HBeAg or HBsAg seroconversion), virologic (reduction of HBV-DNA level or undetectable level), biochemical (normalization of serum ATL level) and histologic (improvement of necroinflammation and fibrosis) improvement. A course of antiviral drugs lead to sustained response after treatment by direct blocking of HBV replication in variable steps of replication. Among antiviral drugs, the effectiveness and resistant profiles to treatment are variable. In addition, the sustained response to antiviral drugs are not durable.Most HBeAg-negative patients and HBeAg-positive patients without HBeAg seroconversion should be continued treatment after 1 year for retaining effectiveness in absence of drug resistance. However, the side-effects of antiviral drugs are mild and acceptable for treated patients (1, 130). Lamivudine is the first antiviral drug which is discovered its anti-HBV activity in HIV/HBV coinfected patient treated with lamivudine (131) and confirmed its action in HBV monoinfection (132). However, the resistance to lamivudine in treated patients is usually found through point mutation at YMDD motif at catalytic center of the viral reverse transcriptase (133). Viral mutants with drug resistance are rising up with time-dependence of therapy (134). The second antiviral drug approved is adefovir which is one of the nucleotide analogues. It is an inhibitor of viral polymerase. Adefovir is very effective to inhibit HBV mutants in lamivudine-resistant patients and it is the least potent, the slowest to clear HBV and the least to develope HBeAg seroconversion (1). Tenofovir was approved for HIV treatment but it also has anti-HBV activity to HBV polymerase. It can reduce circulating HBV DNA level in HBV patients including lamivudine-resistant patients (130). Even though anti-viral drugs have low adverse effect and low cost, the

patients have to treat with long term resulting in the increase of drug resistance by strategy of viral evasion.

4.2 Immunomodulatory therapy

IFN alpha is effective after a relatively short course of treatment (6 months to 1 year) and, unlike antiviral therapy, has not been associated with drug resistance. Peginterferon alpha (PegIFN), created by attaching a polyethylene glycol molecule to interferon alpha, diminished side effects, has less frequent administration only once a week and has more potent antiviral activity than conventional IFN. PegIFNs is effective in reducing HBV DNA levels, normalizing levels of ALT and improving histological activity. PegIFNs also induce high sustained response rates both in HBeAg positive and HBeAg negative chronic hepatitis B (135). The sustained response to HBV treatment requires induction of a host immune response that can be achieved with immunomodulatory therapy to eliminate the virus and preventing disease progression without drug resistance. However, currently the treatment with PegIFN in chronic hepatitis B has been unsuccessful in the majority of patients. Only 30% of the patients have sustained responses (136). Both viral and host factors are important determinations of response to IFN in chronic HBV patients. Moreover, the side effects of IFN therapy are vigorous that make it difficult in a number of patients.

Due to the important role of specific CTL, the effort to activate effective HBV-specific T cells by active immunization or adoptive immunotherapy might be useful in chronic HBV patients. However, .there was a report that T cells exposed to antigen for a long time in chronic stage might have permanent functional change. This phenomenon was demonstrated in chronic HCV patients treated with PegIFN-α/ribavirin that the functional T cell cannot be completely restored even after sustained virologic response (137). Additional strategies which directly target to activate the defective HBV-specific T cells is an alternative way to achieve the complete restoration of the HBV-specific immune function as shown in table 1(5).

Table 1 Strategies of immune-base therapy (Modified Bertoletti A. Gut, 2012 (5))

Strategies	**Effect**
Inhibition of viral replication	Partial restoration of anti-viral HBV-specific T cell functions
Blockage of negative regulatory pathways (co-stimulatory molecules, inhibitory cytokines, Tregs)	Partial restoration of HBV-specific T-cell function
Anti-apoptotic drugs	Reduction of HBV-specific T cell apoptosis
TCR redirect T cells/liver cell targeting by heterologous T cells	De novo reconstitution of functionally active HBV-specific T cell/activation of heterologous T cells
TCR-like antibodies	Targeted delivery of cytokines to HBV-infected cells
TLR agonists	Induction of type I IFN/pro-inflammatory cytokines

CHAPTER III

MATERIALS AND METHODS

PART 1
(Characterization of a new HLA-C-restricted HBV epitope)

Patient population

Subjects were primarily screened by questionnaire to select the expected recovered HBV subjects. Peripheral blood mononuclear cells (PBMC) of fifty-four candidate subjects were collected and kept in liquid nitrogen for this study. Their sera were used to detect hepatitis B surface antigen (HBsAg) and antibodies for HBsAg and HBcAg to clarify the resolved HBV infection. The diagnosis was based on serological evidence including the absence of hepatitis B surface antigen (HBsAg) and presence of IgM anti-HBc and anti-HBs antibody determined by commercial enzyme immunoassay kits (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany). The Institutional Review Board (IRB) of faculty of Medicine, Chulalongkorn university, Bangkok, Thailand approved the trial. All patients gave the written informed consent and were serologically negative for antibody to HIV and HCV. We calculated a sample size of recovered HBV subjects by formula as described below.

Sample size calculation :

No. of samples = pq/SE2

when p = frequency of HLA*08:01 × frequency of the highest response to HBV epitope

= 0.2 × 0.8 = 0.16

q = 1-p = 0.84

SE = 0.05

No. of samples = (0.16 × 0.84)/0.052

= 53.76 (~ 54 samples)

Synthetic peptides

A panel of 313 synthetic peptides with 15 amino acids overlapping by 10 residues covered whole HBV sequence in genotype C from database were purchased from Chiron Mimotopes (Victoria, Australia) or synthesized at the peptide synthesis facility of Massachusetts General Hospital using 9-fluorenylmethoxy carbonyl chemistry. The pools of core and X peptides were made in a 9 by 8 matrix, containing eight or nine peptides/pool, respectively whereas envelope peptide were pooled in a 9 by 9 matrix containing nine peptides/pool as well as polymerase peptides pooling in a 14 by 12 matrix containing 12 or 14 peptides/pool, respectively (138). The known HBV epitopes restricted by HLA-A2, -A11, -A24 and -B51 were purchased from Proimmune (Oxford, United Kingdom) and from GenScript (Piscataway, NJ).

DNA extraction and HLA typing

DNA from PBMCs was extracted by QIAamp DNA extraction kit (Qiagen, Hilden, Germany). The required quantity of DNA for sequencing is to be need at least 10 µg for quantity, ≥30ng/µl for DNA concentration, 1.7 ~ 1.9 for OD 260/280 ratio and 1.7 ~ 2.2 for OD 260/230 ratio. HLA typing for HLA class I in loci A, B and C of patients was performed by HLA-SBT High Resolution (BGI, Kong Hong, China).

Isolation of PBMC

Peripheral blood mononuclear cells (PBMCs) from patients were isolated from resolved HBV patients. Twenty milliliters (ml) heparinized blood samples were collected and processed within 2 hours of collection. Whole blood was diluted in RPMI at ratio 1:1. PBMC were isolated by Ficoll-Hypaque reagent (Robbins Scientific Corporation, Sunny vale, CA) at ratio 3:4 (v/v; Ficoll-Hypaque reagent : diluted blood). This solution was centrifuged at 1,500 rpm for 30 minutes at room temperature. For density-gradient centrifugation technique, separated PBMCs in interface by specific density were collected and washed twice in 10 ml of phosphate buffered saline (PBS) by centrifugation at 1,500 rpm for 10 minutes at 4◦C. PBMCs were suspended with freezing media, 10% DMSO in fetal bovine serum (FBS) and kept in liquid nitrogen tank.

Expansion of PBMCs

PBMCs were *in vitro* expanded with peptides for 10 days before immunologic assay. For full proteome screening, 20% of PBMCs were pulsed with 10 μg/ml of all overlapping peptides for 1 h in incubator, then washed the pulsed cells twice with HBSS (Gibco BRL Laboratories, Gaithersburg, MD) and cocultured with the remaining PBMCs (80%) in AIM-V medium with 2% human AB serum and 20 U/ml of IL-2 (R&D Systems, Abingdon, UK) for 10 days. For single peptide expansion, PBMCs were directly induced with HBV peptide (5 μg/ml) for 15 mers peptide or 1 μg/ml for 9-10 mers peptide for 10 days.

Intracellular cytokine staining (ICS) and degranulation assays.

The 10-day expanded PBMCs *in vitro* were stimulated with 15 mers HBV peptide (5 μg/ml) or or 1 μg/ml for 9-10 mers peptide or medium alone (AIM-V medium with 2% human AB serum) as a control with brefeldin A (10 ug/ml) and CD107a antibody that is a degranulation marker (BD Pharmingen San Diego, CA) for 5 h. After that cells were washed and stained with CD3 and CD8 antibody (BD Pharmingen San Diego, CA) for 30 min in 4°C and then fixed and permeable for 20 min on ice before staining with IFNγ antibody (BD Pharmingen San Diego, CA) 30 min on ice. The cells were washed 3 times and analyzed by flow cytometer. The Phorbol-Myristate-Acetate (10 ng/ml) and Ionomycin (100 ng/ml) was used as a positive control.

IFNγ Elispot assay

Fifty thousand cells of 10-day expanded PBMCs were incubated with pools of 313 overlapping-synthetic peptides (5 ug/ml) as described above in 96-well ELISPOT plate (Multiscreen-HTS; Millipore, Billerica, MA) coated with IFNγ MAb (1DIK; Mabtech, Sweden) and blocked with AIM-V with 10% FBS overnight, then washed the plate with PBS and incubated with Biotinylated anti-human IFNγ MAb (7B6-1; Mabtech, Sweden) for 2 h in room temperature. After washing the plate, Streptavidin-alkaline phosphatase was add on the plate for 1 h at room temperature in the dark, plate was washed again and incubated with Alkaline phosphatase substrate (5 bromo-4-chloro-3-indolyl

phosphate-nitro blue tetrazolium chloride (BCIP-NBT); KPL, Gaithersburg, MD) for 15-20 min in the dark to develop blue spots and then stopped the reaction by washing with distilled water. The plate was air-dried and counted the spots by automated ELISPOT reader (ImmunoSpot; CTL, Cleveland, OH). The number of IFNγ-producing cells was calculated by subtracting with unstimulated control. The positive well was accessed when it's shown in more than 5 SFU two times above the mean of negative control (unstimulated wells). The Phorbol-Myristate-Acetate (10 ng/ml) and Ionomycin (100 ng/ml) was used as a positive control.

HLA restriction, fine specificity and peptide affinity assay

The short-term cell lines of patient PBMCs were generated by re-stimulating the 10-day expanded PBMCs at least once or twice with autologous specific peptide-pulsed PHA blast cells as the antigen presenting cells and allogeneic fresh PBMCs as the feeder cells which both of them were irradiated with 2,500 radians.

For HLA restriction assay, each allogeneic EBV-transformed B-LCLs having HLA class I matched with each HLA class I alleles of tested patient were pulsed with 10 µg/ml of the specific peptide for 1 h in incubator, then washed twice the pulsed cells with HBSS. The short-term cell lines were co-cultured with each pulsed EBV-transformed B-LCLs in AIM-V medium plus 2% human AB serum with brefeldin A (10 µg/ml) and CD107a antibody for 5 h. A negative control is co-culturing the short-term cell lines with unpulsed-HLA matched EBV-transformed B-LCLs while direct stimulation with specific peptide is as a positive control. IFNγ- and CD107a-producing $CD8^+$ cells were examined by flow cytometer.

To examine the fine specificity of the peptide (15 mers), the truncated peptides (8-10 mers) covered 15 mers-specific peptide was tested the specific $CD8^+$ cell response by ICS assay. The allogeneic HLA-matched EBV-transformed B-LCLs (as known HLA-restriction of such specific peptide) were pulsed with each truncated peptides (1 µg/ml) for 1 h. Next, the pulsed B-LCLs were co-cultured with the short-term

cell lines of patient PBMCs and brefeldin A (10 μg/ml) and CD107a antibody for 5 h. IFNγ- and CD107a-producing $CD8^+$ cells were examined by flow cytometer.

The affinity of the fine peptide was determined using various doses of the minimal optimal epitope in 1 μg/ml, 100 ng/ml, 1 ng/ml, 100 pg/ml and 1 pg/ml.

The allogeneic HLA-matched EBV-transformed B-LCLs (as known HLA-restriction of such specifically minimal optimal epitope) were pulsed with minimal optimal epitope for 1 h. Next, the pulsed B-LCLs were co-cultured with the short-term cell lines of patient PBMCs and brefeldin A (10 μg/ml) and CD107a antibody for 5 h. The dose response of the HBV peptide-specific CTL was measured in IFNγ and CD107a production $CD8^+$ cells by flow cytometry.

PART 2
(Gene expression profile in PBMCs of chronic HBV infection)

Patient selection

Eligible subjects were previously untreated (naive) patients, who were 65 years old. All patients have positive HBsAg (HBeAg + or -) and abnormal serum ALT (>UNL but ≤10XUNL) concentrations for at least six months before the beginning of the protocol, and a liver biopsy specimen collected within 12 months of entry in the study, with histopathological confirmation of chronic hepatitis B. Patients were excluded from the study if they had hemoglobin values < 12 g/dL, neutrophils < 1,500/mm^3, platelets < 90,000/ mm^3, human immunodeficiency virus (HIV) infection, co-infection with hepatitis C, decompensated liver disease, seizure disorders, previous organ transplantation, significant cardiovascular dysfunction, chronic pulmonary disease, poorly controlled diabetes, other cause of liver disease, pre-existing psychiatric disease, hemoglobinopathies, hemophilia, clinically significant retinal abnormalities or immunologically-mediated disease. Laboratory values for serum bilirubin, prothrombin time, albumin, creatinine and alpha-fetoprotein have to be within the normal range. All patients have an ultrasonography evaluation before entry in the study with no evidence of liver nodules or hepatocellular carcinoma. Patients are also excluded if they are pregnant, consuming alcohol or using intravenous drugs on a regular basis.

Patients who meet the inclusion criteria received peginterferon α-2b for 48 weeks. Peginterferon α-2b (Schering Plough, Kenilworth, NJ) was used at a dose of 1.5 mg/kg per week subcutaneously. All patients were evaluated for safety and tolerance at the end of weeks 2, 4, 8, 12, and every 4 weeks during treatment. Biochemical (BUN, creatinine, ALT, AST, bilirubin, alkaline phosphatase, albumin) and complete blood count were made at every visit. Thyroid function (TSH and free T4) was assessed at baseline. Serum HBV-DNA levels was quantitatively assessed by quantitative real-time PCR (qRT-PCR) at baseline, 24, 48, 72, and 96 weeks after the end of treatment).

Therapeutic end points that were used to assess treatment response included normalization of ALT levels, inability to detect HBV DNA in the peripheral blood by qualitative assays and/or loss of HBeAg or HBsAg and improvements in liver histology. After informed consent was obtained from the patients, PBMC were collected from the patients at base line and each visit (base line, 12, 24, 48 weeks after the end of treatment) and collected in Liquid Nitrogen and -70 degree Celsius, respectively.

After follow up until 96 weeks, patients were classified as sustained virological response (SVR) with both HBeAg positive patients that have HBV DNA <10,000 copies/ml and HBeAg seroconversion, and HBeAg negative patients that have HBV DNA <1,000 copies/ml. Another group, patients were defined as non responder (NR) at the end of treatment (48 weeks) with HBV DNA >10,000 copies/ml and HBV DNA >1,000 copies/ml in HBeAg positive patients and HBeAg negative patients, respectively. This study was approved and conducted according to the regulations stipulated by Ethic Committee for Human Research, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Sample Preparation and microarray analysis

Total RNA was isolated from 2 million PBMCs using RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). Total RNA was quantified by NanoDrop 1000A spectrophotometer. The ratio of absorbance reading at 260 nm and 280 nm (A260/A280) was used to evaluate the purity of RNA. Pure RNA has an A260/A280 ratio of 1.9-2.1. Furthermore, RNA integrity was evaluated by microfluidics analysis using the Agilent® 2100 bioanalyzer and an RNA 6000 Nano LabChip® kit (Agilent Technologies, Santa Clara, CA), which allows 5 ng of total RNA for analysis. Primarily full-length RNA will display a ratio of 28S to 18S rRNA bands that approaches 2:1.

For Sentrix HumanRef-8 Expression BeadChip which. Briefly, 250 ng RNA was used to prepare cRNA for hybridization by Illumina TotalPrep RNA Amplification Kit (Ambion, Grand Island, NY). This system consists of reverse transcription with an oligo (dT) primer containing a T7 promoter and in vitro transcription with T7 RNA polymerase (amplification and labeling step). Finally, the biotinylated cRNA, antisense RNA copies

of each mRNA in a sample, were generated. From the standard Illumina protocol, 750 ng of cRNA was used for hybridization to the Sentrix HumanRef-8 V2 Expression BeadChip (Illumina, San Diego, CA). After completing the hybridization, BeadChip was scanned on Illumina's BeadArray Reader.

Microarray Data analysis

Raw gene expression data were obtained by using Illumina's BeadStudio. Normalization was performed using a robust spline normalization (RSN) technique in the 'lumi' R package. The data were normalized separately depending on the objectives, including (1) only pre-treatment samples, and (2) both pre- and post-treatment samples. Genes that has low variability or had expression level for the majority of samples that was below the level of detection. We use IQR < 0.267 (20th percentile of IQR) as a first criteria and 75th percentile < 5 as second criteria to discard low variation and unexpressed genes. These two thresholds were selected based on the statistics of the data.

Student's t-test was used to screen for significant differences in expression level between each group of patients. To elucidate the change due to the treatment, averaged signal intensity for significant genes was clustered using agglomerative hierarchical clustering.

Statistical analysis.

Data from patient demographic and microarray were statistically analyzed by using Student's T-test.

PART 3
(The association of SNPs of cytokine and cytokine receptor genes with HBV chronicity)

Patient population

This study protocol had been reviewed and approved by the ethics committee of the faculty of Medicine, Chulalongkorn University. This case–control study was comprised of 131 Thai chronic hepatitis B patients (male:female = 75 : 56, mean age = 50.2 ± 12.7, age range = 25-81) at the Chulalongkorn Memorial Hospital and 142 ethnically and geographically matched healthy controls (male:female = 82 : 60, mean age = 30.8 ± 10.7, age range = 20-55) among the blood donors at the Thai Red Cross Society. After potential subjects completed an informed consent form, the presence of HBsAg, the level of serum alanine transaminase (ALT) and aspartate transaminase (AST) and the histopathology of their samples were examined. Apart from a positive test for HBsAg in a commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratories, Chicago, IL) for at least six months, individuals whose samples showed an abnormal level of ALT and AST and the characteristic histopathology were categorized as chronically infected with hepatitis B. None of the chronic hepatitis B patients have hepatocellular carcinoma. All the samples were negative for anti-HIV and anti-HCV antibodies.

DNA extraction

Genomic DNA was isolated from peripheral blood leukocytes via a standard salting-out method that use ethylene diamine tetra acetic acid (EDTA) as an anticoagulant (139). Briefly, buffy coat was added with 200 µl of nuclei lysis buffer (NLB), 50 µl of 10% SDS and 10 µl of proteinase K (10 mg/ml in H_2O stored frozen) subsequently mixing with pipette and vortex. Then, this solution was incubated at 65ºC for 2 h. After that, 175 µl of 5.3 M NaCl was added for protein precipitation. After centrifugation at 10,000-12,000 rpm for 15 minutes, solution phase was collected and precipitated DNA by adding 1 ml of cold absolute ethanol and following centrifugation at

10,000-12,000 rpm for 10 minutes. The DNA pellet was washed with 1 ml of cold 70% ethanol and centrifuge 1-2 minutes at 10,000-12,000 rpm. The DNA pellet was dried at 37ºC to evaporate the ethanol and dissolved with sterile distilled water. DNA should be kept in -20ºC.

Genotyping study

The genotyping study of 22 cytokine SNPs from 14 cytokine and cytokine receptor genes was performed by LIFECODES Cytokine SNP Typing kit to analyze *IL1A* -889T/C (rs1800587), *IL1B* -511C/T (rs16944), *IL1B* +3962T/C (rs1143634), *IL1R*(pst1 1970 C/T (rs2234650), *IL1RA* mspa1 11100 T/C (rs315952), *IL4RA*+1902 G/A (rs1801275), *IL12* -1188 A/C (rs3212227), *IFNG*+874 A/T (rs2430561), *TGFB* codon 10 T/C (rs1800470), *TGFB* codon 25 G/C (rs1800471), *TNFA*-308 G/A (rs1800629), *TNFA* -238 G/A (rs361525), *IL2*-330 T/G (rs2069762), *IL2*+166 G/T (rs2069763), *IL4*-1098 T/G (rs2243248), *IL4* -590 C/T (rs2243250), *IL4*-33 C/T (rs2070874)), *IL6* -174 G/C(rs1800795), *IL10*-1082A/G (rs1800896), *IL10*-819C/T (rs1800871), *IL10* -592C/A (rs1800872) and *IL18* -137C/G (rs187238). LIFECODES Cytokine-SSO typing kits utilize sequence-specific oligonucleotides (SSOs) to genotype the cytokine loci present in the PCR amplified sample. The loci included in the kit are putative functional SNPs. It is based on the hybridization of labeled single stranded PCR product to SSO probes. Firstly, the all cytokine loci were amplified by dividing in four amplification reactions that use the cytokine master mix1-4. Then the amplified DNA product was hybridized with probe. The probe mix contains two SSOs for each SNP that preferentially hybridizes to one of the alleles of a locus that may or may not be present in the amplified DNA. A different SSO probe can be attached to each color microsphere having up to 100 different populations of Luminex microspheres by its unique fluorescence signature or color so that can be mixed together and analyzed by the Luminex instrument. After hybridization step, the R-Phycoerythrin conjugated Streptavidin (SA-PE) was immediately added in the reaction and moved to the Luminex instrument for analysis.

Statistical analysis

Hardy–Weinberg equilibrium (HWE) was examined by 2 × 2 χ^2 test comparing observed and expected numbers. To perform allele and genotype frequencies in the case-control association tests, the PLINK v1.07 program (140) was used to calculate the odds ratios, 95% confidence interval and *p* value. The *P* value of less than0.05 was considered to be statistically significant. Haplotype frequencies were estimated by a PHASE calculation software (141).

CHAPTER IV

RESULTS

PART 1
(Characterization of a new HLA-C-restricted HBV epitope)

Identification and characterization of novel HLA-C-restricted HBV epitope

Because of low frequency of HBV-specific T cell in peripheral blood of HBV patients especially in chronic infection, the direct detection *ex vivo* by MHC-peptide tetramer staining, intracellular cytokine staining and Elispot assay is difficult. The *in vitro* expansion is a powerful strategy to overcome this problem. To identify new HBV epitope recognized by HBV-specific CTL, PBMC of HBV patient was tested by *in vitro* Elispot assay. PBMC expanded with a panel of overlapping HBV_{genC} peptide mixtures for 10 days. The Env171-185-specific IFN-γ-producing T cells were detected by Elispot assay. This observation was subsequently confirmed by ICS and degranulation assays that detected $CD8^+IFN\text{-}\gamma^+$ and $CD8^+CD107a^+$ T cells, respectively (Fig. 1A). HLA class I-matched B-LCLs were used for Env171-185-HLA restriction testing. The T-cell response was found in Cw*08:01-shared B-LCLs (Fig. 1B). Moreover, fine specificity was shown that Env171-180 is the minimal optimal epitope within Env171-185 restricted to Cw*08:01 (Table 2). The binding affinity for genotype B (F position 171) and genotype C (L position 171) are similar (Fig. 1C). It is suggested that the Env171-180 genotype B and C are HLA-Cw08:01 restricted peptides and have a similarity of Env171-180 specific-CTL affinity.

The immunogenicity of HLA-Cw*08:01-restricted response of Env171-180 peptide was examined in fourteen Thai resolved HBV patients having HLA-Cw*08:01 and one Cw*08:22 patient as shown in table 1. After 10-day *in vitro* stimulation with single Env171-180 peptide or overlapping HBV_{genC} peptide pool, Env171-180-specific T-cell responses were detected by IFN-γ production using Elispot or ICS. For single

Env171-180 peptide expansion, the Env171-180-specific CD8+ T-cell response to genotype C (8/15) is much more than that to genotype B (4/15) (Table 3). Furthermore, the magnitude of CD8+ T-cell response against Env171-180$_{genC}$ is stronger than that against Env171-180$_{genB}$ (Fig.2A, 2B). These responses in 8/15 samples can be confirmed in full HBV protein induction with a HBV$_{genC}$ peptide panel by Elispot assay (Table 3, Fig.2C). Four out of eight positive samples were also confirmed by ICS detecting IFN-γ production or degranulation assay (Fig.2D). We further investigate and found that the cross-reactivity could be observed between Env171-180 genotype B and C (Fig.2E). Specific CD8 response to Env166-180 were undetectable in non-HLA-Cw*08 patients (Fig.2C).

