The effect of histone methylation in trained innate immunity of cord blood monocytes in newborn infants of chronic HBV-infected mothers

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ผลของการเติมหมู่เมทิลของฮิสโตนต่อกระบวนการ trained innate immunity จากเซลล์โมโนไซท์ของสายสะดือในทารกแรกเกิดของแม่ที่ติดเชื้อไวรัสตับอักเสบบีเรื้อรัง

น.ส.เพ็ญนภา พลายพงษา

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เพ็ญนภา พลายพงษา : ผลของการเติมหมู่เมทิลของฮิสโตนต่อกระบวนการ trained innate immunity จากเซลล์โมโนไซท์ของสายสะดือในทารกแรกเกิดของแม่ที่ติดเชื้อไวรัสตับอักเสบบีเรื้อรัง. (The effect of histone methylation in trained innate immunity of cord blood monocytes in newborn infants of chronic HBV-infected mothers) อ.ที่ปรึกษาหลัก : ดร.พิมพเยาว์ สดใส, อ.ที่ปรึกษาร่วม : รศ. ดร.พญ.รังสิมา เหรียญตระกูล,ศ. ดร. ธนาภัทร ปาลกะ

เด็กแรกเกิดที่สัมผัสเชื้อไวรัสตับอักเสบบีจากมารดาผู้ที่ติดเชื้อไวรัสตับอักเสบบีระหว่างตั้งครรภ์ ในเด็กทารกแรกเกิดเหล่านี้ พบว่า มีการพัฒนาของเซลล์ภูมิคุ้มกันที่มีมาแต่กำเนิดอย่างปกติและมีการตอบสนองที่เพิ่มขึ้นเมื่อมีการกระตุ้นครั้งที่สองด้วยเชื้อชนิดอื่น เช่น แบคทีเรีย รูปแบบนี้ถูกเรียกว่า “trained immunity” หรือ “innate immune memory” Trained immunity ถูกควบคุมด้วยการเปลี่ยนแปลงทางอีพิเจเนติกส์ โดยเฉพาะการเปลี่ยนแปลงฮิสโตน ปัจจุบันนี้ยังไม่มีการรายงานอิพิเจเนติกส์ต่อการควบคุม trained immunity ในเชื้อไวรัสตับอักเสบบี ดังนั้น การศึกษานี้จะทดสอบระดับไซโตไคน์และการเปลี่ยนแปลงฮิสโตนเอนไซม์ยีนในโมโนไซท์จากสายสะดือในทารกแรกเกิดที่ สัมผัสไวรัสตับอักเสบบีที่กระตุ้นด้วย TLR8 agonist นอกจากนั้นยังศึกษาฮิสโตนเมทิลเลชันที่ตำแหน่ง H3K4me3 ที่ควบคุมยีน *IL-12p40 IL-6 IL-10 TNF-a* และ *IL-1b* ในโมโนไซท์จากสายสะดือในทารกแรกเกิดที่สัมผัสไวรัสตับอักเสบบี จากผลการทดลองพบว่าโมโนไซท์จากสายสะดือในทารกแรกเกิดที่สัมผัสไวรัสตับอักเสบบีหลั่งปริมาณ IL-12p40 ออกมาสูงกว่าอย่างมีนัยสำคัญเมื่อเทียบกับโมโนไซท์จากสายสะดือในทารกแรกเกิดที่สุขภาพดีหลังจากกระตุ้นด้วย TLR8 agonist นอกจากนั้น การแสดงออกของการเปลี่ยนแปลงฮิสโตนเอนไซม์ไม่มีความแตกต่างในระหว่างโมโนไซท์จากสายสะดือในทารกแรกเกิดที่สัมผัสไวรัสตับอักเสบบีและโมโนไซท์จากสายสะดือในทารกแรกเกิดที่สุขภาพดีหลังจากกระตุ้นด้วย TLR8 agonist ที่ 18 ชั่วโมง ในขณะที่ปริมาณของฮิสโตนเมทิลเลชันที่ตำแหน่ง H3K4me3 ในยีนต่างๆ ไม่ได้สนับสนุนการ trained immunity ในไวรัสตับอักเสบบี จากการทดลองแสดงให้เห็นว่าบทบาทของอิพิเจเนติกส์ผ่านทางการเปลี่ยนแปลงฮิสโตนตำแหน่ง H3K4me3 น่าจะไม่มีความสำคัญในการควบคุม trained immunity ในโรคไวรัสตับอักเสบบี อย่างไรก็ตามยังคงต้องการการศึกษาเพื่ออธิบายกลไกนี้อีกมาก

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Newborns contract Hepatitis B virus (HBV) through exposure *in utero* from mothers who are infected with HBV. In newborn infant from chronic HBV-infected mothers, the immune system displays innate immune cell maturation and enhances immune response upon restimulation with unrelated pathogens such as bacteria. This pattern is called “trained immunity” or “innate immune memory”. Trained immunity is regulated by epigenetic programming, especially histone modification. To date, there is not report on the epigenetics to regulate trained immunity in HBV. Therefore, this study investigated the cytokines levels and expression levels of histone modification enzyme genes in HBV-exposed cord blood monocytes. Moreover, we demonstrated histone modifications at position H3K4me3 that regulated *IL-12p40, IL-6, IL-10, TNF-a*, and *IL-1b* genes in monocytes from HBV-exposed cord blood. The expression of IL-12p40 was significantly higher in HBV-exposed cord blood monocytes than healthy cord blood monocytes after restimulation with TLR8 agonist. In addition, mRNA expression of histone modification enzymes was not significantly different between HBV-exposed cord blood monocytes and cord blood monocytes. Moreover, the level of histone methylation at position H3K4me3 of cytokine genes did not support trained immunity in this study. It is likely to be that epigenetic programming through histone modification at position H3K4me3 might be not involved in HBV-trained immunity.

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Hepatitis B virus (HBV) infection is a major global health problem and has a high prevalence in Asian countries (1). There are 296 million people worldwide who have chronic HBV infection and 820,000 die each year from HBV or related diseases such as hepatocellular carcinoma (HCC) and cirrhosis (2). HBV mostly transmits from mothers to newborns during birth and delivery, as well as from contact with blood or body fluids (including tears, saliva, vaginal secretions and semen) (3). After 30-60 days, HBV infection may be detected and progresses into a chronic stage through the induction of an 'immunotolerant state' of infants (4).

To date, immune response of HBV exposed newborns *in utero* was not defective. Antiviral cytokines, such as IL12p40 and IFN-α2, were higher in HBV exposed cord blood plasma compared to cord blood plasma from healthy mothers (5). Th1 maturation increased in HBV-exposed cord blood and newborn immune cells of HBV-exposed cord blood could be activated by unrelated microbes (*uropathogenic Escherichia coli* (UPEC), *Salmonella typhimurium*, *Acinetobacter baumanii* and *Listeria monocytogenes)* and showed stronger response after secondary stimulation compared to healthy cord blood cells (5). This phenomenon is called trained immunity.

Trained immunity is regulated by epigenetic programmings such as histone modification that can be specifically bound by recognition domains that are composed of different protein implicated in transcriptional control (6). Many recent studies investigate epigenetic modification in trained immunity. A component of *Candida albicans,* ß-glucan, can induce trained immunity through epigenetic rewiring in monocytes by altering trimethylation of histone H3 on lysine-4 (H3K4me3) (7). The production of pro-inflammatory cytokine results from epigenetic reprogramming that enhanced immune response upon secondary stimulation (8). Moreover, trained

immunity does not only display trained or enhanced immunity, but also tolerized immunity or immune-paralysis upon induction by LPS or TNF as well (9).

Currently, data about epigenetic reprogramming in trained immunity in HBV is not shown information. Therefore, this study will investigate trained immunity in HBV-exposed newborn *in utero* and validate genes *IL-6, IL-1ß, TNF-α, IL12p40* and *IFN-γ* genes that are regulated by histone methylation at H3K4me3 in human monocytes from cord blood of chronic HBV-infected mothers.

The results from this study will help to understand the pathogenesis of HBV immunity and help to develop new generations of vaccine.

RESEARCH OBJECTIVE

To study histone methylation at position H3K4me3 that may regulate trained immunity in cord blood monocytes from healthy mothers and infected with HBV mothers *in vitro*

CHAPTER II

LITERATURE REVIEW

Hepatitis B virus (HBV)

Hepatitis B virus (HBV), a small double-stranded DNA virus, is classified within the Hepadnaviridae family and can be divided into two genera, which are *Orthohepadnavirus* and *Avihepadnavirus* (10). There are ten genotypes (A-J) that are well-known genotypes of HBV genome (11). Despite an effective preventive vaccine, it is estimated that 300 million people worldwides will have hepatitis B virus (HBV), with Asia and Africa having the highest frequency (12). In 2015, there are patients living with chronic HBV infection in the world accounting for 296 million people (2). HBV and HCV infected patients who were not treated can progress into having to hepatocellular carcinoma (HCC) (470,000 deaths per year) and cirrhosis (720,000 deaths per year) (13). Ordinarily, HBV transmits from mother to newborn during birth and delivery, as well as contact with blood or other body fluids with an infected partner in adulthood (2). The main routes of HBV transmission are vertical transmission (from mother to child) and horizontal (early childhood) transmission (13). The highest risk of chronic HBV development is found in children. After infection with HBV within 30-60 days, HBV may be detected and it then progresses into a chronic stage through the induction of an 'immunotolerant state' of infants or in some cases can have spontaneous clearance of the virus (4, 13).

HBV diagnosis

Viral biomarkers, the essential tools for monitoring chronic hepatitis B virus (HBV) with or without treatment and the diagnosis of acute and chronic HBV, can be divided into (i.) serological markers (antigen and antibody detection and hepatitis B surface antigen quantification, hepatitis B early antigen quantification, hepatitis B core related antigen quantification) and (ii.) molecular markers (nucleic acid testing) (14, 15).

Serological diagnosis for HBV

Hepatitis B Surface Antigen (HBsAg) Quantification, the first serological marker to appear in active infection or acute HBV and chronic HBV infection (persistence for more than 6 months), can be measured in the serum of HBV-infected patients. There are three proteins of HBsAg, which are a small protein (sHBsAg), a middle protein (mHBsAg) and a large protein (lHBsAg) (Figure 1) (14-17). The level of HBsAg is controlled by immune system activity. If there are low levels of HBsAg, this will make the immune system function better. In contrast, if there are high levels of HBsAg, immune system cannot control or inactive (16).

Hepatitis B Early Antigen (HBeAg), a small polypeptide, is not required for viral DNA replication, but indicates active viral replication and HBV infectivity (Figure 1) (18). After the detection of HBsAg in the serum, levels of HBsAg and HBeAg increase quickly, and increased HBsAg and HBeAg levels show viral replication. Chronic HBV infection phase is determined by anti-HBe antibodies and the level of viral replication (the appearance of anti-HBe can indicate) (19).

Hepatitis B Core Related Antigen (HBcAg or HBcrAg), the nucleocapsid protein, is detected in liver tissues in both acute and chronic HBV-infected patients (Figure 1) (20). This marker will rise in patients positive for HBeAg when compared to patients who are negative for HBeAg (14).