Interestingly, Env171-180 restricted to HLA-Cw*08:01 could also stimulate the patient's PBMC carrying HLA-Cw*08:22 (R32 patient) with high Env171-180-specific CTL response (Fig.2B and 2C). The HLA-Cw*08:01 is closely related to Cw*08:22 shown by phylogenetic tree (146). The HLA-Cw*08:22 differs from Cw*08:01 by A to G at position 2557 in exon 6. This change results in amino acid substitution from cysteine (C) to tyrosine (Y) at position 321 in the mature protein (Fig.3) (147). However, this single nucleotide polymorphism is not located on peptide-binding cleft (IMGT/2Dstructure-Database) that might be the explanation of the sharing the bound peptide (Env171-180) with HLA-Cw*08:01.

Table 2. HLA class I alleles of patients with resolved HBV infection

Samples	HLA Typing					
	A		B		C	
R1	A*11:01	A*24:02	B*13:01	B*15:02	C*03:04	**C*08:01**
R9	A*02:07	A*11:01	B*15:02	B*46:01	C*01:02	**C*08:01**
R10	A*02:07	A*24:07	B*15:02	B*46:01	C*01:02	**C*08:01**
R12	A*03:01	A*24:02	B*15:02	B*15:02	**C*08:01**	C*12:02
R16	A*24:07	A*68:02	B*15:02	B*15:10	C*03:04	**C*08:01**
R17	A*11:01	A*24:07	B*15:02	B*52:01	C*07:02	**C*08:01**
R21	A*02:01	A*11:01	B*15:02	B*39:01	C*07:02	**C*08:01**
R26	A*11:01	A*68:01	B*15:02	B*15:32	**C*08:01**	C*12:03
R29	A*11:01	A*33:03	B*15:02	B*58:01	C*03:02	**C*08:01**
R32	A*24:02	A*31:01	B*38:02	B*40:40	C*07:02	**C*08:22**
R33	A*11:01	A*11:01	B*15:02	B*51:01	**C*08:01**	C*14:02
R35	A*11:01	A*24:02	B*40:06	B*52:01	**C*08:01**	C*12:02
R41	A*11:01	A*24:02	B*15:02	B*39:01	C*07:02	**C*08:01**
R43	A*02:03	A*11:01	B*15:02	B*18:01	C*07:04	**C*08:01**
R48	A*02:07	A*11:01	B*15:02	B*46:01	C*01:03	**C*08:01**

N, negative; P, positive; ND, not determined

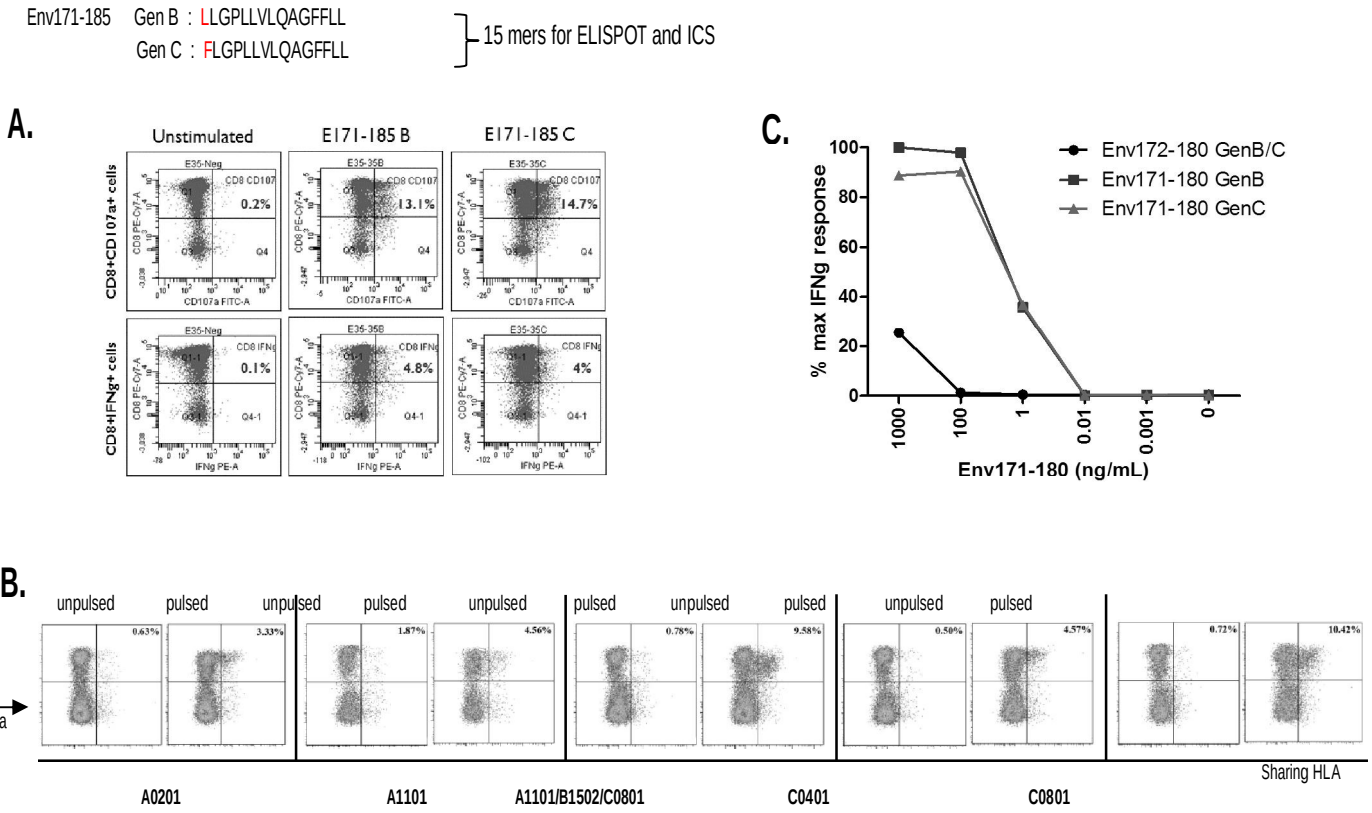


Figure 1. Analysis of specific-CTL response to $HBV_{Env171-185}$ peptide in PBMC of acute HBV patient. (A) PBMC was stimulated with overlapping peptide mixtures *in vitro* for 10 days and $HBV_{Env171-185}$-specific CTL response was determined in IFN-γ production and CD107a expression by ICS. (B) HLA restriction of $HBV_{Env171-185}$-specific CTL response was tested using HLA class I-matched B-LCLs prepulsed with Env171-185 peptide or media for 1 h before co-culturing with the short-term cell line of patient PBMCs. IFNγ- and CD107a-producing $CD8^+$ cells were examined after co-culturing for 5 h by flow cytometer. (C) To test the Env171-180 specific-CTL affinity, a dose titration of the peptides (0, 0.001, 0.01, 1, 100 or 1000 ng/ml) was used for stimulating the HLA-Cw*08:01-positve short-term cell line for 5 h and the response was estimated by detecting the IFN-γ-producing CD8 T-cell.

Table 3. Characterization of fine specificity of HLA-Cw*08:01-restricted to $HBV_{Env171-185}$ peptide[a].

AA no.		Amino Acid Sequence	%CD8+IFNg+	%CD8+CD107a+
166-180	15 mer HBVC	STTSGFLGPLLVLQA	33.1	58.2
171-185	15 mer HBVC	FLGPLLVLQAGFFLL	37.4	58.9
170-179	10 mer HBVB	GLLGPLLVLQ	1.8	19.5
170-180	11 mer HBVB	GLLGPLLVLQA	45.9	60.0
	11 mer HBVC	GFLGPLLVLQA	58.0	61.2
171-180	10 mer HBVB	LLGPLLVLQA	56.7	59.8
	10 mer HBVC	FLGPLLVLQA	57.6	59.5
172-180	9 mer HBVB/C	LGPLLVLQA	7.3	36.1
171-181	11 mer HBVB	LLGPLLVLQAG	58.5	61.1
	11 mer HBVC	FLGPLLVLQAG	58.8	60.2
172-181	10 mer HBVB/C	LGPLLVLQAG	0.1	0.2
173-181	9 mer HBVB/C	GPLLVLQAG	3.6	26.0

[a] The expanded HLA-Cw*08:01-positve short-term cell line with $HBV_{Env171-185}$ peptide *in vitro* for 10 days was induced with prepulsed matched-HLA-Cw*08:01 B-LCLs against each truncated peptide between residues 166-185 of HBV envelope. After 5 h the specific CD8 response was quantified the IFN-γ and CD107a production by flow cytometry.

Table 4. $CD8^+$ T-cell response against Env171-180 genotype B or C in HLA-Cw*08 patients[a]

Peptide	Amino acid sequence	No. of positive patients/total no. of HLA-Cw08 patients		
		single peptide	full HBV Gen.C proteome	
		ICS	Elispot	ICS
E171-180B	LLGPLLVLQA	4/15	ND	ND
E171-180C	FLGPLLVLQA	8/15	11/15	4/8

[a] PBMC of fourteen Thai patients with resolved HBV infection having HLA-Cw*08:01 and one Cw*08:22 were expanded with single Env171-180 peptide in genotype B or C differing first amino acid sequence (as indicated in underline) or with all HBV_{genC} overlapping peptides for 10 days. The Env171-180-specific T-cell responses were analyzed by ICS detecting IFN-γ production or degranulation assay for single Env171-180 peptide expansion while IFN-γ producing T-cells were detected using Elispot and confirmed by ICS or degranulation assay for all HBV_{genC} overlapping peptide *in vitro* expansion.

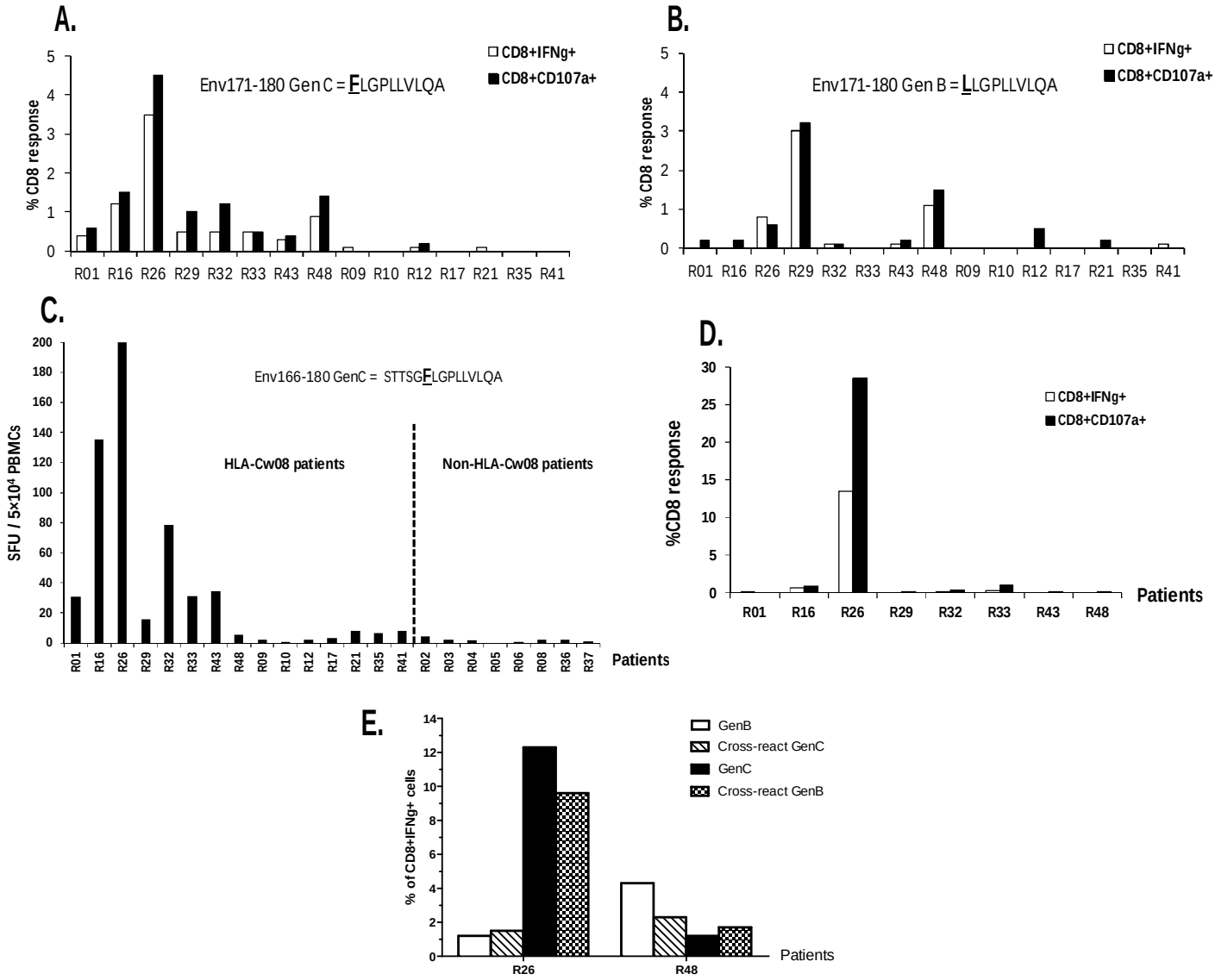


Figure 2. The CD8 response against HLA-Cw*08:01-restricted Env171-180 peptide. The expanded PBMC of the patients with resolved HBV infection with single Env171-180 peptide in genotype B (A) or genotype C (B) *in vitro* for 10 days were examined the Env171-180-specific $CD8^+$ response by degranulation assay and ICS detecting IFN-γ production. Confirmation specific $CD8^+$ response was investigated in the 10-day-expanded PBMC with mixtures of all 15-mers overlapping HBV_{genC} peptides including Env166-180 by IFN-γ Elispot assay (C) and all positive responses were reconfirmed with optimal Env171-180 induction by ICS and degranulation assay (D). (E) Cross-reactivity between genotype B and C of Env171-180 peptide was investigated in PBMC expanded with Env171-180 in genotype B for 10 days and were stimulated with Env171-180 in genotype B or genotype C (cross-react to Gen.C) for testing specific CD8 response by ICS and vise versa for genotype C expansion.

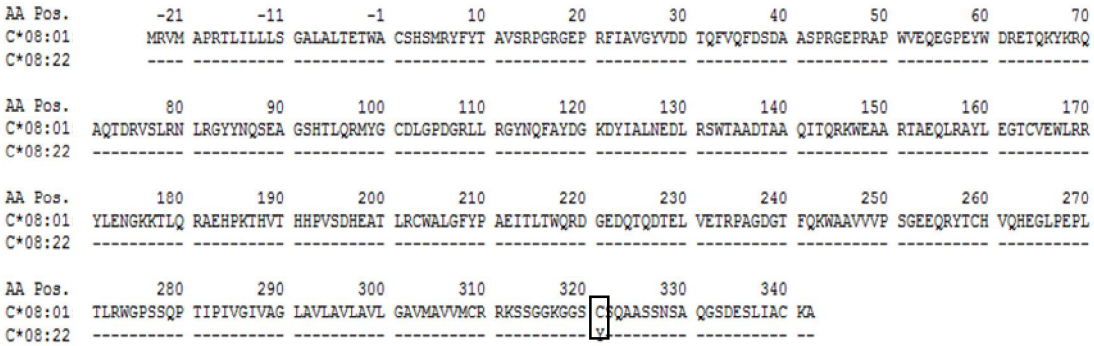


Figure 3. Sequence alignment between HLA-Cw*08:01 and HLA-Cw*08:22 in IMGT/HLA database. Only one single nucleotide polymorphism at position 2557 in exon 6 (*G*>*A*) results in amino acid change at residue 321 (C>Y).

The frequency and magnitude of HLA-Cw*08:01-restricted E171-180 CTL responses compared to well-known characterized epitopes

We subsequently analyzed the frequency and magnitude of specific CTL response against Cw*08:01-restricted Env171-180 epitope compared to well-known characterized epitopes after in vitro expansion with individual peptide (Table 4 and Fig 4A). The frequency of CTL response against HLA-Cw*08:01-restricted Env171-180 is similar to the HLA-A*02-restricted Pol455-463 CTL response (2/4 of both Env171-180 and Pol455-463). One subject, R43 responded to both Env171-180 and Pol455-463. We did not find any response to other known HLA-A*02-restricted epitopes. The frequency of HLA*Cw08:01-restricted Env171-180 CTL response (6/11) is higher than HLA-A*11-restricted C88-96 epitope response (3/11). None responded to P654-663 epitope. Most patients who responded to HLA*Cw08:01-restricted Env171-180 did not respond to any HLA-A*11-restricted epitopes. The frequency of CTL response against HLA-Cw*08:01-restricted Env171-180 and HLA-A*24-restricted epitopes was not different (3/7 of Cw*08:01 vs 4/7 of A*24 epitopes). Three out of four responded A*24 patients are restricted to both HLA-A*24-restricted epitopes (C117-125 and P756-764). One patient having both Cw*08:01 and B*51 only gave positive result with Cw*08:01 restricted Env171-180 but not to B51 restricted C19-27. It seems that the frequency of HLA-Cw*08:01-restricted Env171-180 response is greater or at least comparable to known HLA-A*02, A*11, A*24 and B*51 epitopes (Table 4). The magnitude of HLA-Cw*08:01-

restricted Env171-180 response is stronger than A*02, A*11 and B*51 epitopes but not in A*24 epitopes (Fig 4A). It should be noted that the above known-epitope responses could not be detected upon *in vitro* pooled-$HBV_{gen.C}$ peptide stimulation (data not shown). However, using pooled peptide stimulation, one known A*11:01 restricted epitope, core137-151 could be detected in 7/11 subjects by Elispot assay (Fig.4B). Interestingly, 5 out of 7 responded to both A*11:01 restricted core137-151 as well as Cw*08:01-restricted Env166-180 epitope (Fig.4B). These responses could be confirmed in 2 subjects by ICS and showed higher magnitude of response to Env171-180 than Core137-151 (Fig.4C). Therefore, the magnitude of HLA-Cw*08:01-restricted Env171-180 is higher than most known epitopes restricted by HLA-A or -B.

Table 5. The comparison of CD8 T-cell response to HLA-Cw*08:01-restricted Env171-180 epitope with known HLA-A or -B-restricted epitopes[a].

		peptide	amino acid sequence	No. of positive patients/ total no. of patients
HLA-C0801 vs HLA-A02	C0801	E171-180B	LLGPLLVLQA	1/4
		E171-180C	FLGPLLVLQA	2/4
	A02	C18-27	FLPSDFFPSI	0/4
		E183-191	FLLTRILTI	0/4
		E335-343	WLSLLVPFV	0/4
		E348-357	GLSPTVWLSV	0/4
		P455-463B	GLSRYVARL	2/4
		P455-463C	GLPRYVARL	0/4
HLA-C0801 vs HLA-A11	C0801	E171-180B	LLGPLLVLQA	3/11
		E171-180C	FLGPLLVLQA	6/11
	A11	C88-96	YVNVNMGLK	3/11
		P654-663	LMPLSACIQA	0/11
HLA-C0801 vs HLA-A24	C0801	E171-180B	LLGPLLVLQA	0/7
		E171-180C	FLGPLLVLQA	3/7
	A24	C117-125	EYLVSFGVW	3/7
		P756-764	KYTSFPWLL	4/7
HLA-C0801 vs HLA-B51	C0801	E171-180B	LLGPLLVLQA	0/1
		E171-180C	FLGPLLVLQA	1/1
	B51	C19-27	LPSDFFPSV	0/1

[a] The *in vitro* expanded PBMC of Cw*08:01 patients with single Env171-180 or other known epitopes were detected the specific CTL response by degranulation assay.

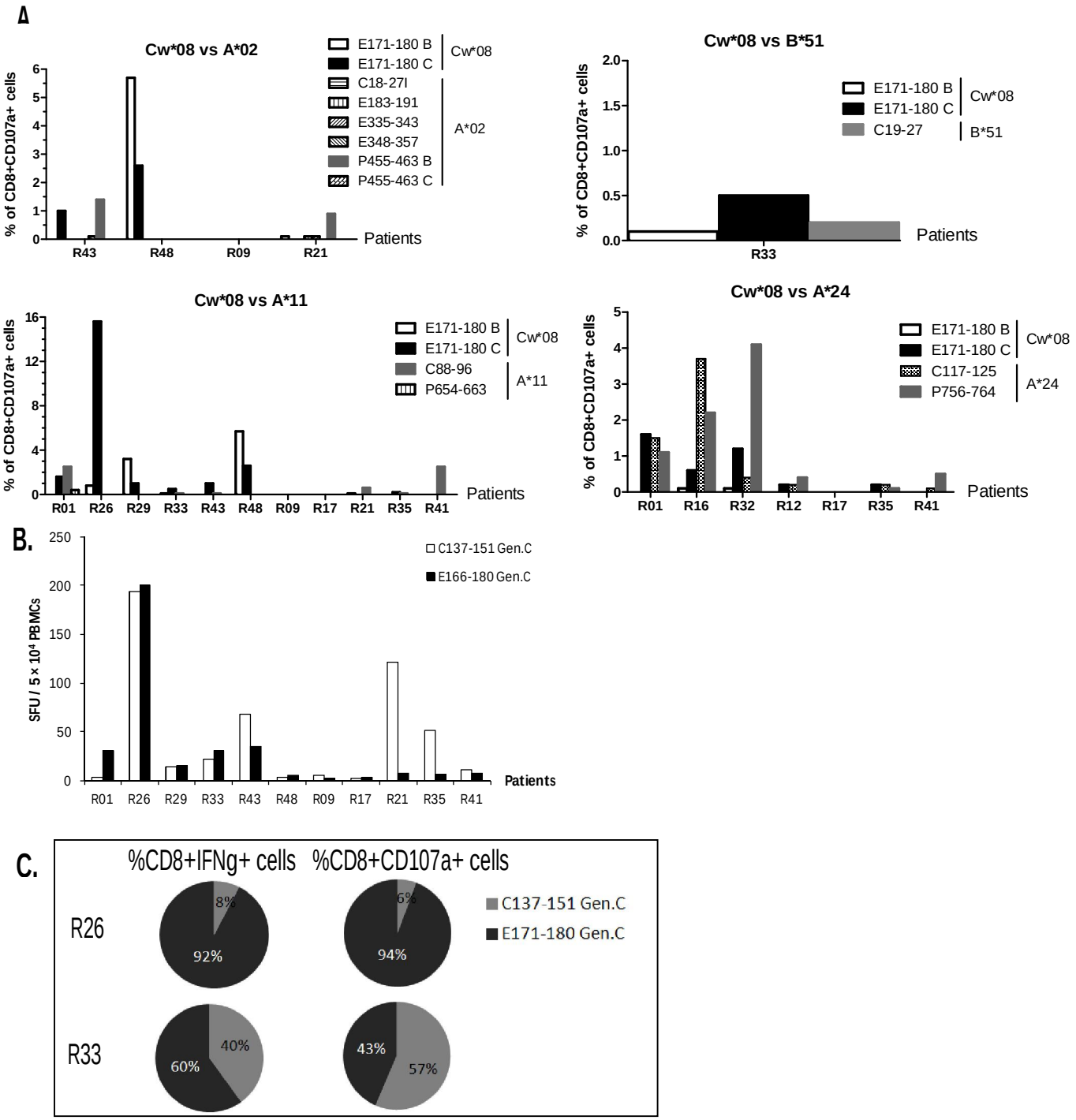


Figure 4. Comparison the HLA-Cw*08:01-restricted Env171-180 CTL response with known-HLA class I-restricted epitopes. (A) *In vitro* 10-day expanded PBMC of Cw*08:01 patients with single Env171-180 or known epitopes restricted by HLA-A*02, A*11, A*24 or B*51 in 4, 11, 7 or 1 patients, respectively were detected the magnitude of specific CTL response by degranulation assay. (B) The magnitude of specific CTL response of HLA-Cw*08:01 and known HLA-restricted epitopes with including in full overlapping $HBV_{gen.C}$ peptide pool stimulation were evaluated by Elispot assay and (C) confirmed by IFN-γ producing CTL and degranulation assay.

Discovery of the new HBV Polymerase epitope

In addition, we screened the PBMC from 23 Thai patients with resolved HBV infection with full 15 mer-overlapping $HBV_{gen.C}$ peptide pool stimulation and found the specific CTL response against the putative novel epitopes in pol561-575 and pol566-580 by Elispot assay (Fig 5A). These responses could be confirmed by IFN-γ producing CTL and degranulation assay that elucidated the stronger specific Pol561-575 CTL response than Pol566-575 CTL response (Fig.5B). Based on the overlapping sequences between the 2 peptides, the optimal minimal epitope restricted by HLA class I within these polymerase peptides were shown in figure 5C. Furthermore, the HLA restriction for Pol561-575 peptide was examined using HLA class I-matched B-LCLs (Fig.5D). This response seems to be restricted by HLA-A*24:07. However, we did not test the restriction for HLA-A*68:02 and HLA-B*15:10 due to the lack of HLA-matched B-LCLs with rare frequency (< 1% in Asian population). We also did not B-LCLs carrying closely related HLA with A*68:02 and B*15:10. Interestingly, the specific Pol561-575 CTL response had higher magnitude than HLA-Cw*08:01-restricted Env171-180 CTL response in the same patient (Fig.5B).

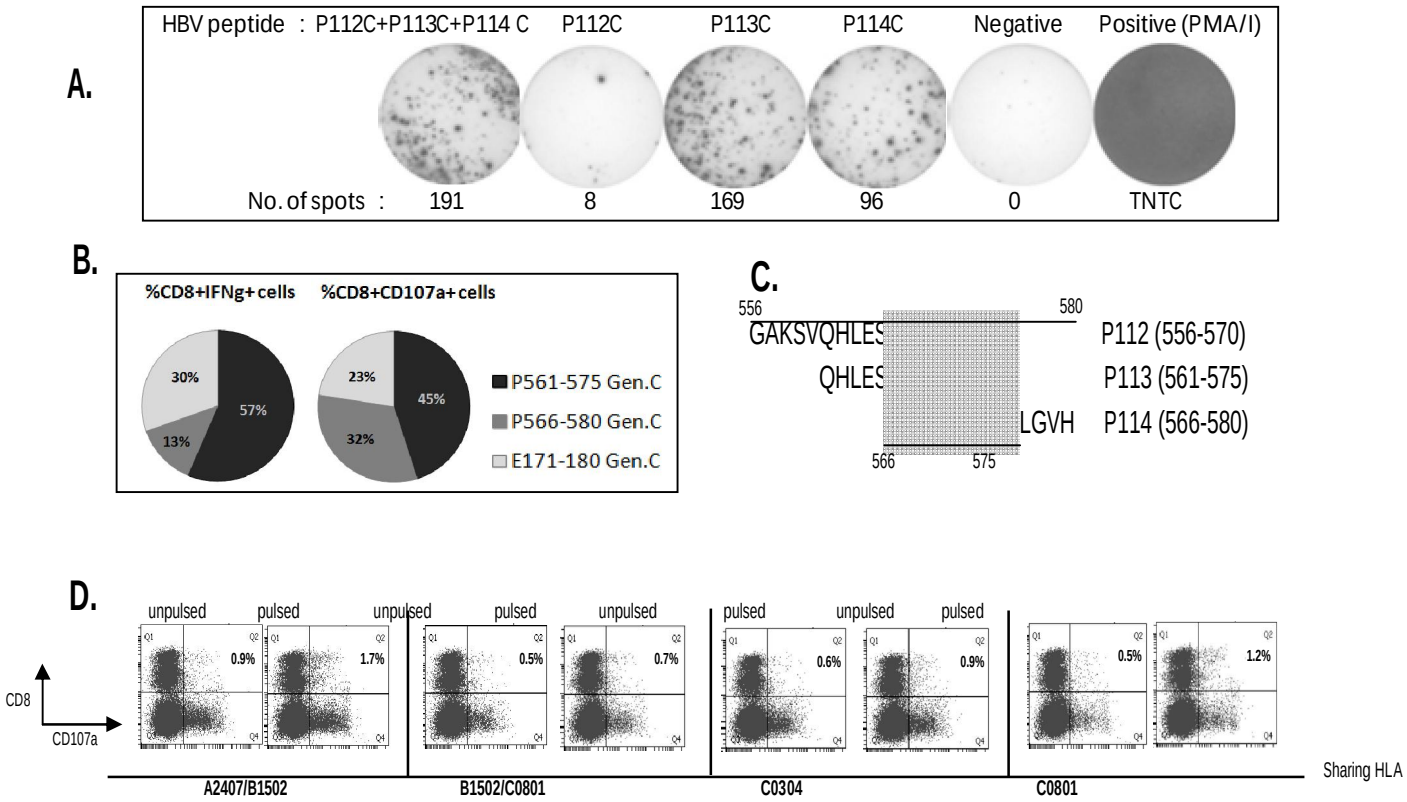


Figure 5. New characterized polymerase peptide. (A) The specific CD8 T-cell responses in expanded PBMC with full $HBV_{gen.C}$ overlapping peptide were analyzed by Elispot assay (unstimulated well as a negative control and PMA/I well as a positive control) and (B) Pol561-575 and Pol566-580-specific CTL responses were re-confirmed and compared the magnitude with $HBV_{Env171-180}$-specific CTL response by IFN-γ and CD107a production. (C) Overlapping amino acid sequences of P112(556-570), P113(561-575) and P114(566-580) were elucidated to predict a fine specific epitope as in the yellow box. (D) HLA restriction of $HBV_{Pol561-575}$-specific CTL response was tested using HLA class I-matched B-LCLs prepulsed with Pol561-575 peptide or media for 1 h before co-culturing with the short-term cell line of patient PBMC. IFNγ- and CD107a-producing $CD8^+$ cells were examined after co-culturing for 5 h by flow cytometer. TNTC, Too Numerous To Count.

PART 2
(Gene expression profile in PBMCs of chronic HBV infection)

Microarray analysis was performed from individual sample of sustained virological responder (SVR) and non responder (NR) groups of chronic HBV patients to analyze the gene expression profile in PBMC of NR and SVR groups at pre-PegIFN-α treatment and during treatment week24. There are evidences of the role of HBeAg in immune regulation acting as a tolerogen in chronic HBV infection. HBeAg can reduce TLR2 expression on monocytes leading to inefficient triggering cascade of inflammatory component production such as TNF- . This event results in imbalance of Th1/Th2 ratio and supports IL-4 and IL-10 production to suppress CTL response. This concept can use to explain the progression of acute rather than chronic infection in the infant who has vertical transmission from mother having chronic infection with HBeAg-negative mutant.

Due to effect of HBeAg in immune regulation as described above, chronic HBV patients in our study were separated in 2 groups, HBeAg positive and HBeAg negative groups, for gene expression analysis.

Gene expression profile between SVR and NR groups of chronic HBV patients with positive HBeAg.

The PBMC expression microarray profile of chronic HBV patients with positive HBeAg was compared between 3 SVR and 5 NR patients at pre-PegIFN-α treatment and during treatment. Table 6 was shown the demographics of all chronic HBV patients with positive HBeAg. The levels of gene expression significantly differences between SVR and NR with fold change > 1.3 and p value <0.05 were summarized in figure 6A. There were 2,744 genes that expressed at higher level in the SVR group and 93 genes that expressed at higher level in the NR group at pre-PegIFN-α treatment. While there were 3,210 genes that expressed at higher level in the SVR group and 46 genes that expressed at higher level in the NR group at week24 after treatment. Accordingly, a number of genes expressed differently since pre-treatment until during treatment at

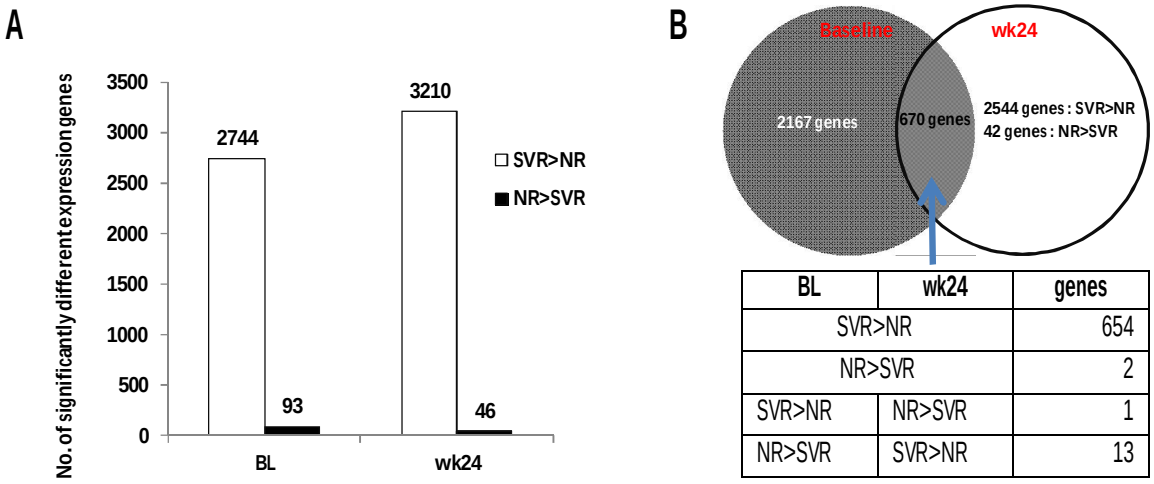
week24. Whereas some genes expression were significantly different between SVR and NR group only at pre-treatment or during treatment (Figure 6B). We are particularly interested in the significantly different expression genes at both pre-treatment and during treatment (670 genes). Among these, group of genes with higher expression in SVR compared to NR group were shown in table 8 (654 genes). The functional annotation of genes in table 8 was categorized in table 9. The immune-related terms include ubiquitin mediated proteolysis (UBE2B, UBE2Q1, UBE2R2), RIG-I-like receptor signaling pathway (TRADD), RIG-I-like receptor signaling pathway (TRADD), cytokine-cytokine receptor interaction (CCL25, CXCL14), T cell activation (IL-7R), natural killer cell mediated cytotoxicity and inflammatory response. Another group of 13 out of 670 significant different expression genes at both pre-treatment and during treatment which had significantly higher expression in NR at pre-PegIFN-α treatment but lower expression in the NR group after PegIFN-α treatment were shown in table 10. These genes have no function in any antiviral function. There were previous report of many genes which were up-regulated upon PegIFN-α treatment refered to as interferon-inducible genes and have an anti-HBV activity. Therefore, the genes which were previously reported from other microarray data or reported to have anti-viral function in HBV or HCV infection were intersected with the 665 significant different expression genes at pre-treatment and during treatment at week24 (Table 11). The functional annotation from genes in table 11 was shown in table 12. The immune terms that were shown from the analysis include T cell activation (FYN, LCK, STAT5B, DPP4, SPN), inflammatory response (AIF1, CLU, STAT5B) and immune response (LAT, TCF7, GBP5, CLU, GBP3, SPN, IFI16). Out of these list, GBP3 and IFI16 were previously studied to have antiviral action which will be described in more detail in the discussion part. The list of intersected genes between the previously reported genes by microarray data of HBV or HCV infection and the 2544 significantly different expressed genes during PegIFN-α treatment but not at pre-PegIFN-α treatment was indicated in table 13. The intersected genes in table 13 were also categorized for functional annotation in table 14. Interesting genes are proteasomal protein catabolic process (TBL1XR1, PPP2R5C,

BUB3, PSMB8), Toll-like receptor signaling pathway (TLR2), and chemokine signaling pathway (CCL2).

Table 6. Patient Demographics of chronic HBV patients with positive HBeAg used in microarray analysis

	SVR	NR
Number	3	5
Age (year)	38.33 ± 15.53	37.4 ± 6.43
Sex (Male/Female)	1/2	2/3
ALT	160.67 ± 152.11	97.2 ± 85.95
HBV DNA (cp/ml)	$10.17\times10^6\pm8.63\times10^6$	$20\times10^6\pm0$
Genotype (B/C)	1/2	2/3

SVR, sustained virological response; NR, non response; HBeAg, hepatitis B "e" antigen, ALT, alanine aminotransferase



BL	wk24	genes
SVR>NR		654
NR>SVR		2
SVR>NR	NR>SVR	1
NR>SVR	SVR>NR	13

Figure 6. Significant different expression genes between SVR and NR groups of chronic HBV patients with positive HBeAg at pre-PegIFN-α treatment and during treatment. (A) The number of significant different expression genes at pre-PegIFN-α treatment and during treatment between SVR and NR groups was indicated (white bar, higher expression genes in SVR than NR group; black bar, higher expression genes in NR than SVR group). (B) A number of significant different expression genes only pre-PegIFN-α treatment (black color) or during treatment (white color) or both pre-PegIFN-α treatment and during treatment were shown.

Gene expression profile between SVR and NR groups of chronic HBV patients with negative HBeAg.

The PBMC expression microarray profile of chronic HBV patients was compared between 4 SVR and 2 NR patients at pre-PegIFN-α treatment and during treatment. Table 7 showed the demographics of all chronic HBV patients with negative HBeAg. In chronic HBV infection with negative HBeAg, the number of genes significantly differences between SVR and NR at pre-PegIFN-α treatment and during treatment was shown in figure 7A. There were 2,419 genes that expressed at higher level in the SVR group and 323 genes that expressed at higher level in the NR group at pre-PegIFN-α treatment. Whereas there were 2,845 genes that expressed at higher level in the SVR group and 212 genes that expressed at higher level in the NR group during treatment at week24. Some genes had persistently different expression since pre-treatment until 24 week after treatment, while some genes expression were significantly different between SVR and NR group only at pre-treatment or during treatment (Figure 7B). The significantly different expressed genes at both pre-treatment and during treatment were 513 genes. We were interested in the group of genes expressed at higher level in SVR than NR at both time points as shown in table 15 (415 genes). The functional annotation of genes in table15 was categorized in table16.The immune-related terms include antigen processing and presentation (PSME2, HLA-DPB1), natural killer cell mediated cytotoxicity (KIR3DL1), proteasome (PSME2, PSMB8), regulation of viral reproduction (APOBEC3F) and Toll-like receptor signaling pathway (STAT1, TLR7). A group of genes with different expression at both pre-treatment and during treatment with higher expression in NR at pre-PegIFN-α treatment but lower expression during PegIFNα treatment (75 genes) were shown in table 17. These genes could not be categorized in any terms of the functional annotation. The genes which were reported in microarray data or studying of the anti-viral function in HBV or HCV infection were intersected with the 513 significant different expression genes at pre-treatment and during treatment at week24 (Table 18). Their functional annotations were categorized in table 19 and the immune-related terms include proteasomal protein catabolic process (PSME2,

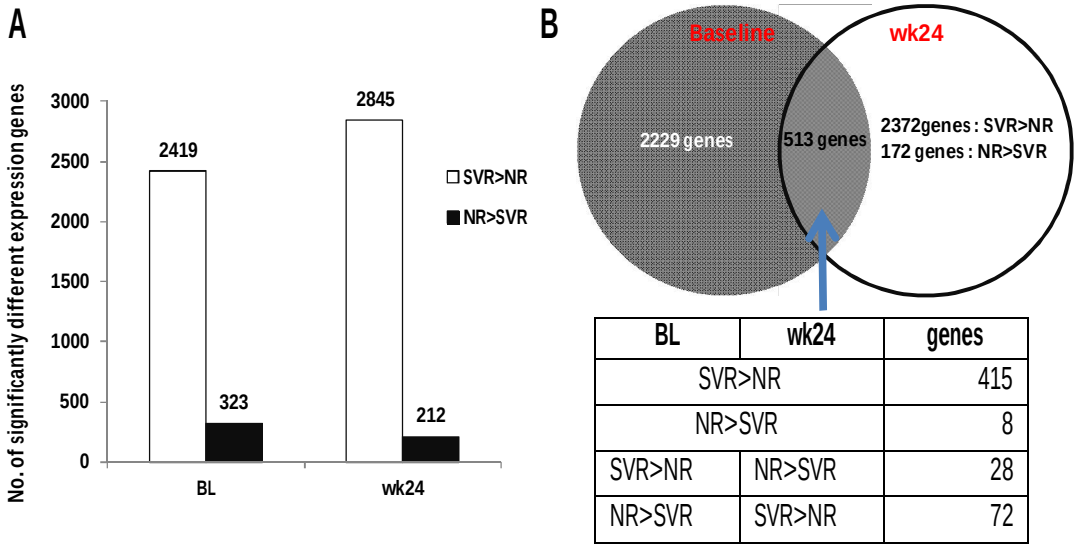
PPP2R5C, PSMB8), antigen processing and presentation (HLA-DPB1, PSMB8) and Toll-like receptor signaling pathway (STAT1, TLR7). In addition, the list of intersected genes between the previously reported genes by microarray data of HBV or HCV infection and the 2372 significantly different expressed genes during PegIFN-α treatment but not at pre-PegIFN-α treatment was shown in table 20. The functional annotation in table 21 include the immune-related terms such as T cell activation (SOX4, IL15), proteasome (PSMA4, PSMD6), Natural killer cell mediated cytotoxicity (TNFSF10, KLRC3, FCGR3A), response to virus (IFI16, MX1, MX2).

From the result several immune-related genes were predominately induced and upregulated in responder groups compared to non-responder groups at pre-treatment and during treatment with PegIFN- or both conditions. Moreover, some of these genes have antiviral function to control viral replication.

Table 7. Patient Demographics of chronic HBV patients with negative HBeAg used in microarray analysis

	SVR	**NR**
Number	4	2
Age (year)	45.75 ± 3.59	41 ± 24.04
Sex (Male/Female)	4/0	1/1
ALT	52.25 ± 9.46	64 ± 16.97
HBV DNA (cp/ml)	$12.87\times10^6\pm9.43\times10^6$	$17.45\times10^6\pm3.6\times10^6$
Genotype (B/C)	1/3	0/2

SVR, sustained virological response; NR, non response; HBeAg, hepatitis B “e” antigen, ALT, alanine aminotransferase



BL	wk24	genes
SVR>NR		415
NR>SVR		8
SVR>NR	NR>SVR	28
NR>SVR	SVR>NR	72

Figure 7. Significant different expression genes between SVR and NR groups of chronic HBV patients with negative HBeAg at pre-PegIFN-α treatment and during treatment. (A) The number of significant different expression genes at pre-PegIFN-α treatment and during treatment between SVR and NR groups was indicated (white bar, higher expression genes in SVR than NR group; black bar, higher expression genes in NR than SVR group). (B) A number of significant different expression genes only pre-PegIFN-α treatment (black color) or during treatment (white color) or both pre-PegIFN-α treatment and during treatment were shown.

Table 8. Significantly different expression of 654 genes between SVR and NR with higher expression in SVR at both pre-PegIFN-α treatment and during PegIFN-α treatment in chronic HBV patients with positive HBeAg.

Gene symbol[a]	Baseline		wk24		Gene symbol[b]	Baseline		wk24	
	P-value	FC	P-value	FC		P-value	FC	P-value	FC
ACOT11	0.0158	1.34	0.0188	1.35	AADAT	0.0201	1.55	0.0007	1.31
ACOT7	0.0139	1.36	0.0147	1.39	ACAD8	0.0400	2.27	0.0009	1.85
ACOX1	0.0040	1.36	0.0408	1.55	ACAN	0.0199	1.35	0.0232	1.35
ACRC	0.0095	1.69	0.0145	1.71	ACSL5	0.0139	1.57	0.0025	1.53
ADAM22	0.0113	1.33	0.0372	1.43	AGMAT	0.0423	2.13	0.0098	1.35
ADD3	0.0033	1.55	0.0066	2.26	AGPAT1	0.0109	1.52	0.0327	1.34
ADD3	0.0293	1.78	0.0099	1.88	ALAD	0.0497	1.78	0.0079	1.58
AFTPH	0.0416	2.56	0.0001	3.00	ALKBH8	0.0029	2.22	0.0049	1.41
AGL	0.0188	1.60	0.0286	1.63	ALS2CL	0.0247	1.46	0.0118	1.37

AIF1	0.0178	1.45	0.0009	1.56	ANKS1B	0.0300	1.51	0.0117	1.36
AKAP1	0.0146	1.61	0.0030	1.72	ANUBL1	0.0108	1.92	0.0020	1.63
AKTIP	0.0249	1.85	0.0026	2.02	AP1M2	0.0144	1.52	0.0001	1.35
AMMECR1	0.0001	1.91	0.0114	1.95	APOC2	0.0452	1.37	0.0014	1.32
AMMECR1L	0.0009	1.69	0.0012	2.09	APOL5	0.0076	1.62	0.0049	1.44
ANKRD27	0.0190	1.36	0.0004	1.76	AQP3	0.0281	2.08	0.0236	1.89
AP1S3	0.0277	1.39	0.0325	1.69	ARRDC3	0.0059	2.97	0.0002	2.68
ASB15	0.0205	1.44	0.0069	1.67	ASF1A	0.0111	2.18	0.0278	2.00
AZI2	0.0231	1.60	0.0051	2.10	ATP11C	0.0311	2.04	0.0028	1.78
BBS7	0.0003	1.51	0.0261	1.58	ATP8B2	0.0032	2.30	0.0095	1.59
BPTF	0.0336	1.56	0.0004	1.58	B3GALT3	0.0303	1.45	0.0241	1.41
BRD4	0.0184	1.58	0.0270	1.59	BAZ1B	0.0055	1.83	0.0136	1.41
BTBD7	0.0219	1.32	0.0089	1.37	BBS5	0.0256	1.99	0.0332	1.42
C10ORF88	0.0330	1.84	0.0042	2.28	BCAS3	0.0221	1.46	0.0042	1.32
C11ORF16	0.0337	1.34	0.0387	1.45	BGLAP	0.0443	1.42	0.0080	1.32
C12ORF32	0.0153	1.64	0.0063	1.88	BOLA1	0.0291	1.54	0.0325	1.35
C12ORF48	0.0425	1.36	0.0006	1.39	C10ORF119	0.0077	1.53	0.0120	1.34
C13ORF7	0.0170	1.84	0.0013	2.74	C10ORF35	0.0179	2.51	0.0421	1.77
C14ORF103	0.0115	1.37	0.0026	1.80	C10ORF68	0.0438	1.47	0.0148	1.44
C14ORF140	0.0104	1.38	0.0004	1.45	C11ORF17	0.0212	1.91	0.0327	1.37
C17ORF81	0.0430	1.34	0.0373	1.66	C11ORF63	0.0409	1.69	0.0184	1.35
C1ORF156	0.0124	1.47	0.0424	1.52	C14ORF105	0.0346	1.39	0.0348	1.36
C1ORF164	0.0109	1.57	0.0005	1.62	C15ORF29	0.0348	2.30	0.0035	1.66
C1ORF19	0.0396	1.55	0.0281	1.67	C15ORF40	0.0022	2.23	0.0013	1.90
C1ORF9	0.0377	1.78	0.0002	2.23	C16ORF46	0.0010	1.60	0.0115	1.42
C1QTNF2	0.0411	1.33	0.0038	1.47	C17ORF25	0.0075	1.48	0.0168	1.48
C2ORF52	0.0322	1.35	0.0035	1.37	C1ORF63	0.0355	2.09	0.0381	2.08
C3ORF15	0.0211	1.40	0.0058	1.41	C1ORF71	0.0477	1.54	0.0138	1.43
C4ORF16	0.0463	1.85	0.0011	2.11	C20ORF19	0.0099	2.12	0.0370	1.77
C6ORF120	0.0134	1.55	0.0121	1.98	C20ORF94	0.0340	1.98	0.0036	1.66
C6ORF167	0.0348	1.35	0.0008	1.50	C2ORF15	0.0221	1.50	0.0037	1.45
C9ORF5	0.0206	1.68	0.0024	2.03	C5ORF3	0.0083	1.40	0.0318	1.34
C9ORF90	0.0178	1.47	0.0019	1.51	C6ORF118	0.0127	1.62	0.0345	1.34
CACNB4	0.0354	1.44	0.0424	1.47	C6ORF134	0.0115	1.73	0.0046	1.44
CAMSAP1L1	0.0162	1.67	0.0012	2.21	C6ORF188	0.0283	1.49	0.0022	1.36
CASP4	0.0140	1.85	0.0022	2.03	C8ORF49	0.0022	1.66	0.0316	1.50
CAV1	0.0022	1.34	0.0496	1.45	C9ORF24	0.0326	1.94	0.0051	1.45
CBY1	0.0473	1.39	0.0441	1.39	C9ORF68	0.0329	1.57	0.0149	1.39
CCBL2	0.0341	1.60	0.0009	1.95	CARS	0.0097	2.24	0.0035	2.11