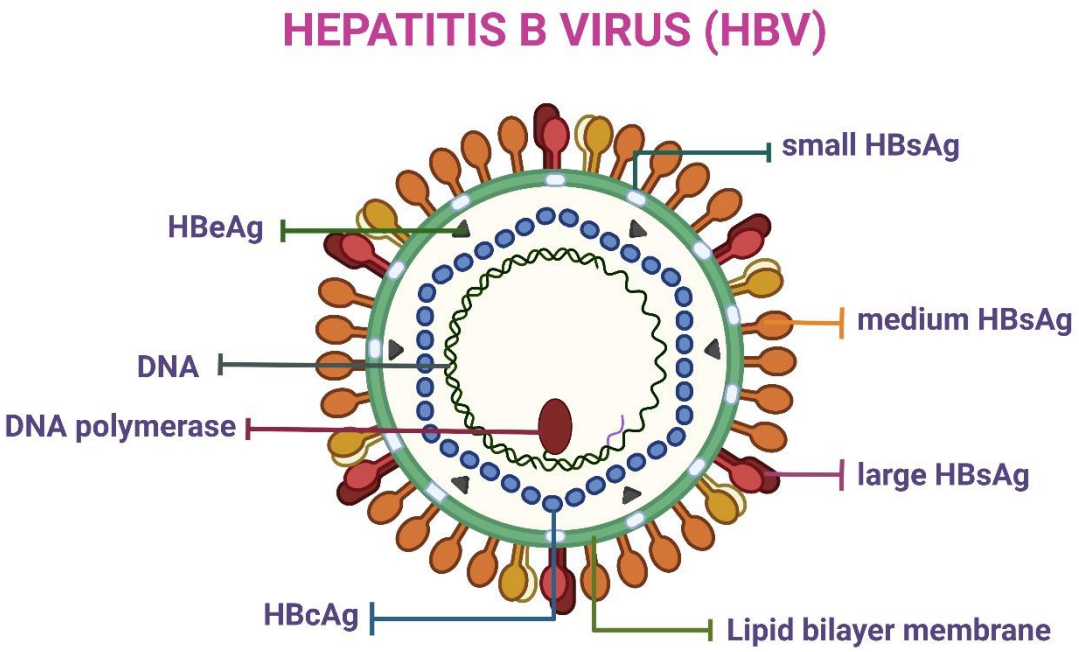


Figure 1. Hepatitis B virus (HBV)
(created with biorender.com)

Natural course of chronic HBV infection

Chronic hepatitis B is diagnosed based on the existence of HBsAg for at least six months (21). It is important to note that chronic hepatitis B is a dynamic disease and patients with it can shift between various clinical phases with varying levels of serum ALT activation, HBV DNA and HBV antigens (21). Decisions regarding the start of treatment as well as the effectiveness of treatment are influenced by the levels of serum ALT and HBV DNA as well as liver fibrosis, which are significant predictors of long-term outcomes (21). The levels of HBV DNA, HBeAg, alanine aminotransferase (ALT) values, and the presence or absence of liver inflammation have all been used to divide chronic HBV infection into five phases that are (i.) the immune-tolerant phase, (ii.) the immune reactive phase, (iii.) the inactive chronic carrier phase, (IV.) the immune reactivation phase, and (V.) HBsAg-negative phase (Table 1.) (21, 22).

The age at with individuals contract HBV infection is a major factor in chronic infection and 90% of infected infants and neonates develop chronic infection (23). Children who are vertically infected typically are asymptomatic and anicteric, but acute HBV infections can occasionally cause serious symptoms and fulminant hepatitis in both adults and children (23). Moreover, Chronic HBV infection can cause liver disease and complications (cirrhosis and hepatocellular carcinoma, which typically show in adults), as well as extrahepatic manifestations, which can also occur in infants and young children (23).

Immune-tolerant phase (HBeAg-positive chronic HBV infection)

This phase is characterized by the persistance of serum HBeAg, HBV DNA (at very high levels), and ALT consistently it within normal range (upper limit of normal [ULN] approximately 40 IU/L]) (22, 24). Although there is little to no liver necroinflammation or fibrosis in the liver, there is a high degree of HBV DNA integration and clonal hepatocyte expansion, which may indicate that hepatocarcinogenesis has already started during this early phase of infection (22).

When newborns contract HBV through mother-to child transmission (MTCT), the initial immunotolerant phase is typically discovered after neonatal infection (25). Because this infection is normally asymptomatic and anicteric, it typically goes undetected unless screening is done (25). There is minimal hepatic inflammation during this phase and most patients have normal to slightly raised serum alanine aminotransferase levels (ALT). The virus is also actively replicating, with high serum HBV DNA levels and positive HBeAg status. 90% or more of the subjects who contract HBV-infected individual during infancy and after birth go on to develop into chronic infection (26).

Immune reactive phase (HBeAg-positive chronic hepatitis B)

A phase of HBeAg-positive chronic HBV known as the immune clearance phase or Immune reactive phase is defined by the presence of serum HBeAg, persistent elevations of ALT level, and high amounts of HBV DNA although not as high as in the immunological tolerant phase of chronic HBV (27, 28). A moderate to severe liver necroinflammation and an accelerated fibrosis progression are present in the liver (22). Most patients are able to reach the HBeAg-negative infection phase after achieving HBeAg seroconversion and HBV DNA suppression (22).

Inactive chronic carrier phase (HBeAg-negative chronic HBV infection)

Clinical remission of chronic HBV and entry into the "immune control" phase, also known as the "inactive carrier state," are indicated by elimination of HBeAg, the generation of serum antibodies to HBeAg (anti-HBe or HBeAb), the normalization of ALT according to normal cut-off values (ULN approximately 40 IU/L), the lowering of HBV DNA levels (<2,000 IU/ml) to undetectable or very low levels, low hepatic necroinflammatory activity, and minimal fibrosis (22, 27).

In this phase, patients have a low chance of progressing to cirrhosis or HCC, but progression to chronic hepatitis B, typically in HBeAg-negative patients, is possible. These individuals typically have low serum HBsAg levels (<1,000 IU/ml) (22).

Immune reactivation phase (HBeAg negative chronic hepatitis B)

The reactivation phase is characterized by the absence of HBeAg, typically with measurable anti-HBe, as well as by persistent or fluctuating moderate to high levels of HBV DNA (usually lower than in HBeAg-positive

individuals) and fluctuating or consistently raised ALT levels. Moreover, inflammation and fibrosis are noticeable in the liver histopathology (22, 28).

HBsAg-negative phase (occult HBV infection)

Negative HBsAg, positive HBcAg (anti-HBc) antibodies, and with or without detectable HBsAg antibodies (anti-HBs) are features of the HBsAg-negative phase. The ALT levels of patients in this phase are normal and HBV DNA levels are usually undetectable (but not always). In the liver, HBV DNA is commonly found. Loss of HBsAg prior to the onset of cirrhosis is linked to a reduced chance of HCC and an increased rate of survival (22). However, patients still run the risk of developing HCC if cirrhosis sets in prior to HBsAg loss, so HCC surveillance should persist (22).

Vertical transmission of hepatitis B virus

Mother-to-child transmission (MTCT) during pregnancy is the main mode of infection and causes an extraordinarily high rate of chronic infection. The majority of chronic HBV carriers are the results of MTCT (26). As a result, children of HBV carrier mothers who are infected become a source of infection for future horizontal infections in society, and female carriers also pass the virus further on to their own children (vertical transmission) (26).

Vertical transmission can happen at any point during pregnancy (intrauterine, peripartum, or postpartum, from *in utero* to breastfeeding). Vertical transmission is more likely to occur under all circumstances that result in maternal-fetal microtransfusions of blood from HBV-infected mothers. Microtransfusions may take place intrauterinally, throughout labor, or during birth. Maternal microtransfusions may be linked to placental leakages brought on by threatened preterm birth or abortion, chorionic villus sampling or amniocentesis, or extended uterine contractions (29).

A major risk factor for vertical transmission of HBV is maternal viraemia, which can be detected through the detection of HBV DNA or through the positivity of its surrogate markers: HBsAg and HBeAg (29). However, the detection of vertical transmission may be impacted by the transplacental transfer of maternal antibodies. The rate of infants with serological markers for HBV that are positive has decreased over time (30). The positive rates of HBsAg and/or HBV DNA were demonstrated to be comparable among infants at the ages of 6, 7, and 12 months and network meta-analyses; furthermore, these rates were considerably lower than the positive rates at birth (30). Additionally, hepatitis B surface antigen (HBsAg) or HBV DNA positivity in infants at 6 to 12 months of age is a sign of vertical transmission of HBV, which is defined as transmission happening during pregnancy and during the perinatal period from HBV-infected mothers to the foetus or to the child, respectively (29, 30). In general, vertical transmission of HBV is a highly effective, with a range of 70% to 90% for mothers who are HBeAg positive and 10% to 40% for mothers who are HBeAg negative in the absence of any preventive treatment (29).

Table 1. The evaluation of patients with a chronic HBV infection

	Immune tolerant	**Immune reactive HBeAg positive**	**Inactive chronic carrier**	**HBeAg negative chronic hepatitis**	**HBsAg-negative phase**
HBsAg	High	High/intermediate	Low	Intermediate	Negative
HBeAg	Positive	Positive	Negative	Negative	Negative
HBV DNA	> 10^7 IU/ml	10^4-10^7 IU/ml	<2,000 IU/ml	>2,000 IU/ml	undetectable (but not always)
ALT	Normal	Elevated	Normal	Elevated	Normal
Liver disease	None/ minimal	Moderate/severe	None	Moderate/severe	None

Immune responses to HBV infection

It is generally known that the progression of chronic hepatitis B infection is caused by failure of the host's immune system to clear the virus (18).

Innate immune responses in HBV infection

Innate immunity is the first immunity that defends our body from viral infections (including HBV) by viral recognition with surface or intracellular expressed PRRs and produce inflammatory cytokines or antiviral cytokines. The activity of many viral proteins allows HBV to actively dampen the innate immunity rather than evading it (31). The PRRs can recognize HBV by TLR-2 binding to HBV proteins, RIG-I binding to the epsilon stem loop of the HBV pre-genomic RNA, cytosolic DNA sensor (CDS) detecting HBV DNA and other PRRs (32-35). The human liver, a site of HBV replication is enriched with innate immune cells, for instance NK cells, Kupffer cells (liver-specific macrophages) and others (36).

Monocytes

Monocytes make up about 10% of mononuclear cells in human peripheral blood and are one of the main APCs (37-39). Monocytes play a role in the early phase of infection with HBV because they can clear the virus and/or dead cells and have antiviral response (37). When HBV components are exposed to monocytes, monocytes are induced robustly to produce pro-inflammatory cytokines, such as IL-6 and TNF-α (40). However, HBV can persist in the body and trigger monocytes and macrophages to secrete anti-inflammatory or immunoregulatory cytokines.

HBV could stimulate monocytes and macrophages to secrete TGF-β and IL-10 and inhibit IL-10 and tumor necrosis factor α (TNF-α) secretion from toll-like receptor (TLR2) (39, 41). In a humanized mouse model of HBV infection, HBV causes human monocytes/macrophages to differentiate into M2 macrophages, which produce IL-10 (42). Patients with persistent HBV infection have substantially greater levels of anti-inflammatory cytokines (TGF-β and IL-10), and inhibitory cell surface molecules (human leukocyte antigen (HLA)-E) and programmed death ligand 1 (PD-L1)) expressed on the monocytes than normal control subjects (43). Recently, the production of inflammatory cytokines from monocytes is induced by HBV through TLR2/MyD88/NF-KB signaling and STAT1-Ser727 phosphorylation and inhibits interferon (IFN)-α-induced stat1, stat2, and ch25h expression through the inhibition of STAT1-Tyr701 phosphorylation and in an IL-10-dependent, partially autocrine manner (39).

The study of *Boltjes A., et al.,* showed that monocytes exposed to HBsAg *in vitro* produced significant amounts of IL-6 and TNF. TLR-induced monocyte cytokine production did not differ across chronic HBV infected patients with different serum concentrations of HBsAg, HBV early antigen

(HBeAg), and HBV DNA. Importantly, patients and healthy controls produced identical amounts of cytokines in response to HBsAg, demonstrating that earlier *in vivo* exposure to the antigen had no impact on *in vitro* responses. Moreover, they also demonstrate that IL-10 can prevent HBsAg-induced monocytes from producing cytokines. In conclusion, monocytes can identify and react to HBsAg, leading to active *in vitro* production of pro-inflammatory cytokines by monocytes or monocyte-derived DC (44).

The liver resident macrophages known as Kupffer cells (KCs), which are found in the liver, are thought to play a major role in liver damage during viral hepatitis because of their pro-inflammatory activity. Kupffer cells are activated by HBV components and produce anti-inflammatory cytokines (Alternative activated macrophage or M2) e.g. IL-10, that are able to reduce liver inflammation and suppress responses of HBV-specific T cells (33). In contrast, Kupffer cells are also able to promote inflammation by producing inflammatory cytokines (Classical activated macrophage or M1) that can contribute to liver injury (45).

Adaptive immune responses in HBV infection

Adaptive immunity specifically recognizes and kills HBV that infects hepatocytes through functional maturation and expansion of B and T cell clones. In acute HBV infection, antibodies are capable to neutralize free viral particles and prevent reinfection (46). When HBV viral load is low, HBV-specific CD4+T cell response is active (47). A crucial immune cell in controlling and resolving HBV infection are HBV-specific CD8+ T cells which correlate with HBV clearance during acute infection through cytolytic and noncytolytic mechanisms (36, 48).

Viral-specific T-cell responses in chronic HBV infection are undetectable or less expressed than those in acute HBV infection (49).

The viral load and persistent antigenic exposure have an influence on the function and phenotypes of HBV-specific CD8+ T cells (48). When there are so many HBV-infected hepatocytes compared to virus-specific CTL, the comparatively ineffective one-on-one mechanism by which CTL kill their target cell may not be able to clear these viruses (48). Hepatocytes that express high HBV antigen loads cause inactivation or exhaustion of HBV-specific T cells, in which express co-inhibitory molecules such as CTLA-4 (Cytotoxic T-lymphocyte associated protein 4), HAVCR2 (Hepatitis A virus cellular receptor 2), TIM-3 and PD-1 (Programmed cell death ligand 1). Moreover, inactivated HBV-specific T cells have defective proliferation and cytokine production and lead to persist of HBV (49-52).

HBV persistence in the offspring was dependent on the mother's HBV positivity (53). In infants born to HBV-positive mothers, the increased expression of programmed death 1 (PD-1) in HBV-specific CD8+ T cells and PD-L1 in Kupffer cells led to a reduced ability of CD8+ T cells to respond to HBV. In mice and in chronic HBV patients, the T-cell exhaustion regulator PD-1 can adversely affect the CTL response to HBV. Thus, patients with persistent hepatitis B had a weaker HBV-specific CTL response (53).

It's possible that mother HBeAg conditions Kupffer cells while an infant is still in the utero. HBeAg could pass through the placenta. After birth and throughout adulthood, kupffer cells can sustain themselves without the need for blood monocyte replenishment because they mature in the fetal liver. They have a long lifespan and have the capacity to suppress HBV-specific CD8+ T cells, which may be the reason why HBV can remain in patients for decades after vertical transmission (53).

Immunity in the newborn

The characteristics of innate immunity in the neonate are different from that of an adult (Figure 2) (54). Newborns experience a variety of antigenic challenges from early life from infections, commensal and harmless environmental antigens (54). Neonatal survival depends on innate immunity. In terms of adaptive immunity, compared to adult blood, cord and neonatal peripheral blood have considerably more CXCL-8-producing T cells, but low level of IFN-γ and IL-17A-producing T cells (55). Neonates are more susceptible to infection, sepsis, brain injury and neurodevelopmental abnormalities because of immunosuppressive, tissue-protective processes. Neonatal immune responses are also significantly influenced by rapidly altering postnatal exposures to the environment and microbes, as well as through epigenetic reprogramming and innate immunological memory (54).

Monocytes and Macrophages

Through their phagocytic, antigen-presenting, and cytokine-secreting capacities, monocytes are crucial in the identification and eradication of pathogens (56). Low levels of HLA-DR and CD80 are expressed by neonatal monocytes, which impair the presentation of pathogen-derived molecules as well as other antigens (56). They also exhibit lower membrane attack complex-1 and L-selectin expression, which inhibits adherence and infiltration to inflammatory regions. Interestingly, stimulation with LPS increases TLR4 expression as well as TNF-α, IL-6, and IL-10 secretion by neonatal macrophages (57). However, downstream TLR4 signaling pathways are compromised as shown by decreased phosphorylation of NF-p65 and p38, which may explain the overall decreased cytokine responses as compared to adult cells. Moreover, the nucleotide-binding domain and leucine-rich repeat containing protein 3 (NLRP3) inflammasomes are less activated in newborn

monocytes (54). In fact, low levels of caspase-1 cause decreased pyroptosis and decreased release of active IL-1 after NLRP3 stimulation (54).

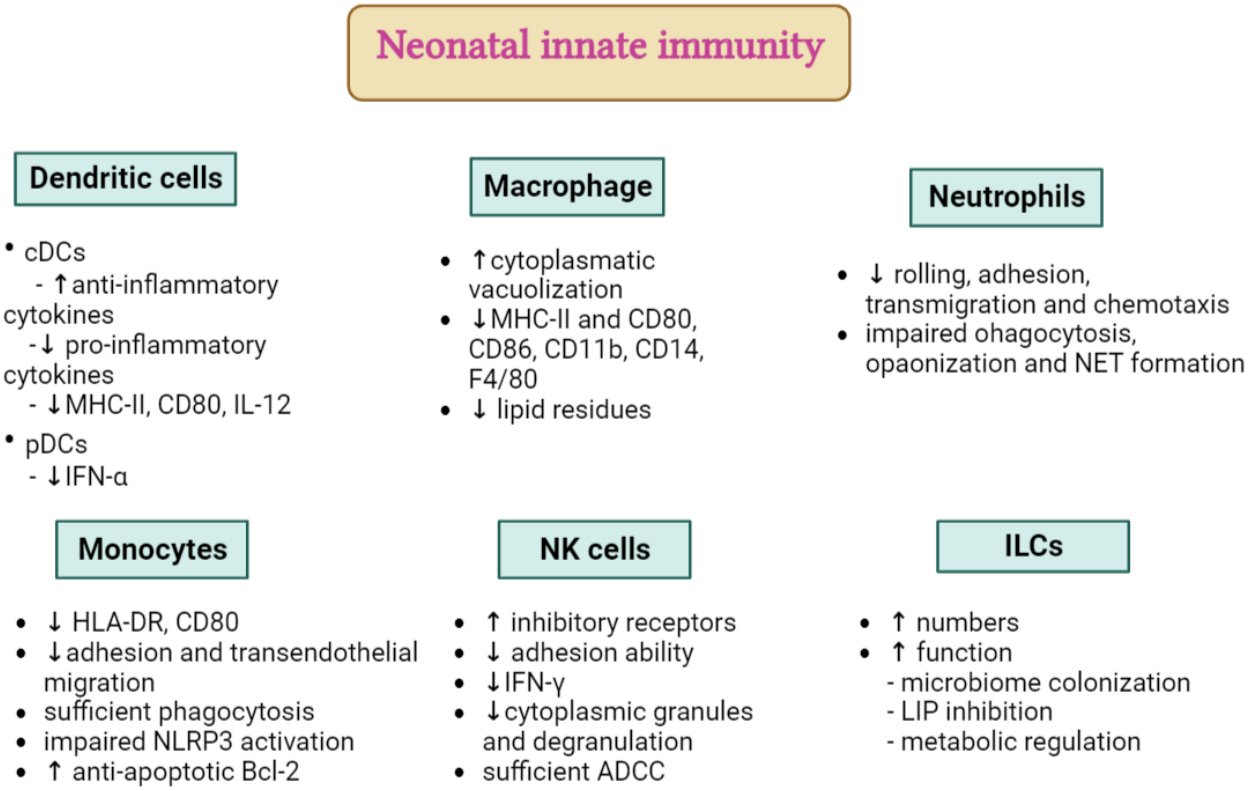


Figure 2. Innate immune responses in neonates (created with biorender.com)

Innate immune memory

When innate immune cells are stimulated for the initial time, they are reprogrammed, which can lead to an increase in effector function upon secondary stimulation (Figure 3), known as trained immunity, or a repress in effector function upon secondary stimulation, known as tolerance (58). Both trained immunity and tolerance are innate immune memory phenotypes that are primarily mediated by the same molecular processes (metabolic and epigenetic reprogramming), though they have different functional outcomes based on the initial stimulus (58). Human trained immunity has an immunological phenotype that lasts for months to

at least a year, but previous research found that vaccination in neonates can have nonspecific effects (After vaccination, innate immune cell, such as monocytes, can be enhanced response by secreting different proinflammatory cytokines that were depend on what is secondary stimulation (It can the same or different from first stimulation)) that last for up to five years (59-61). Induction of trained immunity has been investigated for both microbial and nonmicrobial stimuli more recently. These studies have concentrated on myeloid cells like monocytes and macrophages, but trained immunity can also be induced in innate lymphoid populations like innate lymphoid cells (ILCs) and natural killer (NK) cells (58). Moreover, trained immunity has many features that are different from classical immunological memory or adaptive immunity (Table 2).