CCDC104	0.0099	1.63	0.0001	2.28	CASP6	0.0234	1.96	0.0410	1.46
CCDC25	0.0458	1.92	0.0185	2.03	CASP6	0.0275	3.70	0.0017	1.70
CCDC66	0.0031	1.99	0.0264	2.08	CAST	0.0447	2.90	0.0006	2.67
CCNL1	0.0161	1.90	0.0190	3.28	CAV2	0.0167	1.92	0.0299	1.40
CDH19	0.0063	1.53	0.0128	1.61	CCDC132	0.0071	2.45	0.0302	2.20
CENPE	0.0039	1.62	0.0235	1.63	CCDC77	0.0087	1.58	0.0452	1.44
CEP57	0.0309	2.21	0.0000	2.93	CCL25	0.0326	1.53	0.0050	1.47
CEPT1	0.0274	1.48	0.0262	1.67	CCNB3	0.0237	1.57	0.0093	1.47
CES4	0.0205	1.58	0.0110	1.70	CCT6A	0.0318	1.72	0.0093	1.56
CFH	0.0403	1.41	0.0028	1.53	CD34	0.0311	1.98	0.0079	1.52
CFTR	0.0392	1.39	0.0049	1.40	CD3D	0.0109	2.44	0.0068	1.36
CGN	0.0354	1.54	0.0008	1.64	CD3EAP	0.0189	1.43	0.0098	1.40
CHAC2	0.0308	1.36	0.0225	1.55	CDKN2A	0.0336	1.61	0.0405	1.60
CLDN2	0.0263	1.41	0.0013	1.60	CES7	0.0016	1.62	0.0468	1.46
CLDND1	0.0449	1.43	0.0408	1.54	CETN1	0.0372	1.71	0.0111	1.49
CLEC7A	0.0284	1.73	0.0051	3.36	CETN3	0.0332	1.80	0.0298	1.40
CLTC	0.0284	2.13	0.0001	2.65	CHM	0.0306	1.54	0.0125	1.52
CNOT7	0.0376	1.58	0.0013	1.67	CHRM5	0.0271	1.56	0.0298	1.35
COPS8	0.0199	1.33	0.0315	1.47	CHRNA2	0.0401	1.43	0.0238	1.33
COQ6	0.0111	1.40	0.0481	1.44	CLEC7A	0.0498	3.66	0.0494	1.77
CXCL14	0.0244	1.53	0.0016	1.94	CLU	0.0177	1.62	0.0060	1.33
DACH1	0.0230	1.45	0.0263	1.60	CLYBL	0.0080	1.46	0.0003	1.35
DAPP1	0.0401	2.31	0.0008	2.53	CMTM8	0.0109	2.85	0.0185	2.15
DDHD1	0.0005	1.45	0.0027	1.79	COL4A4	0.0359	2.07	0.0197	1.73
DDX17	0.0355	1.74	0.0290	3.23	CPLX4	0.0304	1.47	0.0209	1.41
DDX27	0.0076	1.45	0.0020	1.78	CREB5	0.0267	1.76	0.0208	1.34
DDX49	0.0248	1.47	0.0075	1.51	CRSP6	0.0472	1.52	0.0199	1.47
DENND1B	0.0375	1.46	0.0024	1.53	CRTAM	0.0363	1.64	0.0012	1.50
DKFZP434N035	0.0323	1.38	0.0232	1.55	CSNK1G1	0.0044	1.66	0.0107	1.46
DKK3	0.0390	1.84	0.0373	2.12	CSTF1	0.0152	1.43	0.0143	1.41
DLGAP4	0.0420	1.33	0.0068	1.51	CTBP2	0.0042	1.47	0.0167	1.40
DMXL1	0.0338	1.94	0.0048	2.01	CTRB2	0.0076	1.51	0.0042	1.41
DNAJB14	0.0030	1.55	0.0013	1.82	CYB561	0.0056	1.91	0.0139	1.32
DNAJB14	0.0498	1.31	0.0136	2.19	CYP19A1	0.0302	1.42	0.0036	1.34
DNASE2B	0.0397	1.37	0.0030	1.52	CYP4Z1	0.0052	1.79	0.0341	1.36
DSCR1	0.0066	1.43	0.0252	1.68	CYSLTR2	0.0221	1.77	0.0216	1.35
EDIL3	0.0444	1.47	0.0113	1.87	DALRD3	0.0171	1.98	0.0390	1.41
EED	0.0142	1.95	0.0008	2.68	DAPK2	0.0004	1.55	0.0114	1.41
EI24	0.0183	1.32	0.0131	1.84	DCAMKL1	0.0208	1.62	0.0178	1.44

EIF2S1	0.0073	1.60	0.0001	1.93	DCP1B	0.0080	1.50	0.0182	1.43
EIF3S10	0.0107	1.97	0.0001	2.25	DCUN1D4	0.0102	2.75	0.0256	1.82
ETAA1	0.0183	1.93	0.0047	2.13	DDB1	0.0011	1.39	0.0499	1.37
EVPL	0.0141	1.41	0.0002	1.57	DDX43	0.0332	1.81	0.0164	1.65
EXO1	0.0134	1.50	0.0316	1.55	DHRS12	0.0087	1.35	0.0240	1.33
FAM122B	0.0365	2.35	0.0030	2.91	DLEU7	0.0317	2.07	0.0387	1.46
FAM47A	0.0027	1.38	0.0020	1.38	DNAL1	0.0458	1.78	0.0132	1.45
FAM73A	0.0376	1.70	0.0004	2.41	DOCK1	0.0097	1.43	0.0267	1.39
FAM98A	0.0375	1.68	0.0001	2.14	DPP4	0.0369	2.69	0.0004	2.26
FBXW10	0.0443	1.33	0.0032	1.49	DSCAM	0.0340	1.46	0.0051	1.37
FBXW5	0.0114	1.46	0.0063	1.52	DUSP13	0.0424	1.43	0.0084	1.32
FGFR1OP2	0.0192	1.90	0.0007	2.41	ECEL1	0.0094	1.43	0.0329	1.39
FLJ27505	0.0015	1.43	0.0064	1.65	EDA	0.0027	1.62	0.0046	1.47
FLJ31951	0.0318	1.47	0.0121	1.48	EEA1	0.0145	1.52	0.0484	1.32
FNDC3A	0.0066	1.91	0.0031	2.57	EFHD1	0.0036	1.75	0.0248	1.31
FNDC8	0.0371	1.31	0.0497	1.39	EGR3	0.0013	1.81	0.0033	1.35
FPRL2	0.0052	1.72	0.0183	2.69	ELF2	0.0076	1.54	0.0286	1.50
FUT11	0.0149	1.49	0.0487	1.77	EPPB9	0.0155	1.61	0.0106	1.38
FXR2	0.0003	1.41	0.0270	1.48	ESCO2	0.0224	1.53	0.0033	1.35
FYN	0.0175	1.37	0.0052	1.78	EVC	0.0078	1.69	0.0011	1.33
FZD6	0.0085	1.81	0.0070	1.84	EVI5L	0.0405	1.70	0.0017	1.36
GCLC	0.0172	1.60	0.0334	1.97	FAM104A	0.0441	1.68	0.0118	1.44
GOPC	0.0047	1.60	0.0035	1.97	FAM29A	0.0384	1.78	0.0247	1.53
GPR12	0.0079	1.32	0.0032	1.36	FAM63B	0.0434	1.97	0.0163	1.31
GPR151	0.0157	1.40	0.0245	1.50	FAM86A	0.0295	1.67	0.0116	1.50
GPR26	0.0307	1.38	0.0018	1.44	FCHO2	0.0475	1.99	0.0171	1.89
GRM6	0.0416	1.46	0.0050	1.52	FGA	0.0090	1.55	0.0064	1.40
GTF2A1	0.0285	1.33	0.0007	1.88	FKBP2	0.0382	1.47	0.0130	1.44
HIF3A	0.0157	1.30	0.0104	1.44	FLAD1	0.0408	1.42	0.0301	1.35
HLTF	0.0392	1.37	0.0184	1.44	FLI1	0.0435	2.91	0.0242	1.81
HNRPLL	0.0310	2.16	0.0035	3.89	FLJ14397	0.0061	1.52	0.0401	1.44
HSPA4	0.0450	2.14	0.0000	3.64	FLJ16478	0.0222	1.35	0.0333	1.34
IFI16	0.0347	2.19	0.0172	2.35	FLJ32447	0.0287	1.59	0.0242	1.31
IFT74	0.0082	1.92	0.0060	2.82	FLJ35429	0.0030	1.50	0.0112	1.42
IHH	0.0263	1.32	0.0047	1.46	FLJ35801	0.0170	1.93	0.0133	1.44
IL1R1	0.0050	1.40	0.0030	4.66	FLJ36492	0.0145	1.58	0.0366	1.43
IL1RAP	0.0441	2.10	0.0022	2.49	FLJ46154	0.0009	1.77	0.0272	1.39
IL6ST	0.0176	1.55	0.0049	1.75	FSHR	0.0128	1.40	0.0132	1.35
IL7R	0.0081	5.70	0.0003	7.55	FUSIP1	0.0241	1.80	0.0021	1.44

INTS2	0.0047	1.58	0.0442	1.70	FXYD3	0.0154	1.36	0.0195	1.33
IQWD1	0.0108	1.38	0.0133	1.58	GALT	0.0012	1.74	0.0057	1.59
ITGA3	0.0424	1.43	0.0093	1.47	GAS2L2	0.0276	1.51	0.0179	1.36
JMJD1A	0.0243	1.58	0.0055	2.83	GBP3	0.0321	2.70	0.0060	2.10
KCNA3	0.0314	1.67	0.0025	1.94	GBP5	0.0138	1.69	0.0150	1.58
KCNJ15	0.0244	1.41	0.0497	1.53	GKAP1	0.0326	1.92	0.0013	1.71
KCNMA1	0.0257	1.36	0.0063	1.57	GLYAT	0.0224	1.54	0.0097	1.32
KEAP1	0.0122	1.31	0.0006	1.48	GNL3	0.0418	1.70	0.0317	1.42
KIAA0251	0.0045	1.34	0.0262	1.50	GNL3L	0.0218	2.28	0.0034	1.54
KIAA0423	0.0380	1.89	0.0080	2.21	GOLGA7	0.0104	1.52	0.0213	1.31
KIAA0564	0.0247	1.89	0.0006	2.49	GPR63	0.0019	2.01	0.0103	1.62
KIAA0737	0.0408	1.39	0.0033	2.01	GTF2H1	0.0085	2.28	0.0284	2.04
KIAA1026	0.0086	1.40	0.0042	1.48	HCFC1	0.0015	1.59	0.0253	1.31
KIAA1033	0.0366	2.18	0.0029	2.51	HHAT	0.0061	1.45	0.0118	1.35
KIAA1794	0.0085	1.85	0.0114	1.86	HIST1H2BA	0.0193	2.17	0.0278	1.38
KLHDC2	0.0235	1.39	0.0339	1.49	HIST1H4L	0.0209	1.80	0.0155	1.53
KLHDC8B	0.0298	1.34	0.0058	1.51	HIVEP2	0.0075	1.90	0.0072	1.64
KLHL32	0.0271	1.31	0.0420	1.50	HOXA13	0.0071	1.52	0.0283	1.34
KPNA2	0.0198	1.98	0.0001	3.52	HYAL1	0.0422	1.32	0.0139	1.31
L3MBTL2	0.0002	1.47	0.0248	1.47	IBTK	0.0384	2.05	0.0061	1.92
LAMC2	0.0207	1.39	0.0030	1.42	IGSF1	0.0107	1.66	0.0286	1.47
LCK	0.0157	1.70	0.0023	2.02	IL11RA	0.0130	1.63	0.0343	1.49
LECT2	0.0057	1.49	0.0024	1.50	ING1	0.0172	1.71	0.0403	1.41
LIG4	0.0187	1.46	0.0140	1.58	ING4	0.0233	2.13	0.0399	1.38
LINS1	0.0337	1.51	0.0025	2.32	INTS8	0.0268	2.12	0.0097	1.89
LOC148137	0.0410	1.40	0.0027	1.51	IQCG	0.0427	1.51	0.0474	1.44
LOC150223	0.0219	1.45	0.0040	1.63	KBTBD7	0.0152	1.82	0.0221	1.43
LOC400707	0.0259	1.32	0.0015	1.37	KCNG4	0.0177	1.48	0.0255	1.43
LOC400986	0.0120	1.34	0.0099	2.47	KCNMB2	0.0192	2.00	0.0095	1.35
LOC401622	0.0220	1.51	0.0443	1.55	KIAA0090	0.0326	1.59	0.0293	1.47
LOC440905	0.0110	1.41	0.0023	1.49	KIAA1012	0.0168	1.80	0.0159	1.77
LOC728554	0.0414	1.66	0.0010	2.05	KIN	0.0036	1.66	0.0409	1.37
LZTS2	0.0059	1.55	0.0089	2.03	KPNB1	0.0072	1.66	0.0159	1.49
MBP	0.0191	1.58	0.0131	2.14	KPRP	0.0353	1.41	0.0426	1.35
MCAT	0.0187	1.33	0.0095	1.51	KRT25	0.0209	1.54	0.0199	1.44
MGC11102	0.0480	1.72	0.0004	2.09	KRTAP9-2	0.0131	1.40	0.0269	1.32
MGC35402	0.0228	1.44	0.0130	1.67	LAT	0.0476	1.56	0.0062	1.44
MGC45800	0.0079	1.51	0.0057	1.71	LBR	0.0008	1.41	0.0278	1.39
MOBKL1A	0.0492	1.67	0.0001	2.34	LCE4A	0.0442	1.53	0.0056	1.49

MRFAP1L1	0.0149	1.50	0.0336	1.74	LDB3	0.0261	1.43	0.0468	1.35
MRPL32	0.0226	1.34	0.0056	1.61	LDHB	0.0443	2.34	0.0203	2.12
MSH5	0.0143	1.33	0.0271	1.35	LIG3	0.0163	2.22	0.0079	1.50
MSTO1	0.0149	2.37	0.0280	2.69	LOC123688	0.0250	1.52	0.0182	1.34
MTF2	0.0042	1.37	0.0244	1.78	LOC153364	0.0160	2.06	0.0018	1.60
MTRR	0.0035	1.73	0.0019	1.91	LOC165186	0.0157	1.47	0.0016	1.42
MUT	0.0490	1.40	0.0294	1.51	LOC283487	0.0090	1.36	0.0380	1.31
MYO10	0.0070	1.77	0.0024	3.27	LOC283932	0.0001	2.02	0.0448	1.49
NAP1L1	0.0437	1.75	0.0002	2.28	LOC285074	0.0076	1.55	0.0125	1.40
NCOA1	0.0152	1.51	0.0022	1.84	LOC340529	0.0373	1.41	0.0364	1.34
NIPA1	0.0273	1.77	0.0074	1.79	LOC340602	0.0382	1.58	0.0321	1.43
NLRC3	0.0242	1.35	0.0053	1.43	LOC347487	0.0380	1.60	0.0272	1.55
NPSR1	0.0033	1.43	0.0007	1.44	LOC387921	0.0063	1.82	0.0415	1.59
NR2C1	0.0200	1.95	0.0022	2.97	LOC388199	0.0304	1.79	0.0338	1.38
NR4A3	0.0256	1.53	0.0057	1.69	LOC90624	0.0314	2.06	0.0103	1.74
NSMAF	0.0038	1.43	0.0215	1.66	LRRC17	0.0450	1.70	0.0096	1.37
NT5DC2	0.0193	1.39	0.0152	1.58	MAL	0.0109	2.43	0.0388	2.17
NUPL1	0.0479	1.57	0.0074	1.81	MALT1	0.0190	1.49	0.0101	1.45
NUT	0.0041	1.41	0.0027	1.49	MAP2K5	0.0099	1.39	0.0063	1.30
OFD1	0.0498	1.75	0.0319	1.99	MAP3K5	0.0147	1.77	0.0112	1.34
OR1S1	0.0006	1.35	0.0003	1.47	MARK2	0.0467	1.68	0.0446	1.38
OR2H2	0.0305	1.43	0.0037	1.51	MBOAT5	0.0042	1.76	0.0284	1.38
OR51F2	0.0121	1.39	0.0241	1.41	MED11	0.0121	1.81	0.0127	1.45
OR6Y1	0.0485	1.41	0.0024	1.42	MEGF8	0.0110	1.76	0.0315	1.31
ORC5L	0.0096	1.41	0.0113	1.80	MEOX1	0.0036	2.50	0.0199	1.33
OSBPL8	0.0375	2.43	0.0000	2.72	METTL2A	0.0161	2.39	0.0151	1.56
PAK2	0.0011	1.43	0.0035	1.67	METTL8	0.0118	1.62	0.0163	1.59
PCBP4	0.0210	1.32	0.0232	1.36	MGAT2	0.0175	1.57	0.0206	1.36
PCDHGA1	0.0035	1.72	0.0261	1.76	MGC40499	0.0288	1.56	0.0408	1.54
PEX3	0.0382	1.52	0.0060	2.24	MINA	0.0167	2.32	0.0022	1.62
PHF20L1	0.0371	1.76	0.0241	2.35	MINPP1	0.0423	2.13	0.0350	1.99
PIK3C3	0.0175	1.54	0.0006	1.76	MIOX	0.0132	1.55	0.0190	1.45
PIK3CG	0.0109	2.04	0.0386	2.14	MLX	0.0243	2.05	0.0240	1.79
PLAA	0.0302	1.93	0.0013	2.13	MPV17L	0.0251	1.86	0.0475	1.37
PLEKHG5	0.0215	1.37	0.0058	1.47	MSH2	0.0268	1.92	0.0344	1.45
PMFBP1	0.0391	1.33	0.0245	1.40	MTO1	0.0225	1.43	0.0363	1.35
POU5F1	0.0383	1.30	0.0099	1.30	MTUS1	0.0419	1.55	0.0085	1.33
PPARBP	0.0067	1.98	0.0053	2.40	MYH14	0.0276	1.52	0.0136	1.48
PPFIA1	0.0470	1.46	0.0172	1.49	MYL9	0.0398	1.71	0.0280	1.36

PPM1B	0.0307	1.57	0.0002	2.65	MYOZ1	0.0174	1.59	0.0009	1.39
PRDM1	0.0448	1.96	0.0131	2.39	NARS	0.0005	2.10	0.0371	1.66
PRKCDBP	0.0008	1.44	0.0194	1.55	NCKIPSD	0.0042	2.06	0.0482	1.48
PRKCI	0.0330	1.95	0.0094	2.14	NDC80	0.0436	1.98	0.0283	1.74
PRKD3	0.0065	1.30	0.0239	1.65	NDUFS2	0.0407	1.68	0.0269	1.39
PSIP1	0.0500	1.56	0.0079	1.74	NEGR1	0.0109	1.59	0.0252	1.49
PSKH2	0.0360	1.34	0.0069	1.47	NEK10	0.0235	1.49	0.0437	1.43
PWP2	0.0112	1.31	0.0198	1.86	NEU3	0.0243	1.53	0.0007	1.32
R3HDM1	0.0164	1.54	0.0030	1.70	NFIA	0.0317	1.70	0.0044	1.67
RAB14	0.0017	1.64	0.0023	2.03	NFIC	0.0133	2.71	0.0272	1.40
RASGRP1	0.0023	2.16	0.0203	2.68	NFS1	0.0471	1.39	0.0115	1.37
RBM12B	0.0295	1.67	0.0313	2.20	NHEJ1	0.0399	1.32	0.0210	1.32
RBM34	0.0174	1.35	0.0007	2.07	NIPSNAP1	0.0321	1.62	0.0198	1.32
RECQL	0.0086	2.00	0.0000	2.63	NLK	0.0470	2.63	0.0059	1.87
REV3L	0.0073	2.15	0.0000	2.66	NLRP10	0.0065	1.75	0.0013	1.58
RFC3	0.0051	1.42	0.0101	1.53	NOLA1	0.0145	1.59	0.0331	1.45
RGR	0.0384	1.41	0.0205	1.51	NPAL3	0.0005	1.95	0.0171	1.50
RGS5	0.0173	1.44	0.0077	1.61	NRG1	0.0320	1.49	0.0465	1.35
RORA	0.0417	1.31	0.0056	1.46	NSBP1	0.0299	1.38	0.0092	1.36
RPS6KA2	0.0040	1.72	0.0138	2.00	NT5C2	0.0288	1.46	0.0018	1.32
RPS6KA3	0.0428	1.73	0.0034	1.89	OR2H1	0.0493	1.38	0.0469	1.35
S100G	0.0346	1.43	0.0081	1.46	OR2T4	0.0097	1.32	0.0416	1.31
SACM1L	0.0318	1.74	0.0040	2.64	OR56A1	0.0284	1.47	0.0053	1.36
SAR1A	0.0285	1.65	0.0046	2.48	OR5AK2	0.0029	1.63	0.0098	1.36
SEC24D	0.0341	2.13	0.0000	2.54	OR8K3	0.0100	1.51	0.0064	1.46
SERINC3	0.0067	1.48	0.0013	2.58	PAGE2B	0.0298	1.61	0.0434	1.51
SFRS11	0.0046	2.55	0.0037	4.26	PCDH1	0.0406	1.60	0.0103	1.30
SFRS12	0.0205	1.65	0.0035	2.89	PCDH7	0.0119	1.48	0.0227	1.31
SFRS16	0.0080	1.58	0.0472	1.64	PCYOX1L	0.0056	2.03	0.0039	1.60
SH3GL1	0.0453	1.63	0.0042	1.65	PDCD10	0.0428	1.59	0.0002	1.43
SLC26A7	0.0126	1.46	0.0059	1.71	PDE8A	0.0047	1.73	0.0042	1.35
SLC33A1	0.0366	1.69	0.0226	1.90	PECI	0.0213	2.35	0.0013	1.52
SLC38A2	0.0015	1.57	0.0002	1.97	PFKP	0.0112	1.74	0.0449	1.38
SLC7A4	0.0305	1.37	0.0499	1.46	PHACTR2	0.0223	2.14	0.0482	1.57
SMARCD1	0.0341	1.38	0.0116	1.39	PHF12	0.0470	1.48	0.0441	1.35
SMN1	0.0414	1.47	0.0007	1.75	PHF14	0.0255	1.76	0.0185	1.58
SNRPD3	0.0184	1.62	0.0017	1.68	PHF14	0.0295	2.13	0.0266	1.38
SOX30	0.0166	1.44	0.0020	1.47	PKIA	0.0180	2.01	0.0065	1.91
SPN	0.0083	1.30	0.0124	1.72	PLEK2	0.0073	1.46	0.0078	1.42

SPON1	0.0013	1.78	0.0115	2.08	PLEKHA6	0.0491	1.51	0.0143	1.30
SRPK2	0.0164	1.55	0.0002	2.07	PLEKHN1	0.0428	1.41	0.0058	1.38
SRPK2	0.0410	1.36	0.0146	1.86	PMS1	0.0239	2.14	0.0047	2.09
STAMBPL1	0.0180	1.51	0.0393	1.53	POLH	0.0023	1.77	0.0221	1.59
STAT5B	0.0498	1.51	0.0050	1.70	POLK	0.0322	2.01	0.0204	1.46
SUHW2	0.0003	1.42	0.0068	1.73	POT1	0.0130	1.83	0.0187	1.32
SULT2B1	0.0120	1.47	0.0041	1.51	POU4F2	0.0233	1.63	0.0089	1.38
SYNJ1	0.0227	1.52	0.0033	2.29	PPARG	0.0026	1.86	0.0031	1.65
SYNJ2	0.0374	1.61	0.0253	1.68	PPIL6	0.0237	1.40	0.0216	1.31
TADA2L	0.0353	1.50	0.0006	1.56	PPP2R1B	0.0008	1.82	0.0087	1.64
TADA2L	0.0373	1.54	0.0014	1.85	PPP3R2	0.0369	1.56	0.0183	1.33
TAF5L	0.0318	1.39	0.0176	1.67	PREPL	0.0188	2.42	0.0436	1.74
TCF7	0.0144	1.40	0.0377	1.72	PROKR1	0.0100	1.56	0.0290	1.31
TCP1	0.0370	1.76	0.0002	2.90	PROP1	0.0072	1.51	0.0213	1.43
TENC1	0.0132	1.41	0.0374	1.59	PRRC1	0.0140	1.73	0.0043	1.72
THBS3	0.0240	1.87	0.0114	2.07	PSG6	0.0005	1.74	0.0226	1.33
THRAP2	0.0077	1.67	0.0263	2.11	PSIP1	0.0278	2.18	0.0016	1.50
THUMPD1	0.0335	1.97	0.0004	2.47	PSMC3IP	0.0345	1.41	0.0239	1.37
TLCD1	0.0411	1.31	0.0443	1.34	PSMC5	0.0423	1.59	0.0070	1.32
TMBIM1	0.0149	1.43	0.0205	1.85	PTK9	0.0034	1.48	0.0324	1.39
TMCO5	0.0235	1.36	0.0023	1.51	PTPRH	0.0011	1.39	0.0208	1.37
TMEM133	0.0250	1.38	0.0334	1.40	RAB37	0.0174	1.86	0.0107	1.33
TMEM26	0.0137	1.35	0.0491	1.41	RABIF	0.0122	1.61	0.0025	1.47
TNC	0.0093	1.36	0.0007	1.48	RAD51L1	0.0087	1.57	0.0142	1.57
TNIP3	0.0246	1.35	0.0351	1.97	RAD9B	0.0071	1.87	0.0284	1.39
TPTE2	0.0000	1.50	0.0081	1.70	RBBP4	0.0174	1.47	0.0182	1.36
TRAPPC2	0.0205	1.42	0.0054	1.89	RBMS3	0.0410	1.48	0.0112	1.37
TRIM29	0.0234	1.39	0.0007	1.49	RBMXL1	0.0281	1.62	0.0176	1.62
TRPS1	0.0001	1.69	0.0045	1.96	RBP3	0.0292	1.40	0.0265	1.32
TTC35	0.0380	1.38	0.0002	1.88	RDH13	0.0441	1.74	0.0134	1.54
TUBD1	0.0091	1.63	0.0016	2.52	REXO1L1	0.0313	2.07	0.0313	1.42
TUBE1	0.0339	1.79	0.0005	3.25	RFP	0.0125	1.80	0.0437	1.37
UBE1DC1	0.0371	1.74	0.0046	1.93	RFWD3	0.0149	1.44	0.0033	1.39
UBE1L2	0.0003	1.51	0.0023	2.27	RGS3	0.0482	1.37	0.0414	1.35
UBE2B	0.0447	1.31	0.0059	1.85	ROCK2	0.0056	1.94	0.0324	1.42
UBE2R2	0.0088	1.50	0.0074	1.69	RPN2	0.0399	1.97	0.0016	1.66
UBE4A	0.0041	1.77	0.0273	1.91	RSPRY1	0.0153	1.62	0.0399	1.57
UCP1	0.0341	1.32	0.0100	1.36	RTN4RL2	0.0014	1.59	0.0128	1.38
USH2A	0.0199	1.36	0.0199	1.50	RUNX1	0.0028	1.86	0.0001	1.53