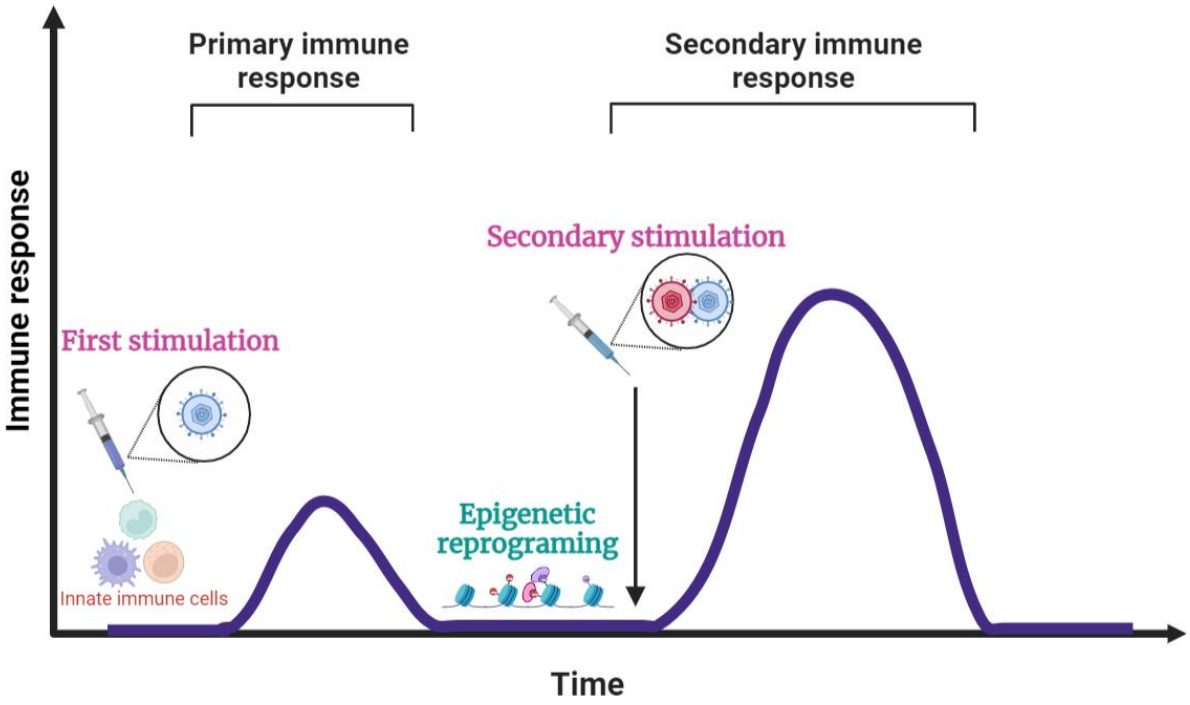


Figure 3. The character of innate immune memory (created with biorender.com)

Table 2. The distinction between innate immune memory and adaptive immune memory (62-64)

Characteristics	Innate immune memory	Adaptive immunity
Specificity	non-specific response	specific response
Cells	Monocytes, Macrophage, NK cells, Dendritic cells, Innate lymphoid cells	T-lymphocyte B-lymphocyte
Type of response	Rapid (same as primary response), either enhanced (“trained memory”) or reduced (“tolerance”)	enhanced/more potent, rapid (much more than the initial response)
Inducers	Pathogens and derived products (PAMPs)	Antigen presentation plus costimulatory signals and cytokines from DCs
Receptors	PRRs	sIg, TCR, receptors for costimulation and cytokines
Memory	Month or year	Many years
Memory mechanism	Epigenetic modifications	Clonal expansion and differentiation

There are many evidence that support trained immunity. For instance, the study of Kleinnijenhuis J., *et al.* demonstrated that Bacille Calmette-Guérin (BCG) can induce trained immunity. Severe combined immunodeficiency (SCID) mice (lack both B and T cells) were vaccinated with BCG or saline injections (control) and restimulated with a lethal dose of *C. albicans* two weeks later (59). The kidneys are the target organ of candidiasis in mice. Results showed that mice vaccinated with BCG and restimulated with *C. albicans* showed significantly higher survival rates than those that were not vaccinated with BCG prior to *C. albicans* challenge mice. These findings firmly establish the ability of BCG to elicit generalized protection against non-mycobacterial infections by functionally reprogramming innate immune cells (eg. monocytes). After that, SCID mice were vaccinated with BCG *in vivo*, the trained immunity of monocytes may also be observed by conducted a further

trial in mice that had received a BCG vaccination or PBS (control). Then, SCID mice were exposed to *C. albicans*. One week later, spleen monocytes were stimulated *in vitro* again with LPS. Mice that received BCG vaccination produced significantly more innate proinflammatory cytokines (TNF-α) when challenged with LPS induced (59).

Furthermore, the study of Quintin J., *et al.* showed trained immunity in human monocytes by priming monocytes with *Candida albicans* for 24 hours and restimulating with the same or unrelated antigen that were chitin synergizes with muramyl dipeptide (LPS and Pam3ys), *Candida albicans* and *Mycobacteria tuberculosis.* The production of proinflammatory cytokines TNF-α and IL-6 in trained monocytes were significantly higher than the untreated control (65). These results demonstrate trained immunity in monocytes.

Another study showed the increased efficiency of human monocytes when trained with chitin from various *Saccharomyces cerevisiae* strains prior to restimulating with bacteria or fungi (66). This trained immunity enhanced the production of cytokines and its level of response depending on the strain of *S. cerevisiae* and amount of chitin used to stimulate the monocytes (66).

The study by Blok B.A., *et al.* demonstrated the potential of proinflammatory cytokine responses in human monocytes stimulated with *Vaccinia* virus (family of *Poxiviridiae*) and MVA (modified *Vaccinia* Ankara) prior to rechallenging with unrelated microbial ligands. The production of pro-inflammatory cytokines (TNF-α and IL-6) monocytes enhanced upon rechallenge (67). In contrast, monocytes trained with MVA had decreased production of pro-inflammatory cytokines upon rechallenge. These results show that the *Vaccinia* and MVA induced trained immunity and innate immune tolerance, respectively (67).

There was trained immunity with enterovirus A71 (EV-A71). Macrophages were trained by enterovirus A71 (EV-A71) and rechallenged with HDM allergen. This trained

immunity showed that macrophages increased in IL-6 and TNF-α cytokine production levels (68).

ChAdOx1 nCoV-19 vaccine (produced by Astra Zeneca), an adenoviral vector COVID-19 vaccine, could induce the character of trained immunity (69). After vaccination, monocytes were isolated from human PBMCs on day –1 (prevaccine), day 14, day 56, and day 83 and were stimulated (ex vivo) with TLR agonists Pam3Csk4 or LPS for 24 hours. The production of IL-6 cytokine in vaccinated-monocytes and restimulation with TLR agonists Pam3Csk4 or LPS was significantly more than pre-vaccine controls at day 14 and day 56. Moreover, vaccinated monocytes and stimulation with LPS showed significantly reduced TNF cytokine on day 56 compared with the prevaccine (69).

Trained immunity in cord blood monocytes

The study by Hong M., *et al*. found that newborn immunity was developed when it exposed to HBV. The antiviral cytokine profile, including IL12p40 and IFN-α2, was observed in cord blood from patients exposed to HBV (Figure 4) (5). Additionally, They showed that HBV-exposed cord blood monocytes stimulated with ssRNA40 (TLR 8 agonist) for 18 hours as a secondary stimulation had significantly higher level of IL-12p40 in HBV-exposed cord blood monocytes than healthy cord blood monocytes (5).

Regulation of innate immune memory by epigenetics

The major mechanism to control innate immune memory is epigenetic programming (64). There are at least three types of epigenetic programming. These include histone modification, DNA methylation and non-coding RNAs (ncRNA) (64). Histone modification, the major of epigenetic modifications that regulate the expression of genes.

Histone modification

Histone modification is important for structure and function of chromatin. There are more than one hundred post-translational modification sites of histone. At the level of chromatin structure, gene expression can be controlled. Histone tails and histone globular domains contain many residues that can be altered in several ways, such as methylation, acetylation, ubiquitylation or phosphorylation of different amino acids such as arginine (Arg or R), serine (Ser or S), lysine (Lys or K) and other amino acid residues (70, 71). Distinct families of histone enzymatic modification target specific site of unstructured N-terminal histone tail and depending on the specific signals and conditions that a cell is exposed (71). The most studied and widely characterizes of histone modification are histone methylation and histone acetylation (71, 72). The acetylation of histone N-terminal tails is thought to promote transcriptional activation either by neutralizing the charge of the tails' interaction with DNA or by creating a binding site for transcription factors with bromodomains, some of which can remodel nucleosomes. The methylation of histone H3 lysine 4 (H3K4), a modification typically linked to transcriptionally active genes and a binding location for a number of factors including histone modifying and remodeling activities (71, 72).

Histone methylation on arginine and lysine residues relies on the enzymes lysine demethylases, lysine and arginine residues methyltransferase, arginine demethylases and arginine deiminases. Moreover, the effect of methylation status on transcription determines the degree of methylation, such as monomethylated (me1), demethylated (me2), or trimethylated (me3). For example, repression of genes is associated with trimethylated (me3) at H3K27 while the activation of transcription is linked to trimethylated at H3K4 (73). Histone acetylation involves the degree of gene expression and it is dependent upon activity of two enzymes, of which are histone deacetylases (HDACs) and histone acetyltransferases (HATs) (73).

The effects of epigenetic regulation on innate immune memory can be divided into tolerance and trained immunity. Tolerance expresses immunosuppressive or hyporesponsive phenotype. Tolerance can protect or limit inflammation that cause tissue damage. Trained immunity can cross protection and increase proinflammatory cytokine production.

The study of Kleinnijenhuis J., *et al.* demonstrated that H3K4me3 on promoters of *tlr4*, *il6* and *tnfα* genes was enriched in monocytes after vaccination with BCG upon compared with before vaccination (59). In addition, the study of Quintin J., *et al*. 2012 demonstrated histone modifications regulated trained immunity in monocytes that primed with β-glucan. The result showed an increase of H3K4me3 on promoter of target genes such as *TNF-α*, *IL-6*, *IL-8, Dectin-1, MYD88* and *TLR4.* In contrast, histone modification at H3K27me3 is not different between trained monocytes with β-glucan and nontrained with β-glucan (65). The report of Bekkering S., *et al.* elucidated training of human monocytes by oxLDL (Oxidized low-density lipoprotein) or β-glucan and observed an enriched H3K4me3 on the promoter of genes (*tnf-α*, *il-6*, *il-18*, *MCP-1*, *MMP2*, *MMP9*, *CD36,* and *sr-a* that encoded for chemokines, proinflammatory cytokines, scavenger receptors or MMPs (matrix metalloproteinases) at 6 days after stimulation (74). These correlate with enhanced proinflammatory cytokines and expression of target genes upon rechallenging of the cells. Moreover, Rizzetto L., *et al.* demonstrated training of human monocytes with *Saccharomyces cerevisiae* or chitin that involved histone methylation (66). Another research demonstrated that trained immunity with MVA (modified *Vaccinia* Ankara) induced innate immune tolerance because of epigenetic changes involving histone methylation, but not involving in H3K4me on the promoters of proinflammatory cytokines (IL-6 and TNF-α) genes (67).