USP16	0.0288	1.39	0.0499	1.54	SACS	0.0074	1.98	0.0015	1.68
USP37	0.0407	1.51	0.0146	1.87	SEPT4	0.0407	1.40	0.0088	1.31
VCAN	0.0255	1.88	0.0093	2.03	SF3B1	0.0007	2.04	0.0042	1.50
WIPI2	0.0066	1.37	0.0268	1.44	SGK3	0.0497	1.72	0.0054	1.32
WSB1	0.0200	2.64	0.0026	2.88	SH3YL1	0.0132	2.78	0.0009	2.21
XKR7	0.0094	1.64	0.0269	1.68	SIRPG	0.0133	1.99	0.0015	1.76
ZBTB11	0.0310	1.95	0.0053	3.23	SIRT5	0.0014	1.62	0.0269	1.36
ZBTB41	0.0235	1.71	0.0000	2.85	SIX3	0.0170	1.36	0.0061	1.35
ZBTB45	0.0244	1.33	0.0120	1.49	SLAIN1	0.0433	1.95	0.0111	1.62
ZDHHC5	0.0160	1.31	0.0001	1.34	SLC25A41	0.0192	1.41	0.0245	1.36
ZFP30	0.0000	1.50	0.0198	1.88	SLC26A8	0.0067	1.47	0.0454	1.30
ZNF12	0.0339	1.34	0.0009	2.86	SLC7A8	0.0052	1.49	0.0158	1.31
ZNF181	0.0249	1.99	0.0004	2.19	SMA3	0.0481	1.39	0.0410	1.31
ZNF238	0.0336	1.51	0.0006	1.59	SMR3A	0.0238	1.56	0.0428	1.41
ZNF254	0.0030	1.40	0.0001	2.60	SNX14	0.0294	1.75	0.0048	1.44
ZNF429	0.0446	1.40	0.0339	1.46	SNX5	0.0110	2.06	0.0466	1.49
ZNF652	0.0161	2.09	0.0000	2.12	SORBS2	0.0194	1.74	0.0381	1.51
ZNF75A	0.0325	1.53	0.0311	2.44	SOX10	0.0034	1.38	0.0185	1.36
ZNF85	0.0091	1.70	0.0094	1.91	SP110	0.0181	2.07	0.0223	1.86
ZWILCH	0.0040	1.33	0.0007	1.82	SPAG16	0.0326	1.72	0.0145	1.36
					SPAST	0.0129	1.81	0.0116	1.32
					SPATA17	0.0224	2.00	0.0263	1.75
					SRY	0.0352	1.34	0.0308	1.31
					SUOX	0.0127	1.76	0.0297	1.42
					SUPT3H	0.0330	2.11	0.0066	1.33
					SURF2	0.0344	1.47	0.0062	1.31
					SVIL	0.0234	2.04	0.0091	1.80
					SYNC1	0.0048	1.71	0.0007	1.66
					SYNE1	0.0174	1.58	0.0447	1.36
					SYT15	0.0177	1.76	0.0172	1.37
					TAL2	0.0284	1.47	0.0192	1.32
					TEDDM1	0.0288	1.35	0.0158	1.31
					TJP3	0.0014	1.80	0.0281	1.51
					TMEM80	0.0330	1.67	0.0097	1.34
					TMPRSS3	0.0348	1.63	0.0230	1.46
					TNFAIP8L1	0.0206	2.20	0.0003	2.07
					TRADD	0.0048	1.92	0.0417	1.46
					TRIM24	0.0458	1.36	0.0204	1.33
					TRIM36	0.0486	1.61	0.0497	1.41

TSLP	0.0313	1.50	0.0388	1.41
TSP50	0.0348	1.50	0.0272	1.38
TSPAN18	0.0311	1.89	0.0013	1.62
TTC9C	0.0021	2.00	0.0152	1.78
TTRAP	0.0307	2.06	0.0000	1.90
TUBB4	0.0150	1.81	0.0092	1.80
TXLNA	0.0171	1.62	0.0409	1.31
TXNDC9	0.0497	2.49	0.0152	2.29
UACA	0.0356	1.66	0.0181	1.30
UBE2Q1	0.0369	1.44	0.0053	1.36
UCP3	0.0431	1.51	0.0429	1.33
UGT2B17	0.0182	1.84	0.0207	1.44
UNC5CL	0.0055	2.19	0.0358	1.54
VPS13B	0.0391	1.49	0.0123	1.43
VTN	0.0132	1.88	0.0208	1.56
WIPF1	0.0288	3.37	0.0002	3.22
WWP1	0.0050	1.66	0.0084	1.54
ZC3H6	0.0186	1.60	0.0013	1.59
ZMAT1	0.0209	2.71	0.0336	1.35
ZMYND11	0.0088	1.96	0.0043	1.83
ZNF322A	0.0060	3.62	0.0204	1.69
ZNF354A	0.0235	1.84	0.0218	1.56
ZNF382	0.0269	1.74	0.0146	1.56
ZNF443	0.0066	1.33	0.0370	1.30
ZNF585A	0.0114	1.79	0.0382	1.52
ZNF620	0.0323	1.46	0.0156	1.34
ZNF623	0.0156	1.60	0.0200	1.36
ZNF658	0.0025	3.42	0.0011	1.81
ZNF714	0.0120	1.70	0.0080	1.42
ZNF793	0.0172	1.57	0.0026	1.35
ZNRF2	0.0205	1.95	0.0204	1.93

All genes significantly expressed in SVR higher than in NR group. [a]list of genes with different expression at pre-treatment lower than during treatment at week24, [b]list of genes with different expression at pre-treatment higher than during treatment at week24.

Table 9. Biological functions of 654 genes listed in table 8

Term	Count	Genes
T cell receptor signaling pathway	9	PIK3CG, LAT, PAK2, CD3D, FYN, RASGRP1, LCK, PPP3R2, MALT1
Ubiquitin mediated proteolysis	7	UBE4A, WWP1, DDB1, KEAP1, UBE2B, UBE2Q1, UBE2R2
B cell receptor signaling pathway	4	PIK3CG, DAPP1, PPP3R2, MALT1
Jak-STAT signaling pathway	6	PIK3CG, TSLP, IL6ST, STAT5B, IL7R, IL11RA
Cytokine-cytokine receptor interaction	9	CCL25, TSLP, IL1R1, CXCL14, IL6ST, IL1RAP, EDA, IL7R, IL11RA
Natural killer cell mediated cytotoxicity	5	PIK3CG, LAT, FYN, LCK, PPP3R2
TGF-beta signaling pathway	3	PPP2R1B, ROCK2, THBS3
Fc gamma R-mediated phagocytosis	3	PIK3CG, LAT, MYO10
Endocytosis	5	WWP1, PRKCI, EEA1, CLTC, SH3GL1
Chemokine signaling pathway	5	PIK3CG, CCL25, CXCL14, ROCK2, STAT5B
RIG-I-like receptor signaling pathway	2	AZI2, TRADD
T cell activation	13	CRTAM, CD3D, STAT5B, MALT1, LIG4, IL7R, NLRC3, FYN, LCK, CLEC7A, NHEJ1, SPN, DPP4
T cell differentiation	7	CD3D, LCK, STAT5B, LIG4, IL7R, NHEJ1, SPN
regulation of T cell mediated immunity	4	MALT1, IL7R, DPP4, SPN
immunoglobulin production	4	EXO1, MSH2, LIG4, IL7R
B cell activation	6	EXO1, MSH2, MALT1, LIG4, IL7R, NHEJ1
regulation of cytokine-mediated signaling	3	CAV1, IL6ST, AGPAT1
activated T cell proliferation	2	CRTAM, FYN
regulation of adaptive immune response	5	IL6ST, MALT1, IL7R, DPP4, SPN
regulation of acute inflammatory response	3	IL6ST, PPARG, SPN
lymphocyte proliferation	4	CRTAM, FYN, MALT1, IL7R
T cell receptor signaling pathway	3	FYN, MALT1, CACNB4
activation of immune response	6	FYN, CLU, CFH, MALT1, CACNB4, CLEC7A
lymphocyte mediated immunity	5	EXO1, CRTAM, MSH2, CLU, LIG4
T cell proliferation	3	CRTAM, FYN, MALT1
regulation of inflammatory response	5	UACA, IL6ST, STAT5B, PPARG, SPN
adaptive immune response	5	EXO1, CRTAM, MSH2, CLU, LIG4
regulation of cell killing	3	CRTAM, STAT5B, IL7R
B cell mediated immunity	4	EXO1, MSH2, CLU, LIG4
innate immune response	7	IL1R1, IL1RAP, CLU, PPARG, CFH, MALT1, CLEC7A
cytokinesis	3	SEPT4, ROCK2, SPAST
cytokine-mediated signaling pathway	4	IL1R1, IL6ST, STAT5B, TRADD

B cell differentiation	3	MSH2, MALT1, NHEJ1
immune response	23	EXO1, TCF7, CRTAM, IL1R1, GBP5, MSH2, CYSLTR2, CLU, PPARG, MALT1, VTN, LIG4, IL7R, MBP, LAT, CCL25, CXCL14, IL1RAP, CFH, CLEC7A, EDA, GBP3, SPN
acute inflammatory response	4	CASP6, CLU, STAT5B, CFH
regulation of interleukin-2 production	2	STAT5B, MALT1
regulation of tumor necrosis factor production	2	CLEC7A, SPN
humoral immune response	3	EXO1, CLU, CFH
inflammatory response	9	PLAA, CASP6, CCL25, AIF1, IL1RAP, CLU, STAT5B, CFH, CLEC7A
phagocytosis	2	DOCK1, CLEC7A
defense response	15	IL1R1, AIF1, MSH5, STAT5B, PPARG, CLU, MALT1, PLAA, CCL25, CASP6, OR2H2, IL1RAP, CFH, CLEC7A, SPN

Table 10. Significantly different expression of 13 genes between SVR and NR in pre-PegIFN-α treatment and during PegIFN-α treatment, higher expression of NR in pre-PegIFN-α treatment and higher expression of SVR during PegIFN-α treatment in chronic HBV patients with positive HBeAg.

Gene	Baseline		wk24	
symbol	P-value	FC	P-value	FC
BCR	0.0350	-2.02	0.0256	1.34
CENPO	0.0248	-1.62	0.0078	1.51
CTNS	0.0070	-1.32	0.0363	1.47
GPR84	0.0253	-1.82	0.0208	2.83
NUP98	0.0428	-2.35	0.0275	1.55
RELB	0.0279	-2.28	0.0310	1.72
RGS16	0.0290	-3.70	0.0355	2.78
SAMSN1	0.0117	-1.76	0.0452	1.95
SLC22A11	0.0484	-1.68	0.0285	1.45
SWAP70	0.0376	-1.69	0.0238	1.34
TP53INP2	0.0498	-2.63	0.0315	1.72
YTHDF3	0.0399	-1.50	0.0389	1.42
ZNF331	0.0180	-2.26	0.0071	3.70

Table 11. The intersection between the published different expressed genes in HBV or HCV microarray reports and the 665 significantly different expressed genes at both pre-PegIFN-α treatment and during PegIFN-α treatment between SVR and NR in chronic HBV patients with positive HBeAg.

Significantly different genes in our study and other HBV papers	Significantly different genes in our study and other HCV papers		Significantly different genes in our study and to be ISGs
GBP3	ADD3	ATP8B2	DDX17
NDUFS2	AIF1	CARS	EIF3S10
	AKAP1	CAST	IFI16
	AMMECR1	CAV2	NR4A3
	CASP4	CETN3	RECQL
	CCNL1	CLU	TRADD
	CENPE	CTBP2	CASP4
	CEPT1	DPP4	LIG3
	CES4	GBP3	
	DAPP1	GBP5	
	DKK3	HIVEP2	
	FYN	IL11RA	
	IFI16	KPNB1	
	ITGA3	LAT	
	KIAA0564	LDHB	
	KLHDC2	MAL	
	LCK	MAP2K5	
	NAP1L1	NT5C2	
	PSIP1	PKIA	
	RORA	PSIP1	
	SEC24D	RABIF	
	SPN	SH3YL1	
	SRPK2	SP110	
	STAT5B	TSLP	
	TCF7	WIPF1	
	THUMPD1	WWP1	
	VCAN	ZMYND11	
	WSB1	ZNRF2	

Table 12. Biological functions of 58 genes listed in table 11

Term	Count	Genes
regulation of immune system process	8	LAT, FYN, LCK, CLU, STAT5B, RORA, DPP4, SPN
T cell activation	5	FYN, LCK, STAT5B, DPP4, SPN
leukocyte activation	6	LAT, FYN, LCK, STAT5B, DPP4, SPN
immune system process	11	LAT, TCF7, GBP5, FYN, LCK, CLU, STAT5B, IFI16, GBP3, DPP4, SPN
cell activation	6	LAT, FYN, LCK, STAT5B, DPP4, SPN
regulation of cell activation	5	LAT, LCK, STAT5B, RORA, SPN
lymphocyte activation	5	FYN, LCK, STAT5B, DPP4, SPN
regulation of immune response	5	FYN, CLU, STAT5B, DPP4, SPN
regulation of T cell activation	4	LAT, LCK, STAT5B, SPN
leukocyte differentiation	4	LCK, STAT5B, IFI16, SPN
T cell differentiation	3	LCK, STAT5B, SPN
induction of apoptosis	5	CASP4, LCK, MAL, IFI16, SPN
immune response	6	LAT, TCF7, GBP5, CLU, GBP3, SPN
activation of immune response	2	FYN, CLU
proteolysis	6	WSB1, CASP4, WWP1, CLU, DPP4, ZNRF2
acute inflammatory response	2	CLU, STAT5B
inflammatory response	3	AIF1, CLU, STAT5B
defense response	4	AIF1, CLU, STAT5B, SPN
cell proliferation	3	ZMYND11, FYN, IFI16
anti-apoptosis	2	CLU, STAT5B
T cell receptor signaling pathway	3	LAT, FYN, LCK
Natural killer cell mediated cytotoxicity	3	LAT, FYN, LCK
Jak-STAT signaling pathway	3	TSLP, STAT5B, IL11RA
Wnt signaling pathway	2	TCF7, CTBP2
Cytokine-cytokine receptor interaction	2	TSLP, IL11RA

Table 13. The intersection between the published different expressed genes in HBV or HCV microarray reports and the 2544 significantly different expressed genes during PegIFN-α treatment but not at pre-PegIFN-α treatment in chronic HBV patients with positive HBeAg.

Significantly different genes in our study and other HBV papers	Significantly different genes in our study and other HCV papers			Significantly different genes in our study and to be ISGs
STK17A	AKAP12	IL4I1	BCL11B	PMAIP1
ALDH1B1	ACSL1	PDCD4	AKT3	PSEN1
MAP4K3	CD164	ATP2B1	CLIP1	ATP1B3
PVRL1	TOP1	ADNP	PLA2G6	RELA
ZNF711	GPD2	FYTTD1	RAP1A	LTBP2
PSMB8	SNX6	CLIP1	SEC31A	PML
UBD	PMAIP1	TGIF1	TNIK	HIF1A
TNKS	PSEN1	MAGED1	CTSL1	SFPQ
	SEPT7	HIF1A	YME1L1	CRYM
	USP25	LMNB1	TFG	HIF1A
	CAPZA1	SMARCA5	SLC2A4RG	C4BPA
	MOBK1B	C3AR1	APOL1	IFNAR2
	C20orf111	ATF3	RGL1	GEM
	CASP7	SPAG9	CREM	PLA2G5
	ANK3	RAP2B	CCL2	ELK4
	SC4MOL	ARNTL	TBL1XR1	PLSCR1
	CYFIP1	SH3GLB1	ING3	HDAC2
	CD69	FLJ11171	PBEF1	ELK4
	ELOVL5	NCOA3	RIN2	MAP3K8
	PASK	BACH1	TLK1	IRF4
	PML	FBXL3	ZNF91	RFC1
	BUB3	PCNX	HMGCR	PSEN1
	TBRG4	DUSP2	PTPRE	STAG2
	HIF1A	CASC3	FLJ20297	PSMB8
	ACTR3	PRC1	SERPINB9	SQLE
	TGM2	ING3	PSEN1	JUN
	DYRK2	UPF3A	ATF3	TMEM1
	PPP2R5C	NR4A2	CHST2	HBE1
	ETV7	B3GNT5	TM9SF2	DSG1
	SNRPN	LILRB2	PSMB8	ALCAM
	CHD7	RNF13	SLC23A2	HLA-G
	TLR2	CYP1B1	TTC3	CASP8
	SEPT6	NFATC3	ZNF588	C1R
	CD274	PLSCR1	BLMH	MKI67
		BIRC2	RNF24	ADAM11

Table 14. Biological functions of 58 genes listed in table 13

Term	Count	Genes
induction of apoptosis	9	MAGED1, PSEN1, SH3GLB1, TLR2, PML, TGM2, STK17A, DYRK2, PMAIP1
apoptosis	11	MAGED1, PSEN1, SH3GLB1, CASP7, PML, TBRG4, STK17A, DYRK2, PMAIP1, BIRC2, PDCD4
proteasomal protein catabolic process	4	TBL1XR1, PPP2R5C, BUB3, PSMB8
proteasomal ubiquitin-dependent protein catabolic process	4	TBL1XR1, PPP2R5C, BUB3, PSMB8
cytokinesis	3	PRC1, SEPT6, SEPT7
immune system process	13	C3AR1, CCL2, TLR2, PML, CD164, PSMB8, LILRB2, CHD7, APOL1, PVRL1, PSEN1, BCL11B, CD274
regulation of cell proliferation	11	MAGED1, HIF1A, ATF3, CCL2, BCL11B, CD274, PML, TGM2, TGIF1, TBRG4, CD164
anti-apoptosis	5	SERPINB9, CCL2, PSEN1, SH3GLB1, TGM2
inflammatory response	6	C3AR1, HIF1A, CCL2, TLR2, CHST2, NFATC3
immune response	9	LILRB2, APOL1, CCL2, PSEN1, PVRL1, CD274, TLR2, CD164, PSMB8
proteolysis	12	TBL1XR1, BLMH, PSEN1, CASP7, PPP2R5C, UBD, YME1L1, USP25, FBXL3, BUB3, PSMB8, CTSL1
defense response	8	LILRB2, C3AR1, APOL1, HIF1A, CCL2, TLR2, CHST2, NFATC3
cell activation	5	PLSCR1, CHD7, PSEN1, BCL11B, TLR2
T cell differentiation	2	CHD7, BCL11B
cytokine-mediated signaling pathway	2	CCL2, TGM2
lymphocyte activation	3	CHD7, PSEN1, BCL11B
lymphocyte differentiation	2	CHD7, BCL11B
innate immune response	2	APOL1, TLR2
NOD-like receptor signaling pathway	2	CCL2, BIRC2
Chemokine signaling pathway	3	CCL2, RAP1A, AKT3
B cell receptor signaling pathway	2	NFATC3, AKT3
Fc gamma R-mediated phagocytosis	2	PLA2G6, AKT3
Toll-like receptor signaling pathway	2	TLR2, AKT3
T cell receptor signaling pathway	2	NFATC3, AKT3
Ubiquitin mediated proteolysis	2	PML, BIRC2

Table 15. Significantly different expression of 415 genes between SVR and NR with higher expression in SVR at both pre-PegIFN-α treatment and during PegIFN-α treatment in chronic HBV patients with negative HBeAg.