Therefore, epigenetic modifications is an important mechanisms to explain trained immunity phenomenon. Recently, there are studies about trained immunity

in HBV infection but no information on epigenetic reprogramming to explain the regulated trained immunity in HBV infection (Figure 5). In this study we will investigate the regulation of trained immunity by histone modification at histone H3 on lysine-4 trimethylation (H3K4me3) and histone H3 on lysine-27 trimethylation (H3K27me3) in monocytes from HBV *in utero*-exposed cord blood of chronic HBV-infected mothers.

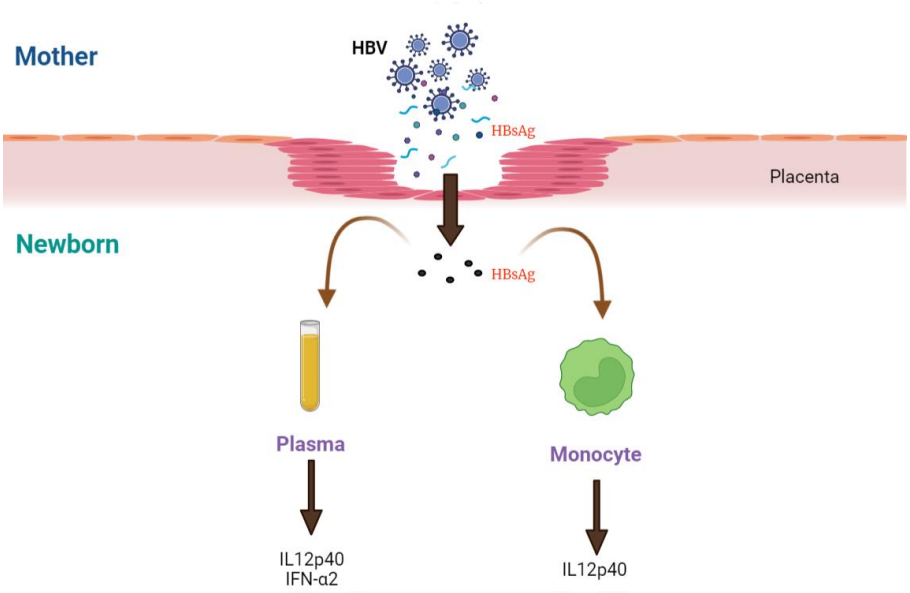


Figure 4. Induction of innate immune memory in human neonates of HBV+ mothers (created with biorender.com)

Histone modification enzymes

The histone tails can be recognized by proteins that harbor histone-binding domains that can "read," "write," or "erase" histone marks (75). Histone modification enzymes are able to add or remove a variety of various histone modifications (75). There were studied several histone-modifying enzymes, such as KDM6B (JMJD3) inhibits IL4i1 from increasing acute lung damage brought on by lipopolysaccharide

through H3K27 demethylation (76), Coronavirus-related coagulopathy and inflammation are mediated by the histone methyltransferase MLL1/KMT2A in monocytes and the absence of Kdm5a reduces mice resistance to Listeria Monocytogenes Infection (77).

CHAPTER III

MATERIALS AND METHODS

Participants

Hepatitis B virus-infected mothers and healthy pregnancy women were recruited in 10 people per group for this study.

All participants were recruited and signed in informed consent. This study was approved by the Institutional Review Board of the faculty of Medicine, Chulalongkorn University (COA No.1164/2019). Participants were categorized into two groups:

Control group

Healthy pregnancy women were recruited.

Inclusion criteria

Healthy pregnancy individuals aged between 20-45 years old with no underlying diseases.

Exclusion criteria

Individuals with pregnancy were excluded from the study if they have human immunodeficiency virus (HIV) infection, autoimmune disease, hepatitis B, co-infection with hepatitis C, decompensated liver disease, other chronic infections and malignancy. Patients were excluded if they were consuming alcohol or using intravenous drugs on a regular basis and during treatment with immunosuppressive drugs.

Patient group

Hepatitis B virus-infected mothers (HBsAg positive mothers) were recruited for this study.

Inclusion criteria

All HBV-infected mothers have positive HBsAg and abnormal serum alanine aminotransferase (ALT) (>UNL but <10XUNL) concentrations for at least six months before the beginning of the protocol

Exclusion criteria

Patients were excluded from the study if they have human immunodeficiency virus (HIV) infection, autoimmune disease, co-infection with hepatitis C, decompensated liver disease, other chronic infections and malignancy. Patients were excluded if they were consuming alcohol or using intravenous drugs on a regular basis and during treatment with immunosuppressive drugs.

Cord blood collection and PBMCs isolation

When umbilical vein was cut, cord blood from umbilical cord was immediately collected (approximate 40 ml) by syringe (50 ml) and contained in heparinized tube. Suddenly, samples were proceeded for cord blood mononuclear cells (CBMC) isolation using Ficoll-Hypaque reagent with ratio of cord blood : RPMI medium : Ficoll-Hypaque at 1 : 1 : 1. Cells were washed for two times with 10% FBS in RPMI before counting. These CBMCs were collected in liquid nitrogen container until used.

Positive selection

Monocytes were isolated from CBMCs by anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). First, CBMCs from liquid nitrogen were thawed and washed with RPMI-1640 medium mixed with 10% Fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). Cells were washed again with sorting buffer containing 1xPBS, 10% FBS and 500 mM EDTA and

centrifuged at 300 xg for 10 minutes. Secondly, these CBMCs were resuspended in appropriate volume of sorting buffer for monocyte sorting. Thirdly, 20 µL of CD14 MicroBeads per 10^7 total cells were added into suspension cells before incubating for 15 minutes in the refrigerator (2–8 ℃). Fourthly, Cells were washed by adding 1-2 ml of sort buffer per 10^7 cells, centrifuged at 300×g, 4 ˚C for 10 minutes, and aspirated supernatant completely. Next, cells were resuspended in 500 µl of sorting buffer. After that, MS Columns was applied on magnetic separator, rinsed with 500 µl of sorting buffer and applied cell suspension onto the column. After washing column for 3 times with 500 µl of sorting buffer, the column was placed on a suitable collection tube, and added 500 µl of sorting buffer. Immediately, the magnetically labeled cell was flushed out by firmly pushing the plunger into the column, and $2X10^5$ monocytes were took for testing the purity by flow cytometry. The remaining of monocytes were resuspended by RPMI-completed medium (RPMI+10%FBS+1%pen-strep) before stimulation.

Negative selection

Monocytes were isolated from CBMC by MojoSort™ Human Pan Monocyte Isolation Kit (BioLegend, San Diego, CA). Firstly, CBMCs from liquid nitrogen were thawed and washed twice with RPMI-1640 medium. Secondly, red blood cells were lysed by RBC lysis buffer that is composed of ammonium chloride. The ratios of CBMCs to lysis buffer volume of 1:9 was used and then incubated at room temperature for 3 minutes before centrifuged at 1500 rpm for 5 minutes at 25 ˚c and supernatant was removed. Thirdly, PBMCs were then resuspended with 4 ml of MojoSort™ Buffer, centrifuged at 300 xg for 5 minutes and resuspended in an appropriate volume of MojoSort™ buffer for counting. Next, 100 µl of 10^7 cells were added with 5 µL of Human TruStain FcX™ (Fc Receptor Blocking Solution) before incubating at room temperature for 10 minutes. After that, 10µL of the Biotin-Antibody Cocktail (antibody against CD3, CD7, CD15, CD19, CD20, CD56, CD57, CD123, CD235ab) were added and incubated on ice for 15 minutes before adding 10 µL of

Streptavidin Nanobeads and incubating on ice for 15 minutes. Next, cells were washed with MojoSort™ Buffer up to 4 ml and centrifuged at 300 xg for 5 minutes at 4 ˚c. Cells were suspended with 2.5 ml of MojoSort™ Buffer. Then, the tube was placed on the magnet stand for 5 minutes and poured the liquid out into a new tube for collecting the purified monocytes. Finally, $2x10^5$ monocytes were tested the purity by flow cytometry and the remaining of monocytes were resuspended by RPMI complete medium.

Monocyte stimulation

The sorted monocytes from positive selection were stimulated with 1 μg/ml of ssRNA40/LyoVec™ (InvivoGen) for 18 hours in the incubator (37˚ C). After 18 hours, cells were harvested, centrifuged at 1,500 rpm for 10 minutes at 25 ˚c, and the supernatant was collected for Luminex assay. Cells were resuspended with RPMI-completed medium and counted for ChIP. While the sorted monocytes from negative selection stimulated as above for 18 hours were determined RNA expression assay

Luminex assay

The supernatants from cell culture were detected cytokines that were IL12p40, IFN-α2, IFN-γ, IL-1β, IL-10, IL-6, IL-12 (p70), and TNF-α. The 96-well plate for Luminex was added with 50 μl of washed beads with 100 μl buffer of two times. Next, the 50 μl of standards, samples, and controls were added to the plate and incubated on a shaker at 850 rpm for 30 minutes at room temperature. Then, the plate was washed with 100 μl of buffer (3 times), added 25 μl of 1X detection antibody, and incubated on a shaker at 850 rpm for 30 minutes at room temperature before washing with buffer 3 times again. After washing, 50 μl of 1X streptavidin PE were added to the plate, and incubated this plate on a shaker at 850 rpm for 10 minutes at room temperature. After that, this plate was washed 3 times with buffer,

resuspended in 125 µl of assay buffer, and incubated on shaker at 850 rpm for 30 sec at room temperature. Finally, this plate took to Luminex instrument for analysis.

RNA isolation

Firstly, stimulated monocytes were collected. Secondly, RLT buffer and 70% ethanol were added to the lysate and mixed by pipetting. Thirdly, the samples were transferred to an Rneasy Mini spin column placed in a collection tube, centrifuged, and discarded the flow-through. Next, RW1 buffer was added to the column, centrifuged, discarded the liquid and PRE buffer were also added to the column (times), centrifuged, and discarded the liquid. Then, the column was placed in a new 1.5 ml collection tube and added 30 µl of Rnase-free water and centrifuged. Finally, RNA was kept in -80˚C for farther assay.

cDNA Synthesis

Mastermix for cDNA synthesis was prepared (Table 3).

Table 3. Mastermix for cDNA Synthesis

Reagent	Volume per reaction (µl)
5X iScript™ Reverse Transcription Supermix for RT-qPCR	4
RNA template (250 ng total RNA)	5 (or variable)
Nuclease free water	11 (or variable)
Total	20

Quantitative PCR (qPCR) assay by Taqman probe

After cDNA synthesis, 2 µl of cDNA were used for real-time PCR per reaction. The components of the mastermix were TaqMan® Fast Advanced Master Mix(2X), TaqMan® Assay(20x), and nuclease-free water (Table 4).

Table 4. The components of the mastermix for real-time PCR

Reagent	Volume per reaction (µl)
TaqMan™ Fast Advanced Master Mix	10
TaqMan® Assay (20X)	1
Nuclease free water	7
Total	18

Chromatin immunoprecipitation (ChIP) assay by CUT&RUN (Cleavage Under Targets and Release Using Nuclease)

CUT&RUN Assay Kit (Cell Signaling Technology Inc., Danvers, MA, USA) was used for Chromatin immunoprecipitation (ChIP). There were six main steps.