Gene symbol [a]	Baseline		wk24	
	P-value	FC	P-value	FC
ABCD3	0.0238	1.33	0.0151	1.37
ADAR	0.0327	1.55	0.0221	1.61
AFTPH	0.0132	2.76	0.0435	3.51
AMY1C	0.0077	1.76	0.0397	2.04
ANKMY2	0.0047	1.75	0.0036	1.75
APBB1	0.0316	1.42	0.0040	1.67
ARMCX6	0.0163	1.39	0.0002	1.75
ARSB	0.0175	2.18	0.0028	2.18
ATE1	0.0158	1.50	0.0154	1.63
ATP1B3	0.0420	2.24	0.0295	2.28
ATP6V1C1	0.0184	1.65	0.0335	1.86
B4GALT2	0.0431	1.44	0.0000	1.49
BAD	0.0226	1.44	0.0012	1.89
BCL10	0.0280	1.65	0.0080	2.64
BMP2K	0.0241	1.64	0.0015	2.83
BMPR2	0.0173	1.96	0.0012	1.96
BRD3	0.0018	1.57	0.0014	1.84
BTN3A3	0.0126	1.99	0.0156	2.09
C19ORF12	0.0060	1.55	0.0012	1.58
C19ORF50	0.0434	1.38	0.0015	1.38
C1ORF183	0.0040	1.39	0.0092	1.51
C1QTNF5	0.0256	1.42	0.0325	1.58
C4ORF16	0.0499	1.36	0.0061	2.37
C6ORF15	0.0234	1.53	0.0326	1.59
C7ORF36	0.0072	1.63	0.0266	1.85
C9ORF41	0.0108	1.63	0.0079	1.65
CAPN7	0.0157	1.50	0.0009	1.62
CASC4	0.0193	1.67	0.0012	2.06
CCDC102A	0.0129	1.76	0.0152	1.87
CCNC	0.0382	1.75	0.0071	1.92
CEP350	0.0193	1.36	0.0156	1.62
CEPT1	0.0178	1.70	0.0121	2.02
CNOT7	0.0375	1.95	0.0042	2.17

Gene symbol [b]	Baseline		wk24	
	P-value	FC	P-value	FC
ABR	0.0239	1.46	0.0219	1.30
ACN9	0.0243	2.61	0.0272	1.66
ADCK2	0.0252	1.63	0.0468	1.33
ADRA1A	0.0009	1.57	0.0104	1.43
ALG1	0.0270	2.46	0.0113	1.38
ALG10B	0.0182	1.99	0.0084	1.53
ALKBH4	0.0363	1.53	0.0120	1.31
ANKRD16	0.0346	1.66	0.0219	1.56
ANKRD36	0.0108	1.99	0.0108	1.53
AP1S1	0.0048	3.31	0.0184	1.87
AP3B1	0.0026	1.69	0.0392	1.39
APAF1	0.0015	2.01	0.0426	1.34
API5	0.0177	1.74	0.0495	1.33
APOBEC3F	0.0076	2.65	0.0066	1.52
APOL1	0.0353	2.07	0.0097	1.38
APPBP1	0.0008	2.25	0.0020	2.14
APPBP2	0.0068	1.42	0.0046	1.39
ARF3	0.0190	1.38	0.0387	1.34
ARHGEF3	0.0087	1.94	0.0035	1.75
ARL6IP6	0.0002	2.79	0.0240	1.35
ARMCX3	0.0369	2.30	0.0303	1.43
ARSB	0.0076	2.02	0.0185	1.53
ARV1	0.0168	1.99	0.0381	1.41
ASB3	0.0315	1.59	0.0154	1.46
ASCC3	0.0139	2.03	0.0491	1.37
ATF4	0.0185	3.91	0.0143	1.34
ATP2C1	0.0094	2.48	0.0014	1.81
ATR	0.0150	1.63	0.0448	1.49
BAD	0.0182	1.66	0.0179	1.41
BANF1	0.0311	1.74	0.0040	1.73
BAT1	0.0095	1.76	0.0166	1.46
BCCIP	0.0430	1.62	0.0239	1.52
BMF	0.0121	1.73	0.0232	1.32

COL8A2	0.0414	1.32	0.0179	1.52	BPTF	0.0450	1.55	0.0025	1.42
CSNK1A1	0.0480	1.77	0.0059	2.62	BRF2	0.0290	1.53	0.0259	1.32
CSTF2T	0.0062	1.44	0.0233	1.46	C10ORF99	0.0077	1.41	0.0013	1.31
CYB5B	0.0197	1.57	0.0055	1.62	C11ORF71	0.0000	1.93	0.0061	1.56
DHRS7B	0.0202	1.31	0.0199	1.48	C12ORF11	0.0142	1.76	0.0500	1.54
DOCK8	0.0031	1.33	0.0431	1.38	C14ORF101	0.0017	2.58	0.0203	1.46
DSCR3	0.0375	1.35	0.0230	1.40	C1GALT1C1	0.0329	2.38	0.0289	1.79
DSN1	0.0417	1.35	0.0384	1.42	C20ORF112	0.0086	1.43	0.0086	1.33
DYNC2LI1	0.0240	1.64	0.0376	2.84	C20ORF117	0.0030	1.55	0.0498	1.38
DYNLT3	0.0306	1.41	0.0448	1.44	C20ORF44	0.0072	1.96	0.0070	1.41
FAM125B	0.0109	1.46	0.0290	1.64	C3ORF23	0.0229	2.09	0.0442	1.58
FBXO4	0.0021	1.69	0.0115	3.09	C4ORF36	0.0056	1.59	0.0123	1.56
FLJ11171	0.0010	1.63	0.0415	1.72	C5ORF22	0.0217	1.73	0.0065	1.72
FLJ44635	0.0381	1.40	0.0170	1.46	C6ORF190	0.0451	2.08	0.0264	1.96
GALE	0.0402	1.54	0.0038	1.61	C6ORF211	0.0216	2.83	0.0385	1.54
GAMT	0.0270	1.50	0.0031	1.69	C6ORF57	0.0115	1.48	0.0181	1.43
GIYD2	0.0279	1.88	0.0203	1.99	C7ORF24	0.0080	2.35	0.0158	1.60
GMFB	0.0105	1.37	0.0097	1.53	C9ORF100	0.0043	1.55	0.0005	1.39
HRASLS3	0.0376	1.65	0.0194	2.03	CASP8	0.0332	3.11	0.0184	2.11
ISY1	0.0272	1.88	0.0104	2.15	CCDC24	0.0043	1.68	0.0061	1.67
ITGB1BP3	0.0285	1.36	0.0157	1.56	CCM2	0.0068	2.08	0.0370	2.07
KIAA1522	0.0407	1.32	0.0131	1.38	CCM2	0.0158	2.07	0.0474	1.47
KLHL7	0.0068	2.44	0.0001	2.79	CCT7	0.0110	4.95	0.0007	1.90
LCMT1	0.0043	2.02	0.0028	3.37	CDC7	0.0014	1.83	0.0174	1.46
LOC728913	0.0248	1.43	0.0069	1.48	CDCA8	0.0191	1.45	0.0079	1.30
LOC732402	0.0129	1.89	0.0041	1.91	CDK10	0.0040	3.23	0.0017	1.45
LRP10	0.0011	1.44	0.0019	2.12	CDKAL1	0.0133	2.40	0.0415	1.41
LY6H	0.0481	1.35	0.0033	1.37	CDKN2D	0.0201	2.17	0.0261	1.44
MAP3K7IP1	0.0482	1.62	0.0371	1.63	CEP192	0.0166	1.55	0.0399	1.52
MRPL22	0.0388	1.44	0.0013	1.58	CHMP5	0.0442	2.42	0.0276	1.34
MRPS18B	0.0098	1.38	0.0048	1.47	CHMP6	0.0430	2.10	0.0308	1.41
MTFR1	0.0202	1.39	0.0475	1.40	CLDN10	0.0354	1.42	0.0398	1.36
NEDD1	0.0106	1.49	0.0032	1.85	CLEC2D	0.0030	3.67	0.0007	2.61
NFATC3	0.0289	1.83	0.0042	2.17	CLSTN1	0.0164	1.86	0.0096	1.60
NHLRC2	0.0017	1.67	0.0049	1.76	CNIH	0.0174	2.00	0.0326	1.50
NPTN	0.0452	1.45	0.0284	2.02	CNOT1	0.0215	1.42	0.0059	1.33
PALLD	0.0139	2.54	0.0109	3.27	COL25A1	0.0182	1.42	0.0397	1.42
PATZ1	0.0275	1.88	0.0125	1.95	COL4A3BP	0.0074	1.71	0.0322	1.43
PNKD	0.0377	1.35	0.0031	1.42	CPSF6	0.0109	1.68	0.0376	1.34

POLR2C	0.0449	1.30	0.0209	1.36	CSNK1G1	0.0078	1.99	0.0104	1.45
PPID	0.0365	2.23	0.0061	2.26	CTBP2	0.0141	2.63	0.0430	1.49
PSME2	0.0357	1.42	0.0174	1.42	CTCF	0.0372	1.49	0.0133	1.40
RBBP9	0.0181	1.87	0.0418	2.27	CUTC	0.0062	2.40	0.0135	1.41
RFC2	0.0126	2.38	0.0001	2.84	CXORF42	0.0004	1.48	0.0184	1.31
RHOT1	0.0047	1.50	0.0096	1.91	CYP20A1	0.0302	1.56	0.0193	1.37
RRAGC	0.0319	1.33	0.0136	1.74	CYP2U1	0.0421	1.80	0.0478	1.55
RSF1	0.0477	1.40	0.0031	1.73	DDX17	0.0428	3.25	0.0046	1.73
SAR1B	0.0134	1.61	0.0000	1.77	DHRSX	0.0385	1.78	0.0282	1.42
SEPHS1	0.0135	1.50	0.0353	1.51	DHX32	0.0192	2.50	0.0416	1.45
SERAC1	0.0214	1.40	0.0218	1.51	DICER1	0.0111	2.03	0.0085	1.86
SKIV2L2	0.0084	1.35	0.0047	1.70	DIDO1	0.0247	2.36	0.0489	1.38
SLA/LP	0.0373	1.34	0.0362	1.56	DNAJC19	0.0300	1.75	0.0224	1.37
SLIC1	0.0231	1.79	0.0229	2.09	DNAJC7	0.0125	1.61	0.0266	1.40
SNAP23	0.0274	2.31	0.0002	3.18	DNAL4	0.0067	1.58	0.0240	1.37
SNRPN	0.0119	2.52	0.0038	2.61	DPP8	0.0050	2.03	0.0235	1.65
SNRPN	0.0291	2.33	0.0160	2.47	DYM	0.0468	1.53	0.0133	1.42
SNX11	0.0036	1.48	0.0025	1.57	DYNC2LI1	0.0035	1.49	0.0046	1.37
SNX13	0.0276	1.53	0.0018	1.53	DYNLRB1	0.0117	2.04	0.0095	1.52
SNX14	0.0201	1.42	0.0095	1.76	DYNLRB1	0.0214	1.97	0.0352	1.36
SORBS2	0.0024	1.34	0.0296	1.54	ENPP4	0.0035	1.96	0.0177	1.71
SOX17	0.0434	1.36	0.0115	1.70	ESD	0.0134	1.64	0.0327	1.31
SP110	0.0089	1.58	0.0001	3.08	FAF1	0.0183	2.86	0.0014	1.68
ST13	0.0359	1.38	0.0394	1.39	FAM18B	0.0009	3.30	0.0007	1.39
SUPT3H	0.0175	1.61	0.0086	1.88	FBXO6	0.0432	2.13	0.0036	1.75
TADA2L	0.0093	1.61	0.0100	1.69	FBXO9	0.0236	1.90	0.0070	1.47
TCF12	0.0179	1.36	0.0002	1.65	FER	0.0063	1.69	0.0339	1.31
TLR7	0.0353	1.85	0.0039	3.11	FEZ1	0.0403	2.01	0.0200	1.87
TM7SF3	0.0114	1.44	0.0024	1.76	FLJ20035	0.0387	4.24	0.0284	1.41
TMEM117	0.0187	1.44	0.0160	1.85	FMNL3	0.0153	1.79	0.0206	1.56
TMPRSS11D	0.0327	1.33	0.0087	1.40	FRAS1	0.0013	1.63	0.0185	1.33
TRPV1	0.0139	1.38	0.0024	1.58	FXYD4	0.0365	1.94	0.0005	1.44
TSGA10	0.0215	1.49	0.0158	1.64	GALK2	0.0042	1.60	0.0312	1.35
UGP2	0.0025	1.63	0.0004	2.34	GCNT2	0.0019	2.83	0.0251	1.93
VHL	0.0128	1.66	0.0004	1.79	GDAP2	0.0020	1.42	0.0293	1.35
XTP3TPA	0.0363	1.59	0.0009	1.78	GIMAP6	0.0050	3.35	0.0233	1.69
ZAP70	0.0232	1.46	0.0149	1.66	GIMAP7	0.0086	2.28	0.0137	1.68
ZBTB26	0.0044	1.39	0.0077	1.51	GLT8D1	0.0178	1.57	0.0166	1.41
ZBTB45	0.0422	1.42	0.0042	1.50	GMPR2	0.0213	1.65	0.0295	1.54

ZMAT3	0.0298	2.13	0.0001	2.34
ZNF454	0.0316	1.31	0.0479	1.31
ZNF552	0.0017	1.58	0.0132	1.77
ZNF816A	0.0375	1.44	0.0189	1.45

GPHN	0.0344	1.45	0.0072	1.38
GPR174	0.0206	3.24	0.0325	1.66
GPX5	0.0191	1.37	0.0061	1.32
GRIN2D	0.0193	1.36	0.0154	1.32
GRSF1	0.0265	1.82	0.0025	1.45
GTF2H5	0.0057	1.76	0.0272	1.37
HCFC1R1	0.0130	1.54	0.0447	1.44
HLA-DPB1	0.0411	1.61	0.0164	1.60
HP1BP3	0.0222	1.74	0.0064	1.64
IFIT1	0.0245	4.03	0.0137	3.63
IFIT3	0.0485	9.33	0.0240	5.96
IFIT3	0.0495	3.89	0.0355	3.61
INADL	0.0034	1.47	0.0045	1.36
INTS12	0.0264	1.51	0.0296	1.34
JAK3	0.0490	1.57	0.0157	1.45
KATNAL1	0.0401	1.70	0.0336	1.36
KBTBD3	0.0252	2.50	0.0207	1.34
KCNMB3	0.0290	1.37	0.0453	1.30
KHK	0.0201	1.56	0.0027	1.48
KHK	0.0423	1.65	0.0332	1.48
KIAA0251	0.0250	2.46	0.0107	1.31
KIDINS220	0.0095	1.90	0.0390	1.33
KIR3DL1	0.0258	2.57	0.0214	2.49
KL	0.0199	1.65	0.0324	1.42
LCMT1	0.0223	1.73	0.0382	1.40
LIN9	0.0060	2.98	0.0042	1.62
LINS1	0.0161	2.79	0.0038	1.62
LOC150383	0.0011	2.50	0.0210	1.36
LOC153364	0.0450	1.96	0.0094	1.66
LOC201181	0.0279	1.76	0.0206	1.32
LOC283155	0.0400	1.86	0.0141	1.46
LOC388524	0.0106	1.70	0.0388	1.50
LOC642897	0.0079	1.63	0.0166	1.40
LOC728944	0.0024	2.09	0.0124	1.84
LYSMD2	0.0209	1.58	0.0425	1.57
MAD2L2	0.0087	1.58	0.0208	1.36
MANEAL	0.0210	1.53	0.0139	1.39
MAP4K4	0.0213	1.98	0.0098	1.49
MAT2B	0.0052	3.57	0.0313	1.82

MDP-1	0.0003	1.83	0.0064	1.42
MFSD5	0.0307	1.61	0.0118	1.58
MGAT2	0.0176	1.65	0.0491	1.40
MGC33212	0.0035	2.04	0.0084	1.64
MGC70863	0.0293	1.77	0.0433	1.41
MGST3	0.0146	1.53	0.0493	1.47
MOCS2	0.0311	2.42	0.0252	1.36
MRE11A	0.0104	2.28	0.0082	1.55
MRPL27	0.0431	1.77	0.0139	1.41
MRPL30	0.0080	2.17	0.0001	1.61
MRPL34	0.0086	1.79	0.0489	1.41
MRPL35	0.0098	1.96	0.0108	1.52
MRPL36	0.0234	1.58	0.0070	1.58
MRPL44	0.0144	1.61	0.0167	1.56
MRPS11	0.0150	1.87	0.0089	1.43
MTFMT	0.0110	2.28	0.0392	1.51
MTX2	0.0048	1.53	0.0016	1.45
NBPF3	0.0271	2.13	0.0195	1.49
NBR1	0.0077	2.36	0.0112	1.85
NCAPH2	0.0279	1.74	0.0119	1.49
NDUFA8	0.0062	1.68	0.0334	1.55
NEK3	0.0392	1.71	0.0323	1.41
NFATC1	0.0368	1.57	0.0069	1.50
NIPA1	0.0069	1.71	0.0331	1.69
NNT	0.0273	2.11	0.0450	1.62
NOVA1	0.0234	1.90	0.0007	1.35
NUP35	0.0457	1.68	0.0240	1.52
OAS2	0.0366	3.34	0.0111	1.70
OGFOD1	0.0117	2.40	0.0317	1.32
OR13C5	0.0131	2.05	0.0100	1.45
OR4K13	0.0284	1.45	0.0213	1.33
OR8D4	0.0049	1.51	0.0244	1.34
ORC5L	0.0011	2.06	0.0004	2.00
OSGEPL1	0.0094	1.58	0.0076	1.49
P2RX5	0.0233	3.89	0.0338	1.36
PCDH7	0.0043	1.44	0.0304	1.43
PCGF6	0.0075	2.27	0.0033	1.34
PCTP	0.0361	1.91	0.0110	1.87
PDCD10	0.0086	1.44	0.0044	1.31

PDCD2	0.0170	1.74	0.0053	1.52
PEX13	0.0435	1.68	0.0164	1.61
PHF14	0.0170	1.83	0.0051	1.72
PIGP	0.0053	1.91	0.0488	1.33
PIGY	0.0151	1.85	0.0187	1.57
PLD3	0.0329	2.27	0.0027	1.72
PML	0.0247	2.09	0.0235	1.35
POLR3K	0.0482	1.64	0.0287	1.33
PPP1R7	0.0312	2.09	0.0037	1.39
PPP2R1B	0.0038	1.50	0.0162	1.39
PPP2R2B	0.0094	1.71	0.0229	1.50
PPP2R2D	0.0495	2.21	0.0479	1.66
PPP2R5D	0.0209	1.64	0.0061	1.62
PPP2R5D	0.0269	3.00	0.0277	1.33
PRIM1	0.0042	2.44	0.0296	1.41
PSEN1	0.0198	2.26	0.0057	1.55
PSMB8	0.0163	3.33	0.0053	1.75
PSPH	0.0262	1.71	0.0198	1.69
PTPDC1	0.0076	3.23	0.0319	1.31
RABAC1	0.0057	1.51	0.0046	1.36
RAD50	0.0226	1.66	0.0077	1.42
RAD51C	0.0092	2.56	0.0046	1.52
RASSF5	0.0228	4.30	0.0051	2.01
RBCK1	0.0075	1.79	0.0034	1.61
REG3G	0.0386	2.25	0.0123	1.47
RFXANK	0.0108	1.54	0.0150	1.34
RHOT1	0.0130	2.15	0.0308	1.49
RIF1	0.0396	1.52	0.0115	1.39
RNF14	0.0001	2.23	0.0134	1.89
RPLP0	0.0053	2.91	0.0398	1.32
RPUSD3	0.0060	2.19	0.0214	1.40
S100PBP	0.0030	1.83	0.0275	1.32
SAMD9L	0.0174	4.12	0.0137	2.80
SATB2	0.0044	1.66	0.0016	1.58
SBNO1	0.0376	1.81	0.0462	1.69
SCAND2	0.0192	2.35	0.0029	1.96
SCP2	0.0181	2.05	0.0365	1.34
SDCCAG1	0.0211	1.80	0.0302	1.40
SDF2	0.0001	1.72	0.0312	1.32

SKIP	0.0125	2.09	0.0239	1.78
SLC25A20	0.0040	5.58	0.0076	1.69
SLC27A6	0.0390	1.74	0.0445	1.30
SLC30A5	0.0059	2.62	0.0345	1.55
SLC33A1	0.0226	1.66	0.0098	1.56
SLC35E3	0.0130	2.23	0.0376	1.90
SLC39A3	0.0248	2.26	0.0498	1.99
SLFN5	0.0302	3.64	0.0439	1.96
SMAP1	0.0128	1.78	0.0328	1.61
SNAP29	0.0118	2.54	0.0269	1.98
SNTB2	0.0316	1.74	0.0311	1.61
SNX12	0.0094	1.42	0.0147	1.39
SNX5	0.0380	1.66	0.0336	1.34
SOCS5	0.0377	1.74	0.0165	1.49
SOX5	0.0184	1.89	0.0339	1.50
SPAST	0.0158	1.99	0.0087	1.62
SPN	0.0400	1.77	0.0197	1.68
SRP9	0.0058	2.75	0.0303	1.32
SRPK2	0.0122	2.42	0.0136	1.85
SRR	0.0137	2.13	0.0235	1.34
SS18	0.0340	2.78	0.0058	1.50
ST8SIA4	0.0118	3.25	0.0010	1.43
STAMBP	0.0164	1.88	0.0323	1.44
STAT1	0.0227	3.00	0.0304	1.57
STK16	0.0353	1.76	0.0026	1.48
STK3	0.0137	2.76	0.0222	1.57
STOM	0.0048	3.10	0.0000	1.82
STUB1	0.0105	1.86	0.0028	1.56
SUDS3	0.0339	1.52	0.0434	1.43
SUGT1	0.0237	1.42	0.0334	1.41
SYNE1	0.0282	1.48	0.0022	1.33
TADA3L	0.0150	3.03	0.0183	1.62
TAF9L	0.0058	1.87	0.0190	1.55
TARP	0.0449	3.34	0.0112	1.50
TBN	0.0101	1.75	0.0484	1.33
TCIRG1	0.0289	3.40	0.0153	2.44
TENC1	0.0127	1.71	0.0240	1.35
TES	0.0265	2.25	0.0304	1.83
TMCC1	0.0273	2.62	0.0001	2.44

TMCO3	0.0271	2.30	0.0412	1.45
TMED10	0.0099	3.55	0.0458	1.43
TMEM103	0.0076	2.33	0.0067	1.34
TMEM14A	0.0160	2.55	0.0006	1.47
TMEM5	0.0155	1.74	0.0213	1.40
TMPO	0.0365	2.39	0.0309	1.65
TP53AP1	0.0157	1.62	0.0356	1.52
TRIM5	0.0191	7.91	0.0248	1.54
TRIM68	0.0034	1.83	0.0387	1.46
TRIP4	0.0215	2.13	0.0027	1.57
TRPT1	0.0259	1.68	0.0429	1.67
TSNAX	0.0177	2.15	0.0323	1.91
TTYH2	0.0189	1.65	0.0183	1.49
UBE2L6	0.0113	4.97	0.0035	1.69
UBL7	0.0111	4.50	0.0279	1.58
UPF3A	0.0111	1.83	0.0092	1.47
VDP	0.0178	1.84	0.0034	1.30
VPS53	0.0160	2.20	0.0400	1.39
VPS72	0.0466	1.53	0.0132	1.34
WDR68	0.0013	3.01	0.0418	1.35
YARS2	0.0400	1.73	0.0150	1.33
ZH2C2	0.0466	2.42	0.0117	1.63
ZKSCAN5	0.0431	1.46	0.0010	1.31
ZMAT5	0.0229	2.14	0.0057	1.66
ZMYM3	0.0159	2.28	0.0150	1.46
ZNF136	0.0316	1.65	0.0239	1.47
ZNF174	0.0016	1.82	0.0134	1.62
ZNF254	0.0005	7.97	0.0198	2.13
ZNF28	0.0194	1.78	0.0022	1.64
ZNF397	0.0205	2.54	0.0183	1.45
ZNHIT2	0.0211	1.62	0.0291	1.31
ZSCAN5	0.0113	1.90	0.0337	1.47
ZXDA	0.0179	1.73	0.0188	1.72

All genes significantly expressed in SVR higher than in NR group. [a]list of genes with different expression at pre-treatment lower than during treatment at week24, [b]list of genes with different expression at pre-treatment higher than during treatment at week24.

Table 16. Biological functions of 415 genes listed in table 15

Term	Count	Genes
Antigen processing and presentation	4	PSME2, HLA-DPB1, RFXANK, KIR3DL1
RNA degradation	3	SKIV2L2, CNOT1, CNOT7
Ubiquitin mediated proteolysis	5	VHL, PML, UBE2L6, FBXO4, STUB1
Natural killer cell mediated cytotoxicity	4	ZAP70, NFATC3, KIR3DL1, NFATC1
Apoptosis	3	CASP8, APAF1, BAD
Endocytosis	5	FAM125B, STAMBP, SMAP1, CHMP5, CHMP6
Proteasome	2	PSME2, PSMB8
Notch signaling pathway	2	CTBP2, PSEN1
Toll-like receptor signaling pathway	3	CASP8, STAT1, TLR7
T cell receptor signaling pathway	3	ZAP70, NFATC3, NFATC1
NOD-like receptor signaling pathway	2	CASP8, SUGT1
B cell receptor signaling pathway	2	NFATC3, NFATC1
TGF-beta signaling pathway	2	PPP2R1B, BMPR2
Jak-STAT signaling pathway	3	JAK3, SOCS5, STAT1
Chemokine signaling pathway	2	JAK3, STAT1
regulation of defense response to virus	3	PML, APOBEC3F, SPN
regulation of T cell differentiation	4	ZAP70, SOCS5, BAD, AP3B1
regulation of T cell activation	5	ZAP70, SOCS5, BAD, SPN, AP3B1
protein ubiquitination	5	VHL, FBXO4, FBXO9, STUB1, RNF14
regulation of immune response	7	PSEN1, ZAP70, PML, SOCS5, APOBEC3F, TLR7, SPN
regulation of protein ubiquitination	4	PSME2, FBXO4, STUB1, PSMB8
regulation of viral reproduction	2	RSF1, APOBEC3F
T cell receptor signaling pathway	2	PSEN1, ZAP70
T cell differentiation	3	ZAP70, PATZ1, SPN
T cell activation	4	PSEN1, ZAP70, PATZ1, SPN
antigen receptor-mediated signaling pathway	2	PSEN1, ZAP70
cell activation during immune response	2	PSEN1, TLR7
JAK-STAT cascade	2	STAMBP, STAT1
activation of immune response	3	PSEN1, ZAP70, TLR7
lymphocyte activation	5	PSEN1, ZAP70, PATZ1, KIR3DL1, SPN
acute inflammatory response	3	KL, TRPV1, REG3G
regulation of immune effector process	3	PML, APOBEC3F, SPN
immune response	14	CNIH, OAS2, TLR7, APOBEC3F, PSMB8, APOL1, PSEN1, COL4A3BP, ZAP70, DPP8, HLA-DPB1, TCF12, KIR3DL1, SPN
regulation of adaptive immune response	2	SOCS5, SPN

cellular defense response	2	TCIRG1, SPN
innate immune response	3	APOL1, APOBEC3F, TLR7
regulation of gene expression, epigenetic	2	DICER1, CTCF
defense response	10	TCIRG1, APOL1, KL, TRPV1, APAF1, REG3G, APOBEC3F, NFATC3, TLR7, SPN
inflammatory response	5	KL, TRPV1, REG3G, NFATC3, TLR7
regulation of cytokine production	2	TLR7, SPN

Table 17. Significantly different expression of 72 genes between SVR and NR in pre-PegIFN-α treatment and during PegIFN-α treatment, higher expression of NR in pre-PegIFN-α treatment and higher expression of SVR during PegIFN-α treatment in chronic HBV patients with negative HBeAg.