The first step was a cell preparation. Monocytes were collected after culturing for 18 hr and fixed with formaldehyde. 2.7 µl of formaldehyde (37%) added to a tube containing 1 ml medium to get 0.1% the final concentration of formaldehyde and swirled briefly before incubating for 2 minutes at room temperature. Next, 100 µl of glycine solution (10X) per 1 ml of fixed cell suspension was added and swirled to mix before incubating for 5 minutes at room temperature. After centrifuging the cell suspension for 3 min at 3,000 x g at 4°C, the cell pellet was washed twice in 1 ml of 1X wash buffer plus spermidine and PIC by gently pipetting up and down, centrifuged for 3 min at 3,000 x g at 4°C. After that, each reaction or input sample were resuspended 100 µl of 1X wash buffer plus spermidine and PIC by gently pipetting up and down. Finally, 100 µl of cell suspension were transferred to a new tube and stored at 4°C for using as the input sample. The remaining cells were proceeded to the next step.

The second step was the binding of concanavalin A beads and primary antibody. Activated-concanavalin A magnetic bead suspension was prepared by gently pipetting up and down of concanavalin A magnetic beads, and transferring 10 µl of the bead suspension per each reaction to a new 1.5 ml microcentrifuge tube.

Concanavalin A bead activation buffer (100 µl) was added and gently mixed beads by pipetting up and down. Next, the tube was placed on a magnetic rack until solution becomes clear for 2 mins and then the liquid was discarded before removing tube from the magnetic rack. The beads were washed a second time and added a volume of concanavalin A bead activation buffer equal to the initial volume of bead suspension to the bead.

The 10 µl activated bead suspension per reaction were added to the remaining cells from the first step. Sample tubes were rotated for 5 min at room temperature, briefly centrifuged at 100 x g for 2 sec, and placed on the magnetic rack until the solution turned clear for 2 mins. Then the liquid was discarded, the tubes were removed from the stand, added 100 µl of antibody binding buffer plus spermidine, PIC and digitonin per reaction, and placed on ice. After that, the cell bead suspension was aliquoted 100 µl into separate 1.5 ml tubes and placed on ice before adding H3K4me3 Rabbit mAb per reaction and mixing gently by pipetting up and down. These samples were rotated at 4°C, overnight.

The third step was binding of pAG-MNase enzyme. After the second step, the sample tube was brought up and briefly centrifuged at 100 x g for 2 sec. The tubes were placed on the magnetic rack until the solution turned clear for 2 mins, removed the liquid, transferred from the magnetic rack, added digitonin buffer plus spermidine, PIC and digitonin (1 ml), resuspended beads by gently pipetting up and down (make sure to collect all beads that are stuck to the tube wall), placed the tubes on the magnetic rack until the solution turned clear for 2 mins, and then removed the liquid before removing tubes from magnetic rack, respectively. Each tube was added 50 µl of pAG-MNase pre-mix, and gently mixed the sample by pipetting up and down. These tubes were taken to rotate at 4°C for 1 hr, and immediately were proceed to the next step.

The fourth step was DNA digestion and diffusion. The tubes were taken from rotation at the third step, briefly centrifuged at 100 x g for 2 sec, placed on the

magnetic separation rack until the solution turned clear for 2 mins, removed the liquid, taken tubes from the magnetic separation rack, added digitonin buffer plus spermidine, PIC and digitonin (1 ml), and resuspended beads by gently pipetting up and down (repeated these step again for washing with digitonin buffer). Next, the tubes were placed on the magnetic rack until the solution turned clear for 2 mins, moved the liquid, transferred tubes from magnetic rack, added digitonin buffer plus spermidine, PIC and digitonin (150 µl) to each tube and mixd by pipetting up and down, and placed tubes on ice for 5 min before digestion.

pAG-MNase was activated by adding 3 µl of cold calcium chloride to each tube and mixing by pipetting up and down, incubated samples at 4°C for 30 min, added 150 µl of 1X stop buffer plus digitonin, RNAse A and spike-in DNA to each sample and mixed by pipetting up and down, incubated the tubes at 37°C for 10 min, centrifuged at 4°C for 2 min at 16,000x g, and placed the tubes on a magnetic rack until the solution was clear for 2 mins. After that, the supernatants were transferred to new 2 ml microcentrifuge tubes (these are your enriched chromatin samples), allowed samples to warm (room temperature) and added 3 µl of 10% SDS solution (0.1% final concentration) and 2 µl of proteinase K (20 mg/ml) to each sample. Finally, each sample was vortexed and incubated at 65°C for at least 2 hr. After incubation, the samples were quickly spined at 10,000x g for 1 sec and equilibrated samples to room temperature for processing DNA purification step.

The fifth step was the preparation of the input sample. Each input sample tube from the first step was added 200 µl of DNA extraction buffer (+ Proteinase K + RNAse A), mixed by pipetting up and down, placed at 55°C for 1 hr with shaking (1200 rpm), placed on ice for 5 mins to completely cool the samples, and lysed the cells and fragmented the chromatin by sonicating. The sonication conditions were 25 %: pulse in 20s, pulse off 30s for 1 min (for 400,000 cells per reaction), and 29%: pulse in 20s, pulse off 30s for 1 min (for 500,000 cells per reaction). After sonicating, the samples were clarified lysates by centrifugation at 18,500 x g for 10 min at 4°C,

removed supernatant to a new 2 ml microcentrifuge tube, and immediately continued to DNA purification.

The sixth step was DNA purification by using spin columns. Each DNA sample was added with 750 µl of DNA binding buffer, and vortexed briefly. 450 µl of each sample were transferred to a DNA spin column in a collection tube and centrifuged at 18,500 x g in a microcentrifuge for 30 seconds. The spin column from the collection tube was removed, and discarded the liquid before replacing the spin column in the collection tube again. The remaining 450 µl of each sample were transferred to the spin column (repeated centrifugation steps) and then added 750 µl of DNA wash buffer to the spin column. Next, each sample was centrifuged at 18,500xg in a microcentrifuge for 30 seconds, and removed the spin column from the collection tube before discarding the liquid and replacing the spin column in the collection tube (repeat this step). The spin column was placed into a clean 1.5 ml microcentrifuge tube and 15 µl of DNA elution buffer were added to each spin column. To elute DNA, each spin column was centrifuged at 18,500 x g in a microcentrifuge for 30 seconds and the purified DNA was continued to qPCR.

Quantitative PCR (qPCR) assay

The interesting genes that are *IL12p40, IL-6, IL-10, IFN-α2, TNF-α*, and *IL-1β* were chosen for validation by qPCR. The master mix of qPCR reaction was prepared. The components of the master mix were Nuclease free water, PowerUp™ SYBR™ Green Master Mix and primer (IL12p40, IL-6, IL-10, IFN-α2, TNF-α, and IL-1β) (Table5). 9 µl of master mix and 1 µl DNA from CUT&RUN assay were added into each well of real time PCR plate. These real time PCR products were detected and analyzed by Qstudio6 Flex Real Time PCR machine.

Table 5. The components of the master mix for real-time PCR

Reagent	Volume per reaction (µl)
PowerUp™ SYBR™ Green Master Mix	5
10µM forward primer	0.25
10µM reverse primer	0.25
Nuclease free water	3.5
Total	9

Table 6. Primer sequences used for qPCR (78-80).

Gene	Forward Primer	Reverse Primer
IL12p40	5'-CCCTCCTCGTTATTGATACACAC-3'	5'-GCTTGGGAAGTGCTTACCTTG-3'
IL-6	5'-TCGTGCATGACTTCAGCTTT-3'	5'-GCGCTAAGAAGCAGAACCAC-3'
IL-10	5'-TAGAGAAGGAGGAGCTCTAAGCAG-3'	5'-AGGGAGGCCTCTTCATTCA-3'
IFN-α2	5'-GGTGAGAAAAACAGCTGAAAACCCA-3'	5'- GACCTTGCTTTGTGCCTAGCCTT-3'
TNF-α	5'-CAGGCAGGTTCTCTTCCTCT-3'	5'-GCTTTCAGTGCTCATGGTGT-3'
IL-1β	5'-GGTGAGAAAAACAGCTGAAAACCCA-3'	5'-GACCTTGCTTTGTGCCTAGCCTT-3'

CHAPTER IV

RESULTS

1. The data of samples for this experiment

The cord blood samples of healthy and patients were collected. 6 patients and 5 healthy donors were used in experiment of cytokine profile assay and expression levels of histone modification enzyme genes. The clinical data and demographic characteristics of these participants were showed in table 7. Moreover, 5 patients and 3 healthy controls were recruited for CUT&RUN assay to determine the effect of histone methylation H3K4me3 on genes of interest. We showed their clinical and demographic data in Table 8.

Table 7. Clinical and demographic characteristics of study group for cytokine profile assay and histone modification enzymes

Sample ID	Clinical diagnostic	Age	HBsAg	HBeAg	Viral load (IU/ml)
CB-HBV 10	HBV	36	Positive	-	-
CB-HBV 15	HBV	34	Positive	-	-
CB-HBV 17	HBV	34	Positive-	-	-
CB-HBV 18	HBV	30	Positive	-	-
CB-HBV 19	HBV	29	Positive	-	-
CB-HBV 22	HBV	31	Positive	Negative	-
CB-NOR 06	Healthy	24	Negative	-	273
CB-NOR 07	Healthy	36	Negative	Negative	-
CB-NOR 08	Healthy	40	Negative	-	-
CB-NOR 12	Healthy	41	Negative	-	-
CB-NOR 18	Healthy	27	Negative	-	-

Table 8. Clinical and demographic characteristics of study group for Chromatin Immunoprecipitation (ChIP) assay

Sample ID	Clinical diagnostic	Age	HBsAg	HBeAg	Viral load (IU/ml)
CB-HBV 30	HBV	42	Positive	Negative	81
CB-HBV 31	HBV	30	Positive	Positive	<10
CB-HBV 32	HBV	42	Positive	Negative	73
CB-HBV 33	HBV	40	Positive	Negative	<10
CB-HBV 34	HBV	21	Positive	Negative	<10
CB-NOR 32	Healthy	25	Negative	-	-
CB-NOR 34	Healthy	27	Negative	-	-
CB-NOR 39	Healthy	42	Negative	-	-

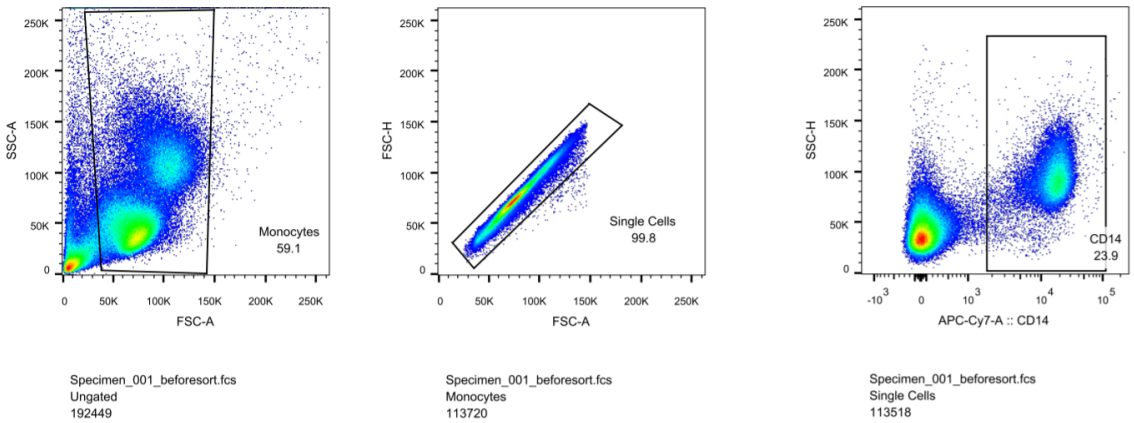
2. The cytokine profiles of HBV-exposed cord blood monocytes after stimulation with ssRNA40 (TLR8 agonist)

The previous report showed that IL-12p40 in cord blood plasma and stimulated cord blood monocytes of HBV-infected mothers was significantly higher than in healthy, which also indicated trained immunity to unrelated pathogens in HBV-infected mothers (5). We would like to validate this cytokine production in our experiment.