Gene symbol	Baseline		wk24	
	P-value	FC	P-value	FC
AKAP7	0.0450	-2.18	0.0305	1.31
B4GALT1	0.0292	-2.05	0.0087	1.68
BAG4	0.0490	-1.71	0.0269	1.38
C1GALT1	0.0361	-2.33	0.0296	2.78
C1ORF108	0.0077	-1.47	0.0068	2.42
C1ORF9	0.0118	-3.35	0.0192	1.98
CCNL1	0.0201	-2.68	0.0229	2.45
CENPM	0.0271	-1.75	0.0343	1.65
CEP27	0.0498	-1.81	0.0140	2.08
CHST4	0.0065	-1.34	0.0027	1.50
CNNM2	0.0218	-1.81	0.0223	1.33
CRSP7	0.0240	-1.63	0.0387	1.52
DNTTIP2	0.0430	-2.87	0.0008	2.42
EDARADD	0.0077	-1.33	0.0035	3.60
ELK4	0.0216	-3.26	0.0327	1.43
ENSA	0.0322	-1.55	0.0001	1.78
FAM98A	0.0304	-1.92	0.0061	2.64
FSHR	0.0255	-1.34	0.0281	1.47
GMNN	0.0063	-1.53	0.0068	2.88
GTDC1	0.0453	-1.39	0.0401	1.50
HOXB1	0.0481	-1.82	0.0148	1.56
IDS	0.0181	-4.39	0.0006	2.98
IL21R	0.0233	-1.85	0.0109	1.60
ING3	0.0448	-3.84	0.0017	2.34
KIAA0564	0.0291	-1.76	0.0193	1.88

KIAA1026	0.0176	-1.46	0.0082	1.50
KPNA2	0.0279	-2.99	0.0125	1.60
LELP1	0.0378	-1.64	0.0063	1.57
LOC652251	0.0079	-1.56	0.0096	1.43
LOC727922	0.0175	-1.60	0.0130	2.16
MAP1B	0.0419	-1.40	0.0032	2.14
MTF2	0.0466	-1.80	0.0019	1.85
MTMR6	0.0165	-2.94	0.0365	2.42
NFE2L2	0.0411	-1.47	0.0107	1.39
NFKBIB	0.0160	-1.78	0.0387	1.43
NFX1	0.0199	-1.68	0.0192	1.45
NR1D2	0.0366	-1.97	0.0422	1.73
NUP54	0.0298	-2.04	0.0057	2.28
PBLD	0.0449	-1.79	0.0153	1.54
PDE8A	0.0359	-1.47	0.0038	1.78
PIK3CA	0.0125	-1.76	0.0069	2.65
PKHD1	0.0071	-1.39	0.0179	1.40
PKN2	0.0200	-1.93	0.0037	1.93
PLG	0.0077	-1.79	0.0068	1.46
PTPN2	0.0469	-1.96	0.0010	2.88
RANBP2	0.0241	-2.18	0.0234	2.01
RAXL1	0.0311	-2.01	0.0396	1.85
REL	0.0122	-2.15	0.0092	3.42
RRN3	0.0163	-2.02	0.0014	1.82
SELV	0.0313	-1.35	0.0062	1.32
SFPQ	0.0492	-2.15	0.0053	1.82
SFRS11	0.0291	-3.75	0.0057	2.92
SFRS12	0.0260	-3.33	0.0044	2.21
SIRT1	0.0385	-1.73	0.0074	1.68
SLC25A32	0.0132	-1.69	0.0049	2.08
SLC6A15	0.0427	-1.38	0.0183	1.46
SULT1A1	0.0350	-1.48	0.0490	1.53
SYAP1	0.0067	-4.06	0.0009	2.56
TAGLN	0.0135	-1.91	0.0105	1.74
TCEAL7	0.0422	-1.40	0.0137	1.35
TCERG1	0.0228	-1.36	0.0135	1.80
THRAP2	0.0370	-2.10	0.0016	1.62
TMEM16G	0.0284	-1.63	0.0352	1.53
TUBA4A	0.0277	-5.60	0.0301	1.85
TUBB4Q	0.0153	-2.90	0.0495	1.56
UAP1	0.0180	-2.07	0.0008	2.55

UBE2D2	0.0377	-2.75	0.0234	1.47
USP49	0.0371	-2.37	0.0451	1.78
WAC	0.0406	-1.35	0.0010	1.68
WWOX	0.0062	-1.56	0.0309	1.44
ZCCHC8	0.0321	-1.40	0.0383	1.58
ZNF91	0.0413	-1.43	0.0022	4.29

Table 18. The intersection between the published different expressed genes in HBV or HCV microarray reports and the 513 significantly different expressed genes at both pre-PegIFN-α treatment and during PegIFN-α treatment between SVR and NR in chronic HBV patients with negative HBeAg

Significantly different genes in our study and other HBV papers	Significantly different genes in our study and other HCV papers			Significantly different genes in our study and to be ISGs
CHMP5	ADAR	FLJ20035	SLFN5	ATP1B4
Ifit1	ATP6V1C1	HLA-DPB1	SPN	BRF3
Ifit3	CEPT1	HP1BP3	SRPK2	CASP9
PSMB8	FLJ11171	IFIT1	ST8SIA4	DDX18
Stat1	GMFB	IFIT3	STAT1	IFIT4
UBE2L6	NFATC3	MAT2B	STOM	OAS3
	PSME2	OAS2	UBE2L6	PML
	SNRPN	P2RX5	UPF3A	PSEN2
	SP110	PML	IMPDH2	PSMB9
	TLR7	PSEN1	PER1	STAT2
	API5	PSMB8	PPP2R5C	ELK5
	APOL1	RAD51C	CCNL1	SFPQ
	ARF3	RBCK1	ING3	
	ARHGEF3	RPLP0	KIAA0564	
	CTBP2	SAMD9L	NFE2L2	

Table 19. Biological functions of 51 genes listed in table 18

Term	Count	Genes
immune system process	9	APOL1, PSEN1, PML, HLA-DPB1, OAS2, TLR7, PSMB8, SPN, IMPDH2
immune response	7	APOL1, PSEN1, HLA-DPB1, OAS2, TLR7, PSMB8, SPN
induction of apoptosis	5	ARHGEF3, PSEN1, PML, STAT1, SPN
proteasomal protein catabolic process	3	PSME2, PPP2R5C, PSMB8
regulation of defense response to virus	2	PML, SPN
apoptosis	5	ARHGEF3, PSEN1, PML, STAT1, API5
lymphocyte activation	3	PSEN1, SPN, IMPDH2
immune system development	3	PSEN1, PML, SPN
response to cytokine stimulus	2	PML, STAT1
antigen processing and presentation	2	HLA-DPB1, PSMB8
activation of immune response	2	PSEN1, TLR7
defense response	4	APOL1, NFATC3, TLR7, SPN
response to virus	2	STAT1, TLR7
T cell activation	2	PSEN1, SPN
innate immune response	2	APOL1, TLR7
anti-apoptosis	2	PSEN1, API5
inflammatory response	2	NFATC3, TLR7
cell proliferation	2	PSEN1, IMPDH2
Notch signaling pathway	2	CTBP2, PSEN1
Proteasome	2	PSME2, PSMB8
Antigen processing and presentation	2	PSME2, HLA-DPB1
Toll-like receptor signaling pathway	2	STAT1, TLR7
Ubiquitin mediated proteolysis	2	PML, UBE2L6

Table 20. The intersection between the published different expressed genes in HBV or HCV microarray reports and the 2372 significantly different expressed genes during PegIFN-α treatment but not at pre-PegIFN-α treatment in chronic HBV patients with negative HBeAg.

Significantly different genes in our study and other HBV papers	Significantly different genes in our study and other HCV papers				Significantly different genes in our study and to be ISGs
Mx1	PLSCR1	MB	ZNRF2	ADD3	PLSCR1
Gbp3	USP48	RABGAP1L	WTAP	DAPP1	GSTZ1
Usp18	CD164	SC4MOL	PPIH	DEXI	HDAC2
PVRL1	AMMECR1	EIF3S12	CASP5	TGFBR3	IFITM1
	PARP9	EVI5	FLJ36166	BUB3	EIF3S10
	EIF2AK2	PCNXL2	RBBP8	NT5C2	SF3B4
	MYCN	EPHA5	RTN1	DECR1	C4BPA
	IFITM1	PPP1CC	FZD1	MX1	PSMD8
	SC4MOL	LILRB2	HESX1	SERPINB9	NMI
	SMARCA5	SEC31A	PRKACB	TCF7	IFI16
	UCHL3	BST2	MT2A	CENTG2	CBFB
	USP25	GBP3	AIM2	CDC42SE2	FGG
	ANXA4	FCGR3A	GP5	WSB1	HLA-G
	PSMA4	CYLD	ABCA5	PNPT1	DSG1
	RPL29	SEPT7	USP18	GNB4	MX1
	H2AFV	PLCB1	RPS7	UBE2G2	RANBP1
	ICAM3	ITM2A	TNFSF10	PLAG1	BST2
	PSMD6	KLK3	SLC25A24	ELOVL6	RECQL
	YME1L1	SLC15A4	OAS3	RABIF	TMEM1
	KIF23	AKAP1	MX2	MTHFD2	PMAIP1
	KIAA0101	SMARCA4	RTP4	LSM14A	MKI67
	ADNP	EEF1B2	IL15	STK39	CYC1
	SF3B4	ATP2A3	TM9SF2	WIPF1	AIM2
	THUMPD1	VDR	SAMD9	AMFR	IRF4
	VRK2	HLA-DQB2	MAF	LYN	TNFSF10
	NMI	ARS2	BBX	NAP1L1	PMPCB
	CPA3	IL4R	KIF23	DGKA	MX2
	IFI16	GPD2	IFI6	RSAD2	IL15
	LDHB	ADD3	SH3GLB1	HNRPF	PRAME
	CDC42	FBXL3	ZNF248	PCGF5	DRAP1
	RRM2	CARS	RGL1	ENPP2	TMEM1

MAPK8IP2	CAST	SOX4	FYTTD1
CSNK1E	MFHAS1	UXT	KLRC3
HIVEP2	DLG7	LAG3	TTC3
GIMAP2	TREX1	DRAP1	KLRC3
PTPRCAP	RPL15	PLA2G6	FBL
NAP1L1	PMAIP1	ATP5G2	MOBK1B
ITM2B	DYRK2	CREM	PSIP1
MRPL19	SLC2A4RG	RPL10A	RUNX3
			CX3CR1

Table 21. Biological functions of 161 genes listed in table 20

Term	Count	Genes
response to virus	7	PLSCR1, BST2, RSAD2, IFI16, MX1, EIF2AK2, MX2
immune response	17	TCF7, BST2, LYN, ENPP2, OAS3, RSAD2, IL15, CD164, AIM2, LILRB2, TNFSF10, PVRL1, IL4R, TGFBR3, FCGR3A, GBP3, IFI6
immune system process	21	TCF7, BST2, LYN, ENPP2, OAS3, RSAD2, SOX4, IFI16, IL15, CD164, AIM2, CDC42, LILRB2, TNFSF10, PVRL1, IL4R, TGFBR3, FCGR3A, GBP3, IFI6, MB
immune system development	8	CDC42, LYN, TGFBR3, SOX4, IL15, IFI16, CD164, MB
proteolysis	17	KLK3, UBE2G2, ZNRF2, WSB1, CASP5, CYLD, USP18, PSMA4, CPA3, YME1L1, UCHL3, USP48, AMFR, PSMD6, USP25, FBXL3, BUB3
virus-infected cell apoptosis	2	PMAIP1, EIF2AK2
cellular defense response	3	LILRB2, KLRC3, CX3CR1
defense response	10	LILRB2, KLRC3, NMI, LYN, CX3CR1, RSAD2, IL15, MX1, PTPRCAP, MX2
anti-apoptosis	4	SERPINB9, SH3GLB1, ANXA4, IFI6
apoptosis	8	CASP5, TNFSF10, SH3GLB1, DYRK2, PMAIP1, ITM2B, EIF2AK2, IFI6
cytokine production	2	MAF, LYN
T cell differentiation	2	SOX4, IL15
lymphocyte activation	3	BST2, SOX4, IL15
T cell activation	2	SOX4, IL15
inflammatory response	3	NMI, LYN, IL15
Chemokine signaling pathway	6	CDC42, LYN, CX3CR1, GNB4, PRKACB, PLCB1
Fc gamma R-mediated phagocytosis	4	CDC42, LYN, PLA2G6, FCGR3A
B cell receptor signaling pathway	3	IFITM1, DAPP1, LYN
Proteasome	2	PSMA4, PSMD6
Natural killer cell mediated cytotoxicity	3	TNFSF10, KLRC3, FCGR3A
Cytokine-cytokine receptor interaction	4	TNFSF10, IL4R, CX3CR1, IL15

PART 3
(The association of SNPs of cytokine and cytokine receptor genes with HBV chronicity)

Here, we reported the effects of 22 SNPs from 14 cytokine and cytokine receptor genes on the susceptibility to HBV-related chronicity among Thai patients with chronic HBV infection and healthy individuals. Most of these SNPs were putative functional SNPs and were previously reported to be associated with chronic HBV infection by individual SNPs or genes but have not been analyzed the relationship between the SNPs of Th1 and Th2 cytokine genes as a network.

The genotypic distributions of each of the cytokine SNPs in chronic HBV and healthy controls group except for *IL6* (-174) in the control group was in Hardy-Weinberg equilibrium. That *IL6* (-174) was not in the equilibrium was possibly because of its small sample size. There were no statistically significant differences in allelic distributions for any of the all SNPs between chronic hepatitis B patients and healthy controls (Table 16). When the results from each genotype were separately analyzed, the frequencies of the heterozygous CA (-592) and CT (-819) genotype of *IL10* gene promoter polymorphisms were significantly higher in chronic HBV patients than that in healthy controls (OR=1.76, 95%CI=1.03-3.01, p=0.028; OR=1.79, 95%CI=1.04-3.06, p=0.024, respectively) (Table 2). In addition, there was a trend of a positive association between chronic HBV and some cytokine gene polymorphisms such as *TNFA* -308A (OR=0.61 95%CI=0.34-1.09, p =0.09) and -590CT compared to TT (OR=0.61 95%CI=0.35-1.06, p =0.063). Interestingly, the TCC (-1098/-590/-33) haplotype frequency of *IL-4* showed an association with chronic hepatitis B as a protective haplotype (OR=0.53, 95%CI=0.32-0.85, p=0.005) as shown in Table 17.

In addition, we analyzed particular functional SNP based on types of cytokines to Th1 and Th2 cytokine and analyzed their distributions in chronic HBV and control groups. Table 18 showed the association of genes based on number of low activity genotypes of Th1 cytokine (*IFNG*, *IL12* and *IL18*) and Th2 cytokine (*IL4* and *IL10*), and

table 19 showed the combined effect of Th1 and Th2 genotypes on the risk of chronic HBV. The analysis revealed that there was not any statistically significant association.

Table 22. Allelic distributions of cytokine and cytokine receptor polymorphisms in patients with chronic HBV and healthy controls

Genes	SNPs	allele	CHB n (%)	Control n (%)	OR (95% Confidence Interval)	*P*
TNFA	-308 (rs1800629)	G A	240(92.3) 20(7.7)	248(87.9) 34(12.1)	1.00 0.61(0.34-1.09)	**0.090**
	-238 (rs361525)	G A	249(95.8) 11(4.2)	276(97.2) 8(2.8)	1.00 1.52(0.60-3.85)	0.370
IL1A	-889 (rs1800587)	C T	239(91.9) 21(8.1)	264(93.0) 20(7.0)	1.00 1.16(0.61-2.19)	0.648
IL1B	-511 (rs16944)	C T	135(51.9) 125(48.1)	147(51.8) 137(48.2)	1.00 0.99(0.71-1.39)	0.970
	+3962 (rs1143634)	C T	253(97.3) 7(2.7)	280(98.6) 4(1.4)	1.00 1.94(0.56-6.69)	0.288
IL1R	pst1 1970 (rs2234650)	C T	179(68.3) 83(31.7)	176(62.0) 108(38.0)	1.00 0.76(0.53-1.08)	0.120
IL1RA	mspa1 11100 (rs315952)	T C	133(0.5) 131(0.5)	151(53.1) 133(46.9)	1.00 1.14(0.81-1.59)	0.46
IL6	-174 (rs1800795)	G C	257(98.1) 5(1.9)	279(98.2) 5(1.8)	1.00 1.09(0.31-3.79)	0.898
TGFB	Codon 10 (rs1800470)	C T	98(50.0) 98(50.0)	137(56.1) 107(43.9)	1.00 1.28(0.88-1.87)	0.199
	Codon 25 (rs1800471)	G C	240(99.2) 2(0.8)	239(99.6) 1(0.4)	1.00 1.99(0.18-22.11)	0.567
IL2	-330 (rs2069762)	T G	155(62.5) 93(37.5)	179(63.9) 101(36.1)	1.00 1.06(0.75-1.52)	0.734
	+166 (rs2069763	G T	157(60.8) 101(39.2)	175(61.6) 109(38.4)	1.00 1.03(0.73-1.46)	0.855

Table 22. Allelic distributions of cytokine and cytokine receptor polymorphisms in patients with chronic HBV and healthy controls *(cont.)*

Genes	SNPs	allele	CHB n (%)	Control n (%)	OR (95% Confidence Interval)	*P*
IFNG	+874 (rs2430561)	A T	191(73.5) 69(26.5)	204(72.3) 78(27.7)	1.00 0.94(0.65-1.38)	0.769
IL12	-1188 (rs3212227)	A C	133(51.1) 127(48.9)	154(54.6) 128(45.4)	1.00 1.15(0.82-1.61)	0.421
IL18	-137 (rs1872380)	G C	224(86.2) 36(13.8)	251(88.4) 33(11.6)	1.00 1.22(0.72-2.09)	0.515
IL4	-1098 (rs2243248)	T G	236(90.8) 24(9.2)	265(93.3) 19(6.7)	1.00 1.42(0.76-2.66)	0.273
	-590 (rs2243250)	T C	204(78.5) 56(21.5)	206(72.5) 78(27.5)	1.00 0.73(0.49-1.08)	0.109
	-33 (rs2070874)	T C	202(77.1) 60(22.9)	206(72.5) 78(27.5)	1.00 0.78(0.53-1.16)	0.220
IL4RA	+1902 (rs1801275)	A G	195(75.0) 65(25.0)	208(74.8) 70(25.2)	1.00 0.99(0.67-1.46)	0.962
IL10	-1082 (rs1800896)	A G	245(94.2) 15(5.8)	267(94.0) 17(6.0)	1.00 0.96(0.47-1.97)	0.915
	-819 (rs1800871)	T C	168(64.1) 94(35.9)	193(68) 91(32)	1.00 1.19(0.83-1.69)	0.344
	-592 (rs1800872)	A C	168(64.6) 92(35.4)	194(68.8) 88(31.2)	1.00 1.21(0.84-1.73)	0.302

Table 23. Genotypic and haplotypic distributions of *IL4* and *IL10* polymorphisms in patients with chronic HBV and healthy controls.

Genes	SNPs	Genotype/ haplotype	CHB n (%)	Control n (%)	OR (95% Confidence Interval)	*P*
TNFA	-308	AA	0(0)	2(1.4)	1.00	
		AG	20(15.4)	30(21.3)	undefined	0.254
		GG	110(84.6)	109(77.3)	undefined	0.157
	-238	AA	0(0)	0(0)	1.00	
		AG	11(8.5)	8(5.6)	undefined	undefined
		GG	119(91.5)	134(94.4)	undefined	undefined
	-308/238	GG	229(88.1)	239(85.4)	1.03(0.80-1.33)	0.853
		AG	20(7.7)	33(11.8)	0.65(0.35-1.21)	0.192
		GA	11(4.2)	8(2.9)	1.48(0.54-4.10)	0.547
IL1A	-889	TT	1(0.8)	1(0.7)	1.00	
		TC	19(14.6)	18(12.7)	1.06(0-42.46)	0.970
		CC	110(84.6)	123(86.6)	0.89(0.02-33.13)	0.937
IL1B	-511	TT	31(23.8)	31(21.8)	1.00	
		TC	63(48.5)	75(52.8)	0.84(0.44-1.60)	0.569
		CC	36(27.7)	36(25.4)	1.00(0.48-2.09)	1.000
	+3962	TT	0(0)	0(0)	1.00	
		TC	7(5.4)	4(2.8)	undefined	undefined
		CC	123(94.6)	138(97.2)	undefined	undefined
IL1R	pst1 1970	TT	13(9.9)	18(12.7)	1.00	
		TC	57(43.5)	72(50.7)	1.10(0.46-2.61)	0.821
		CC	61(46.6)	52(36.6)	1.62(0.68-3.92)	0.235
IL1RA	mspa1 11100	CC	32(24.2)	36(25.3)	1.00	
		CT	67(50.8)	61(43.0)	1.24(0.66-2.32)	0.481
		TT	33(25.0)	45(31.7)	0.82(0.41-1.67)	0.564

Table 23. Genotypic and haplotypic distributions of *IL4* and *IL10* polymorphisms in patients with chronic HBV and healthy controls *(cont.)*

Genes	SNPs	Genotype/ haplotype	CHB n (%)	Control n (%)	OR (95% Confidence Interval)	*P*
IL6	-174	CC	0(0)	1(0.7)	1.00	
		CG	5(3.8)	3(2.1)	undefined	0.236
		GG	126(96.2)	138(97.2)	undefined	0.340
TGFB	Codon 10	TT	20(20.4)	21(17.2)	1.00	
		TC	58(59.2)	65(53.3)	0.94(0.44-2.02)	0.857
		CC	20(20.4)	36(29.5)	0.58(0.24-1.44)	0.197
	Codon 25	CC	0(0)	0(0)	1.00	
		CG	2(1.7)	1(0.8)	undefined	undefined
		GG	119(98.3)	119(99.2)	undefined	undefined
IL2	-330	GG	23(18.5)	19(13.6)	1.00	
		GT	47(37.9)	63(45.0)	0.62(0.28-1.34)	0.183
		TT	54(43.6)	58(41.4)	0.77(0.36-1.66)	0.369
	+166	TT	21(16.3)	24(16.9)	1.00	
		TG	59(45.7)	61(43.0)	1.11(0.53-2.32)	0.775
		GG	49(38.0)	57(40.1)	0.98(0.46-2.10)	0.960
IFNG	+874	TT	12(9.2)	14(9.9)	1.00	
		TA	45(34.6)	50(35.5)	1.05(0.40-2.73)	0.918
		AA	73(56.2)	77(54.6)	1.11(0.45-2.76)	0.813
IL12	-1188	CC	29(22.3)	28(19.9)	1.00	
		CA	69(53.1)	72(51.0)	0.93(0.48-1.79)	0.805
		AA	32(24.6)	41(29.1)	0.75(0.35-1.60)	0.425
IL18	-137	CC	4(3.1)	1(0.7)	1.00	
		GC	28(21.5)	31(21.8)	0.23(0.01-2.39)	0.355
		GG	98(75.4)	110(77.5)	0.22(0.01-2.16)	0.196
IL4RA	+1902	GG	8(6.2)	8(5.8)	1.00	
		GA	49(37.7)	54(38.8)	0.91(0.28-2.92)	0.857
		AA	73(56.1)	77(55.4)	0.95(0.30-2.96)	0.919

Table 23. Genotypic and haplotypic distributions of *IL4* and *IL10* polymorphisms in patients with chronic HBV and healthy controls *(cont.)*

Genes	SNPs	Genotype/ haplotype	CHB n (%)	Control n (%)	OR (95% Confidence Interval)	*P*
IL4	-1098	GG	1 (0.8)	0 (0)	1.00	
		GT	22 (16.9)	19 (13.4)	0.00(0-21.89)	0.358
		TT	107 (82.3)	123 (86.6)	0.00(0-15.29)	0.285
	-590	TT	83(63.8)	75(52.8)	1.00	
		CT	38(29.2)	56(39.4)	0.61(0.35-1.06)	**0.063**
		CC	9(7.0)	11(7.8)	0.74(0.26-2.05)	0.525
	-33	CC	9(6.9)	11(7.8)	1.00	
		CT	42(32.1)	56(39.4)	0.92(0.32-2.68)	0.860
		TT	80(61.0)	75(52.8)	1.30(0.47-3.66)	0.578
	-1098/ -590/-33	TCC	32(12.4)	60(21.1)	0.53(0.32-0.86)	**0.005**
		TTT	198(76.7)	204(71.8)	1.07(0.82-1.39)	0.663
		GCC	23(8.9)	18(6.3)	1.41(0.71-2.79)	0.375
IL10	-1082	GG	1(0.8)	0(0.0)	1.00	
		GA	13(10.0)	17(12.0)	0(0-14.94)	0.263
		AA	116(89.2)	125(88.0)	0(0-16.30)	0.300
	-819	TT	47(35.9)	67(47.2)	1.00	
		CT	74(56.5)	59(41.5)	1.79(1.04-3.06)	**0.024**
		CC	10(7.6)	16(11.3)	0.89(0.34-2.31)	0.796
	-592	AA	48(36.9)	68(48.2)	1.00	
		CA	72(55.4)	58(41.1)	1.76(1.03-3.01)	**0.028**
		CC	10(7.7)	15(10.7)	0.94(0.36-2.47)	0.899
	-1082/- 819/-592	ATA	167(64.2)	191(67.7)	0.95(0.72-1.25)	0.748
		ACC	77(29.6)	70(24.8)	1.19(0.82-1.75)	0.392
		GCC	15(5.8)	17(6.0)	0.96(0.44-2.06)	0.951
		ACA	1(0.4)	3(1.1)	0.36(0.01-3.90)	0.679

Table 24. Distributions of T-helper cytokine genotypes in chronic HBV and healthy controls.