The healthy and HBV patients were used for cytokine profile detection are shown in table 7. All cord blood monocytes from HBV patients and healthy controls were sorted by Pan monocyte isolation kit which is a negative selection sorting. The purity of cord blood monocytes after sorting was investigated by CD14 staining and detected by flow cytometry. The purity of monocytes has range of 85.8-93.2 and average was 90.3. A representative of monocyte purity was shown in Figure 5. The sorted cord blood monocytes of HBV-infected mothers after stimulation with or without ssRNA40 (TLR8 agonist) for 18 hours were

evaluated the levels of cytokines (IL-12p40, TNF-α, IFN-γ, IL-10, IL-2, IL-13, and IL-4) by Luminex assay compared to healthy group. The levels of IL-12p40 from cultured supernatant were significantly higher in HBV-exposed cord blood monocytes after stimulation than in healthy cord blood monocytes (Figure 6). This our result can repeat previous data (5) and refers that HBV-exposed cord blood monocytes in our samples is able to induce trained immunity. Moreover, we also tested the other cytokines (TNF-α, IFN-γ, IL-10, IL-2, IL-13, and IL-4) from cord blood monocytes after stimulation with or without ssRNA40 (TLR8 agonist) for 18 hours. The trend of TNF-α, IFN-γ, IL-10, IL-2, IL-13, and IL-4 cytokines production were higher in HBV-exposed cord blood monocytes than in healthy cord blood monocytes after stimulation, although it was not statistically significance (Figure 6). In unstimulated condition, there was no difference between healthy and HBV-exposed cord blood monocytes (Figure 6).

A.



B.

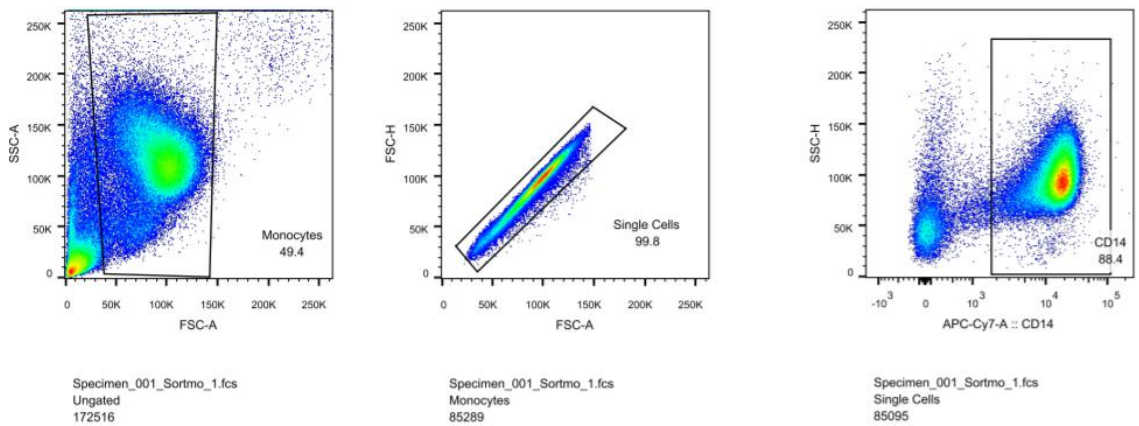
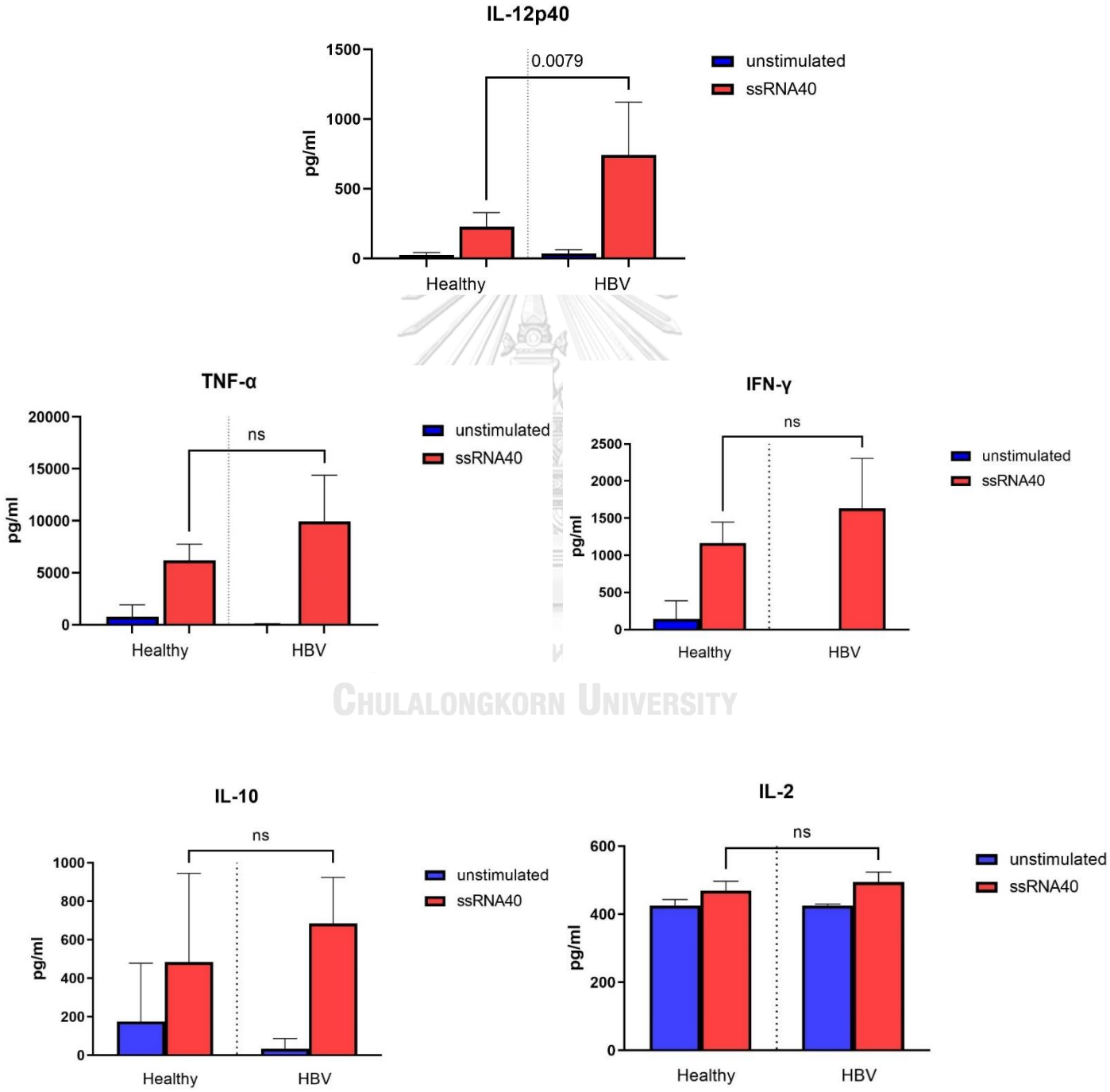


Figure 5. Gating is shown for negative monocyte isolation. (A) Cord blood mononuclear cells before monocyte sorting, (B) sorted monocytes by negative selection (after sorting) were determined the purity by staining with CD14 antibody and investigated using flow cytometry.