Cytokine genotype	No. of cases	No. of controls	OR (95% Confidence Interval)	*p-value*
Th1 genes				
***IFNG* (+874)**				
TT	12	14	1.00	
AT/AA[a]	118	127	1.08 (0.45-2.62)	0.845
***IL12* (-1188)**				
AA	32	41	1.00	
AC/CC[a]	98	100	1.26 (0.71-2.23)	0.408
***IL18* (-137)**				
GG	98	110	1.00	
GC/CC[a]	32	32	1.12 (0.62-2.04)	0.686
Total low-activity Th1 genotypes[b]				
0	3	3	1.00	
1	30	39	0.77 (0.11-5.24)	0.758
2	73	76	0.96 (0.15-6.20)	0.961
3	24	22	1.09 (0.15-7.80)	0.920
Th2 genes				
***IL4* (-590)**				
CT/TT	121	131	1.00	
CC[a]	9	11	0.89 (0.32-2.40)	0.795
***IL10* (-1082 and -819)**				
GG/AG and CC/CT	14	17	1.00	
AA and/or TT[a]	116	125	1.13 (0.50-2.54)	0.755
Total low-activity Th2 genotypes[c]				
0	14	14	1.00	
1	106	120	0.88 (0.38-2.07)	0.757
2	9	8	1.13 (0.28-4.46)	0.848

[a]Putative low-activity genotypes.

[b]Summed across *IFNG, IL12 and IL18* genotypes.

[c]Summed across *IL4 and IL10* genotypes.

Table 25. The combined effect of T-helper1 and T-helper2 genotypes on the risk of chronic HBV.

	Total no. of low activity Th2 genotypes[a]					
	1 or 2			0		
Total no. of low activity Th1 genotypes[b]	**Cases/ controls**	**OR (95% CI)**	***p-value***	**Cases/ controls**	**OR (95% CI)**	***p-value***
0	3/3	1.00		0/0	Undefined	
1	27/35	0.77(0.11-5.31)	0.761	3/4	0.75 (0.05-11.28)	0.797
2	63/69	0.91(0.14-5.93)	0.913	9/7	1.29 (0.14-12.19)	0.793
3	22/19	1.16(0.16-8.43)	0.867	2/3	0.67 (0.03-13.47)	0.740

[a]Summed across *IL4 and IL10* genotypes.

[b]Summed across *IFNG, IL12 and IL18* genotypes.

CHAPTER V

DISCUSSION

PART 1
(Characterization of a new HLA-C-restricted HBV epitope)

The patients during or after acute HBV infection develop a number of HBV antigen-specific CTL responses with HLA class I restricted to multiple epitopes in core, envelope, polymerase and X proteins (8-10, 12, 70, 142). These memory HBV-specific CTLs still persists after clinical recovery from acute and chronic infection. Currently, it is believed that a strong HBV-specific CTL response has a great potential to control HBV infection. Therefore, the immunodominant HBV epitopes are being explored to apply in vaccine development to enhance immune responses in the patients as well as to be applied in immune-based therapies to restore dysfunctional virus-specific T-cell immunity in chronic HBV infection.

In our study, a preliminary analysis of patient with acute HBV infection firstly reveals the sequence within the envelope molecule that can induce HBV-specific T-cell response restricted to HLA-Cw*08:01. This epitope was subsequently tested in 15 resolved HBV Thai patients carried HLA-Cw*08:01. The interesting finding is the identification of this immunodominant epitope (residue Env171-180) which was recognized by 53% (8 out of 15) of our resolved HBV patients. It is possible that non-response to Env171-180 epitope in some patients may reflect the infection by variant hepatitis B virus which have different amino acid sequence compare to the synthetic peptide used to induce the CTL. This problem was previously reported in which there was no response to a prototype HBsAg epitope in the patient who was infected with variant virus (8). Additionally, each patient had HBV-specific CTL response to more than one immunogenic epitopes (Fig.4). We reported that our resolved HBV patients demonstrated specific CTL response to the new HLA-Cw*08:01-restricted envelope epitope as strong as the response to other well known HLA class I -restricted epitopes.

This finding supports a theory of effective control of HBV infection in these resolved patients by a vigorous and multispecific CD8 responses. Several previous reports also revealed specific CTL responses to numerous HBV epitopes during and after acute HBV infection (8, 9, 60, 61).

Since HBV subtypes vary in distinct geographical distribution, different ethnic populations tend to have an infection with different HBV genotypes (26, 143). Viral heterogeneity among genotypes will generate the epitopes with variation. Our novel HLA-Cw*08:01-restricted Env171-180 epitope has a distinction on the first amino acid between HBV genotype B and C (B : LLGPLLVLQA, C : FLGPLLVLQA). Both genotypes were found in Asian population. While the HBV genotype A and D, which were found more common in Caucasians have the same Env171-180 amino acid sequence. However, Env171-180-specific CTL response in resolved patients was explored in both HBV genotype B and C, and cross-activity of these genotypes could also be detected. Interestingly, binding affinity with dose titration of Env171-180 genotype B and C are comparable to each other.

The highly polymorphic HLA molecule is a main limitation for the application of specific epitope in a diverse population. The immunogenic peptide restricted to the common HLA allele in the population will be a preferable target epitope. In this case, HLA-Cw*08:01-restricted epitope is a very good candidate since it has a high frequency in Asian population (10-36%) which still have high HBV prevalence.

One interesting observation is a possibility that the specific CTL Env171-180 epitope is restricted by HLA-Cw*08:01 as well as HLA-Cw*08:22. This is likely because this 2 alleles have identical peptide-binding cleft. However, due to the absence of patient with the HLA-Cw*08 subtype except HLA-Cw*08:22, we do not know if the Env171-180-specific CTL response in this report is restricted only by the HLA- Cw*08:01 allele or whether it extends to other HLA-Cw*08 subtypes. Taken together, the specific CTL response to HLA-Cw*08:01-restricted Env171-180 epitope is one of the interesting HBV immunodominant epitopes.

According to our experiment strategy that used the overlapping synthetic peptides analog to induce T cell activation *in vitro*, the identified synthetic peptide which is recognized by specific CTL might not be generated by endogenous processing and presentation during natural infection. To further answer this question, we need to study the recognition of intracellular synthesized antigen by peptide-specific CTL (8, 12). However, we did not have the hepatocellular cell or B cell line with HLA-Cw*08:01 molecule and expressing envelope antigen, we cannot demonstrate whether the Env171-180 immunodominant amino acid sequence was generated by endogenous processing of native envelope molecule or not.

The identification of immunodominant T cell epitopes could be useful for future strategies to manipulate the immune response to HBV patients who do not spontaneously clear the virus developing to chronicity and hepatocellular carcinoma or use for monitor their progression.

PART 2
(Gene expression profile in PBMCs of chronic HBV infection)

Moreover, we investigated whether gene expression patterns in peripheral blood mononuclear cells (PBMC) were different between sustained virological responder and non-responder to Pegylated-interferon alpha in chronic HBV infection with positive or negative HBeAg. The Illumina Sentrix Humanref-8 v2 BeadChips microarray was used for the analysis of global gene expression. We found that most of the significantly different genes were at higher level in the responder compared to the non-responder groups both at pretreatment and/or during treatment at week 24. Some interesting immune-related genes are previously reported to have anti-viral activity such as response to virus (IFI16, MX1, MX2), viral defense or viral genome sensor (GBP3, IFI16), regulation of viral reproduction (APOBEC3F) and proteasome (PSMB8).

Guanylate-binding protein 3 (GBP3) is a member of Guanylate-binding proteins family. It is induced in response to interferons and hydrolyzes GTP to both GDP and GMP (144). GBP3 is upregulated by IFN-γ, TNF or IL-1-β (145) while its function is unknown. In our study, GBP3 was significantly higher expressed in SVR than NR group of HBV infection with negative HBeAg during PegIFN-α treatment at week24 (p=0.01, FC=2.28) and significantly different in SVR versus NR both pre-treatment and during PegIFNα treatment at week24 of HBV infection with positive HBeAg (p=0.03, FC=2.70 and p=0.006, FC=2.10, respectively). The GBP-1 and GBP-3 were identified as anti-influenza activity. One of three GBP3 isoforms, hGBP-3ΔC also has the most important anti-influenza action. The hGBP-3ΔC has the inhibitory activity to the viral polymerase complex leading to the suppression of viral transcription and replication through globular domain of GBPs which is makeable domain for antiviral function (146). In addition to GBP3, the antiviral activity of GBP1 was investigated in HCV infection. GBP1 can suppress HCV replication through binding to NS5B, a non-structural protein of HCV (147, 148). Moreover, GBP1 was also shown to have the anti-viral action against vesicular stomatitis virus (VSV) (149) and encephalomyocarditis virus. However, GBP1

and other members of GBP family were not found to be significantly different expression between SVR and NR in our study.

Interferon-gamma-inducible protein 16 (IFI16) is a member of IFN-inducible p200-protein family and it has a function as an innate immune sensor of DNA. IFI16 protein can recognize both cytosolic and nuclear dsDNA. For nuclear dsDNA recognition, IFI16 interacts with nuclear DNA and activates the transcriptional factors such as IRF3 or NF-κB through the stimulator of interferon genes (STING) protein in the cytosol. Upon this activation, the expression of type I IFN and proinflammatoty cytokines occurred (5, 150). On the other hand, IFI16 can sense cytosolic dsDNA and activates the transcriptional factor, IRF3, IRF7 or NF-κB. The translocated transcriptional factors induce expression of type I IFN and proinflammatoty cytokines. To activate IL-1β and IL-18 cytokine maturation, IFI16 interacts with the adaptor molecule ASC to form a functional inflammasome which is cytoplasmic sensors of foreign molecules. It functions to induce caspase-1 activation, IL-1β and IL-18 cytokine maturation and cytokine secretion (5, 151). IFI16 which is an interferon-inducible gene was significantly different expressed in SVR versus NR both at pre-treatment and during PegIFNα treatment at week24 of HBV infection with negative HBeAg (p=0.003, FC=1.97) in our study. Even though there was no previous report of the anti-HBV activity of IFI16, IFI16 has been reported to play a role against other viruses. IFI16 recognized the genome of herpes simplex virus (HSV-1) in infected cell nuclei, relocalized, and colocalized with ASC in the cytoplasm. Upon the activation of the IFI16 and NLRP3 inflammasomes by HSV-1, the activation of IRF-3 through cytoplasmic STING resulted in IL-1β maturation (152, 153). DNA sensor of IFI16 was also reported in adenovirus (154) and cytomegalovirus (155).

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) is comprised of 7 members (APOBEC3A-G) which can trigger hypermutation on minus strand cDNA during replication. It is a broad innate immunity against exogenous and endogenous elements. Several experiments reported the action of APOBEC3 to control viral replication. APOBEC3 subtypes that were induced by interferon included APOBEC33B, F and G in the hepatocytes. The transfected hepatocytes with

APOBEC3B, F or G could induce G-to-A hypermutation in a fraction of replicated HBV genome and also inhibited HBV replication especially in APOBEC3G transfectant (156, 157). Interestingly, APOBEC3F was significantly different expressed in SVR versus NR at both pre-treatment and during PegIFNα treatment at week24 of HBV infection with negative HBeAg (p=0.008, FC=2.65 and p=0.007, FC=1.52, respectively). The immune evasion from APOBEC3 was detected in HIV infection in which the Vif protein of HIV could interact with APOBEC3G and induce its ubiquitination and degradation (158). Moreover, APOBEC3C (A3C) overexpression can reduce virus titers of herpes simplex virus 1 (HSV-1) as well as Epstein-Barr virus (EBV) in the tissue culture (159).

Myxovirus resistance protein A gene (MxA) is a key component of the innate antiviral response and has previously been shown to inhibit several RNA viruses. MxA is inducible by IFNα and IFNβ and can block viral nucleocapsid transport or viral RNA synthesis dependent on Mx presented either in cytoplasm or nucleus of viral infected cells (45). Moreover, HBV which is a DNA virus was reported to be controlled by MxA in HBV transfected cell line. MxA independently suppressed the production of hepatitis B surface antigen and HBV DNA without changing the level of hepatitis B core antigen (HBcAg). MxA-HBcAg interaction affected the accumulation of HBcAg by immobilizing HBcAg in the perinuclear structures that may interfere with core particle formation (160). Our experiment demonstrated the significant higher expression of Mx1 in SVR than NR group of HBV infection with negative HBeAg during PegIFNα treatment at week24 (p=0.007, FC=1.49).

Proteasomes are a machinery for antigen processing and antigen presentation. They can be induced by interferon and promoting the immunoproteasome upregulation. This gene was revealed from the gene expression analyses of IFN-induced genes in transgenic mouse livers and hepatocytes (47). In our investigation, proteasome (prosome, macropain) subunit, beta type 8 (PSMB8 or Lmp7) was significantly increased in SVR compared to NR at both before and during PegIFNα treatment of resolved HBV patients with negative HBeAg (p=0.02, FC=3.33 and p=0.005, FC=1.75, respectively). PSMB8 was also differently expressed between SVR and NR group of

HBV infection with positive HBeAg during PegIFNα treatment at week24 (p=0.01, FC=1.4). The IFN-mediated immunoproteasome activity was required for HBV inhibition (161). The IFN-mediated immunoproteasome catalytic subunits LMP2 and LMP7 alter proteasome specificity and influence the pool of peptides responsible for presentation by MHC I molecules. These subunits influenced both the magnitude and specificity of the CD8 T-cell response to the HBV polymerase and envelope proteins (52). Inversely, HBV could activate transcription factor Nrf2 that regulated ARE-mediated cytoprotective genes encoding proteins such as antioxidant proteins and proteasome subunit PSMB5 which supported HBV replication and modulated HBV immune response (162).

In addition to antiviral genes discussed in detail above, a number of other immune genes which have never been reported with anti-viral activity were upregulated in the SVR after treatment. These genes might need to be further explored in the future.

PART 3
(The association of SNPs of cytokine and cytokine receptor genes with HBV chronicity)

Both viral factors (viral load, genotype and genomic mutations) and host factors (age, sex and immune status) contribute to differential clinical outcomes. As a result, both the cytokine polymorphisms that dictate the functionality of cytokine and the immune response might be associated with different outcomes of HBV infection (22). By investigating these factors, healthcare practitioners could predict the severity of the cases and thus suggest the most effective treatment options to the patients.

In this study, we investigated the association between cytokine and cytokine receptor genes and their complication in individuals' susceptibility to chronic HBV infection. Comparisons to previous studies in other populations were summarized below.

Pro-inflammatory cytokines

TNF-α

Early association studies of *TNF-α* showed mixed results. One study suggested that the -308 G/A polymorphism might play a role in the altered *TNF-α* gene expression observed *in vitro* (163), while another study proposed that the position -308 had no functional relevance for *TNF-α* promoter transcription (164). Since then, numerous association studies of its polymorphisms including those related to hepatitis B have been reported, and ethnicity has been reported to influence the severity of HBV infection. At the position of -308 where allele G is more frequently found than allele A, GG genotype (low activity) was less commonly found in chronic patients with OR=0.37 in the Brazilian population (118). On the other hand, G allele and GG genotype was believed to be indicative of high risk of HBV persistence with OR=1.35 among East Asians in the meta-analysis (119). As to *TNF-α* -238G/A, a meta-analysis showed that G is less frequently found in chronic HBV (OR=0.92) but not to a statistically significant extent (119). Our study revealed the same trend in the Thai population as the meta-analysis among the East Asians previously showed.

IL1B

We have previously shown that the CC genotype was reported to be higher in HBV associated HCC patients with OR=1.20 (CI=0.80-1.79)(165) and OR=1.72 (CI=1.04-2.84, P=0.033)(166), whereas, in this study, there was no association between *IL1β* -511 position. However, The previous published study in the Chinese population and our study showed that genotype distributions and allelic frequencies for *IL1β* (-511) promoter polymorphisms in patients with chronic hepatitis B and control subjects were not statistically different (120).

IL6

Regarding the position*IL6* -174 where the GG genotype represented the majority of the population (123), chronic hepatitis patients in Italy were reported to have a higher frequency of G allele than HBcAb negative controls (OR=1.484, CI=0.975-2.260, p<0.05)(167), and a similar result was observed in a study in Brazil (118). However, we did not observe any positive association in our study.

Th1 cytokines

IL2

IL2 -330T allele and TT genotype were associated with an increased risk of persistent HBV (p=0.03, OR=7.14 and p=0.01, OR=2.26) in the Chinese population (121), and the risk of progression and chronic course of viral hepatitis in Caucasian population was also linked with T allele in *IL2* (122). In contrast, we did not observe any positive association in our study.

IFN-γ

The lower *IL4* expression AA genotype was more often found in Asians who are more susceptible to HBV than Caucasians (168). *IFN-γ*+874 AA genotype or A allele was reported to be associated with an increased risk (121, 123, 169). Nevertheless, in this study, we could not find any significant association between polymorphisms of +874 and HBV infection similar to several of the previously reported studies (118, 170).

IL12* and *IL18

There were limited studies on *IL12* polymorphism with chronic HBV. *IL18* -137C was reported to be a protective allele (124). Additionally, two studies have reported that -607A was rather a protective allele (171, 172). Here, we did not find any positive association with *IL12* and *IL18* polymorphism investigated in this study.

Th2 cytokines

IL4

One study in the Chinese population reported *IL4* -590 polymorphism was not associated with the susceptibility to chronic hepatitis B (121). However, the TT genotype of the *IL4* -590 was associated with the risk of progression and chronic course of hepatitis in the Caucasian population (122). In our study, we observed a trend that -590 TT could be a risk genotype. Particularly, our finding substantiated the importance of the -1098/-590/-33 TCC as a protective haplotype for chronic hepatitis B in the Thai population. We hypothesize that the low activity of *IL4* gene promoter resulted in a lower level of Th2 cytokine. A lower suppression to IFN-γ production could thus be a major protective genetic factor for chronic hepatitis B infection in Thai population.

IL10

The effects of *IL10* on HBV infection have also been inconclusive. According to a meta-analysis of approximately 1,500 chronically infected patients and 1,300 controls at position -1082G/A, when compared to GA+GG, AA genotype was reported to be protective of HBV infection (OR=0.684, CI=0.476-0.982, p=0.04) (173). As for the position -592A/C, when compared to AA, AC genotype reported to be risk of HBV infection (OR = 1.343, 95% CI = 1.017-1.684, P = 0.011) (173). Similarly, in another study in the Chinese population, the frequency of AA genotype at position -592A/C was significantly lower in chronic HBV patients (OR=0.67, CI=0.51-0.94, P=0.018) (174). The haplotype ACC of immediate level of IL-10 production was closely associated with chronic liver disease (p=0.004), whereas haplotype ATA and homozygous ATA/ATA (low level of IL-10) were associated with protection (p=0.035) (125).

Anti-inflammatory cytokines

TGF-β

As for *TGF-β*, no statistically significant difference in the genetic ability to produce TGF-β between the HBV group and the controls was previously observed (123). In the same vein, we did not observe any positive association in this study.

In our study, we found a similar trend. When compared to -819 TT and -592 AA, -819 CT and -592 CA were associated with risk to develop chronic hepatitis B. It should be noted that -819 and -592 polymorphisms in the *IL10* gene were in a complete linkage. Possibly due to their small sample sizes, we did not observe any statistically significant association between disease risks with ACC haplotype. Interestingly, IL-10 is not produced only from Th2 cell but another major source was also from regulatory cells. It has a regulatory function to down-regulate HBV specific CD8 T cell response (175). However, the genetic association result consistently reported that the patients with low IL-10 as well as low IL-4 producing allele has lower risk for chronic state suggesting a protective role of Th2 rather than an active regulatory role of *IL10* gene.

Role of the combination of Th1 and Th2 cytokines

Cytotoxic T lymphocytes (CTLs) and Th1 cells are well known to play a central role in the control of viral infection including HBV infection and the defect of their function lead to persistence of HBV infection (2, 21). One possible risk of chronic HBV development is the host genetic factor causing the low levels of Th1 or high level of Th2 cytokine expression(121, 170, 174). However, so far most of the previous studies only analyzed each gene separately and there were limited effort to analyze a combination of cytokine genes grouping as Th1 and Th2. In this preliminary study, after analyzing the combination of Th1 and Th2 genotypes in the way that was performed in the study of genetic risk factor to HBV-related hepatocellular carcinoma (176), we did not observe any significant association. A non-significant association among such combination could have resulted from the limitation of our small sample size. However, the genetic association result with individual genotype and haplotype consistently reported that the patients with low IL-10 as well as low IL-4 producing allele has lower risk for chronic

state suggesting a protective role of Th2. Further study with more samples is needed to completely analyze the role of Th1/Th2 combination in chronic hepatitis B infection.

CHAPTER VI

CONCLUSION

The effective innate and adaptive immunity are necessary to control HBV replication in acute and resolved HBV infection while the defect or exhausted innate and adaptive immune responses cause chronic HBV infection. The specific CTL response against HBV with strong and multispecific response is believed to play as a major role in HBV clearance and several CTL epitopes were identified in acute rather than chronic infection. In our study, we firstly identified novel HLA-C-restricted CTL epitope of HBV antigen from resolved HBV patients. The minimal optimal CTL epitope at position 171-180 of envelope antigen restricted to HLA-Cw*08:01 molecule revealed and the Env171-180-specific CTL response was detected in 53% (8/15) of tested patients with resolved HBV infection. The cross-reactivity of this CTL response was detected in HBV genotype B and C. The frequency and magnitude of HLA-Cw*08:01-restricted Env171-180 response are greater or at least comparable to known HLA-A*02, A*11, A*24 and B*51 restricted CTL responses. For the analysis of global gene expression in chronic HBV infection, most of the significantly different genes were at higher level in the responder compared to the non-responder groups both at pretreatment and/or during treatment at week 24. Moreover, some interesting immune-related genes were found which were previously reported to have anti-viral activity such as IFI16, MX1, MX2, GBP3, APOBEC3F and PSMB8. Furthermore, The analysis of polymorphisms of cytokine and cytokine receptor genes together as the combination of Th1 and Th2 genotypes gave no positive association with chronic hepatitis B infection. Our genotype data showed that the patients with low IL-10 as well as low IL-4 producing allele has lower risk for chronic state suggesting a protective role of Th2. Taken together, host factors affect the distinct immune responses resulting in the different clinical outcomes of HBV infection.

Our result gives us some better understanding of different immune responses to HBV. Particularly, the identification of novel immunodominant CTL epitope restricted to

common HLA-C allele might be useful for future strategies to manipulate the immune response to HBV patients who do not spontaneously clear the virus developing to chronicity and hepatocellular carcinoma or use for monitor their progression.

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APPENDIX

BUFFERS AND REAGENTS

1. Nuclei Lysis Buffer (NLB)

1 M Tris (pH 8.0)	10	ml
5 M NaCl	0.5	ml
0.5 M EDTA (pH 8.0)	0.4	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.

2. 1 M Tris

Tris base	12.11	g
Distilled water	100	g

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes.

3. 5 M NaCl

NaCl	29.22	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilized by autoclaving at 121 ºC for 15 minutes.

4. EDTA

EDTA	37.22	g
Distilled water	200	ml

Adjust volume to 200 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes. Keep refrigerated.

5. 5.3 M NaCl

NaCl	15.5	g
Distilled water	50	ml

Adjust volume to 50 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes. Keep refrigerated.

6. Proteinase K 10 mg/ml

Proteinase K	100	mg
Distilled water	10	ml

Mix the solution and store at -20 ºC

7. 10% SDS

SDS	10	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes.

BIOGRAPHY

Miss Pimpayao Sodsai was born on November 12, 1981 in Chonburi, Thailand. She graduated with Bachelor's of Science in Microbiology (Second Class Honors) from Faculty of Science, Chulalongkorn University in 2004 and Master degree of Science in Medical Microbiology from Chulalongkorn University in 2007. She got Inter-Department of Medical Microbiology, Graduate School, Chulalongkorn University for philosophy degree in 2012.