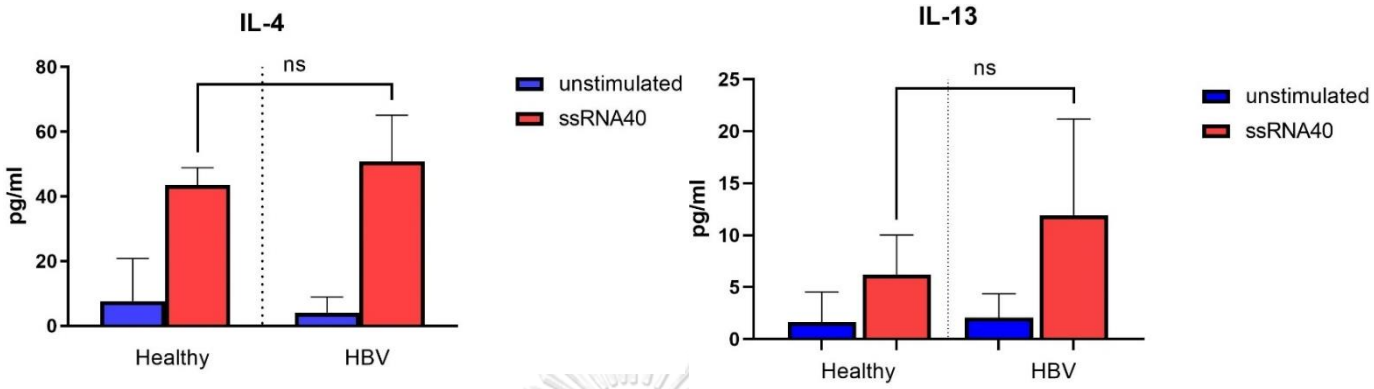


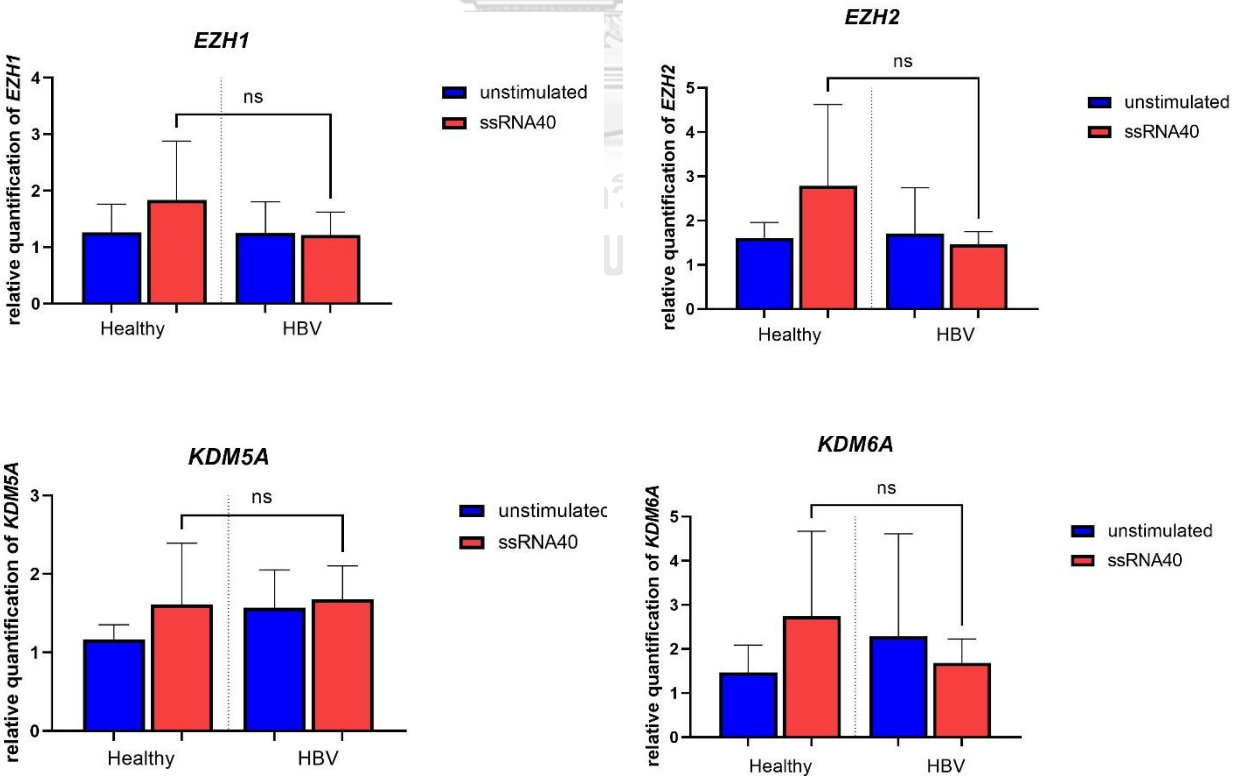
Figure 6 The bars demonstrated that IL-12p40, TNF-α, IFN-γ, IL-10, IL-2, IL-13, and IL-4 levels of monocytes from HBV-infected cord blood mothers and healthy donors after stimulation with ssRNA40 (TLR8 agonist) were determined by Luminex. Mann–Whitney test was used for statistical analysis and p-value<0.05 was considered significantly different.

3. The mRNA expression levels of histone modification enzymes

Histone modifying enzymes play a role in the epigenetic regulation of critical cellular processes by post-translationally altering histone and non-histone substrates. Therefore, histone modification enzymes in HBV-exposed cord blood monocytes and healthy cord blood monocytes after restimulation with ssRNA40 (TLR8) were examined for understanding the enzymatic machinery mediating addition or removal of trimethylation at H3K4 and H3K27 marks. We chose the candidate genes that were *EZH1, EZH2, KDM5A, KDM6A, KDM6B,* and *KMT2A*. The function of *EZH1* and *EZH2* is to transfer of methyl groups to H3K27. Both KDM6A (UTX) and KDM6B (JMJD3) play a role for removing methyl groups from H3K27. The function of *KMT2A* (MLL) is transfer of methyl groups to H3K4, and the function of *KDM5A* (JARID1A, RBBP2) is to remove methyl groups from H3K4. *EZH1, EZH2*, and *KDM5A* involve in suppressing

gene expression and *KDM6A, KDM6B*, and *KMT2A* involve in activating gene transcription (81, 82).

HBV-exposed cord blood monocytes and healthy cord blood monocytes were stimulated with or without ssRNA40 for 18 hours. The mRNA expression levels of histone modification enzymes (*EZH1, EZH2, KDM5A, KDM6A, KDM6B,* and *KMT2A*) were evaluated by real-time PCR technique. The result demonstrated that the expression levels of *EZH1, EZH2, KDM5A, KDM6A, KDM6B, and KMT2A* of HBV-exposed cord blood monocytes stimulated with ssRNA40 were not significant difference compared to healthy cord blood monocytes (Figure 7). However, there is a trend of higher expression of KDM6B and lower expression of *EZH1*, *EZH2, KDM6A,* and *KMT2A* in HBV-exposed cord blood monocytes than healthy controls after stimulation with TLR8 agonist. In addition, there were the trend of higher expression of *KDM6A, and KMT2A* in HBV-exposed cord blood monocytes than healthy controls in unstimulated condition, although it was not statistically significance (Figure 7).



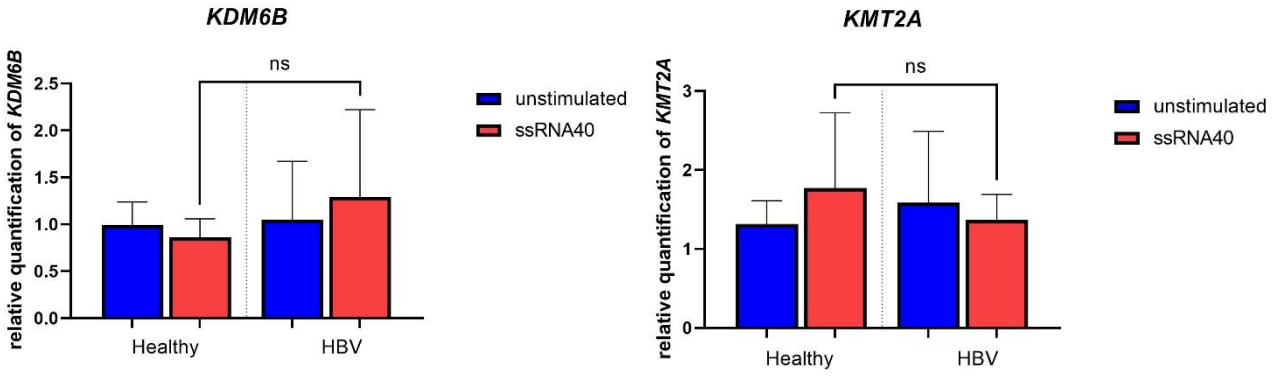


Figure 7. The Bars demonstrated that the mRNA expression levels of histone modification enzymes (EZH1, EZH2, KDM5A, KDM6A, KDM6B, and KMT2A) were quantitatively determined by Taqman probe real-time PCR. Man–Whitney test was used for statistical analysis and p-value<0.05 is significantly different.

4. The effect of histone methylation H3K4me3 on genes of interest

To investigate the effect of histone modification H3K4me3 on candidate genes in HBV-exposed cord blood monocytes, we firstly evaluated cytokine production from stimulated monocytes because we changed technique of monocyte sorting from negative selection to positive selection to get more purity (Figure 8).

4.1 Cytokine profiles of HBV-exposed cord blood monocytes

5 HBV-infected mothers and 3 healthy controls were used for this assay (Table 8). The cord blood monocytes from all these subjects were sorted by CD14 positive monocyte selection kit. The purity of sorted monocytes was examined by CD14 staining and evaluated by flow cytometry. The range of monocyte purity was 97.2%-98.9% and average was 98%. A representative of monocyte purity (CB-HBV32) was shown in Figure 8. The sorted monocytes of HBV-infected mother and healthy control were stimulated with or without ssRNA40 for 18 hours and evaluated cytokine

production (IL-12p40, TNF-α, IL-6, IL-12p70, IL-1β, IL-10, IFN-γ and IFN-α2) in cultured supernatant by Luminex assay. The results demonstrated that the levels of IL-12p40 of HBV-exposed cord blood monocytes stimulated with ssRNA40 was significantly higher than healthy cord blood monocytes (Figure 9). There is a trend of higher levels of TNF-α, IL-6, IL-12p70, IL-1β, IFN-γ, and IFN-α2 cytokines in HBV-exposed cord blood monocytes group than healthy cord blood monocytes group in stimulated condition, although it was not significantly different (Figure 9). But IL-10 levels did not differ between the HBV-exposed and healthy cord blood monocytes group (Figure 9). Interestingly, the levels of IL-6 cytokine in unstimulated condition of HBV-exposed cord blood monocytes were higher than control, although it was not significantly different (Figure 9). That may refer the trained immunity on infant monocytes.

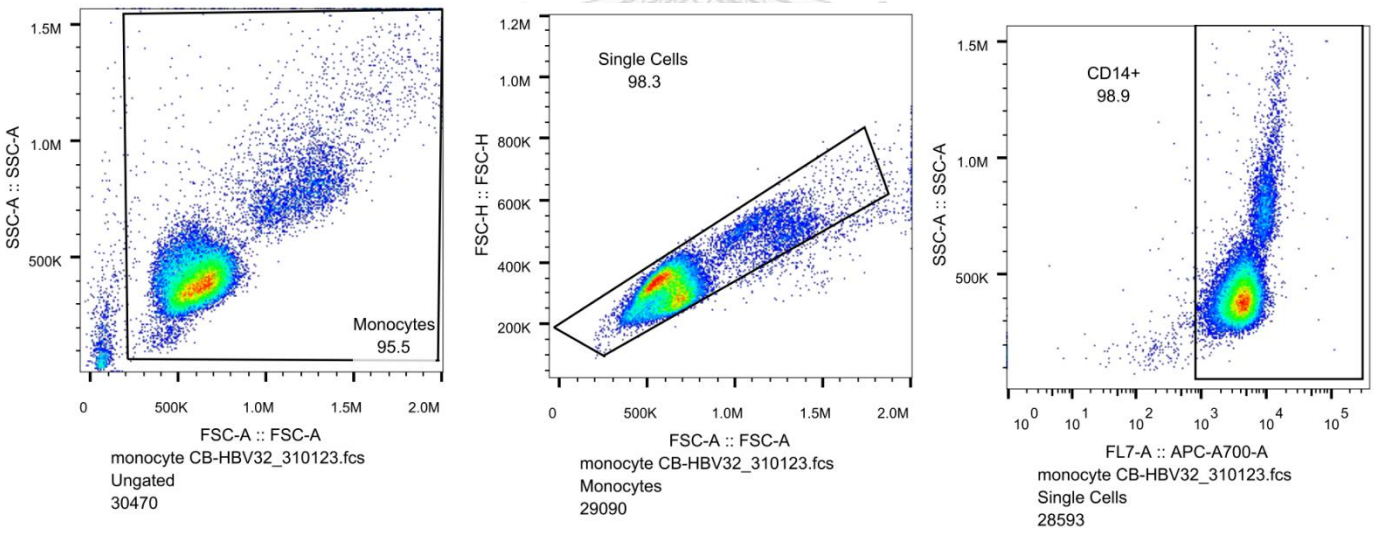


Figure 8. Gating is shown for positive monocyte isolation. Sorted monocytes by positive selection (after sorting) were evaluated the purity by CD14 staining and detected using flow cytometry.

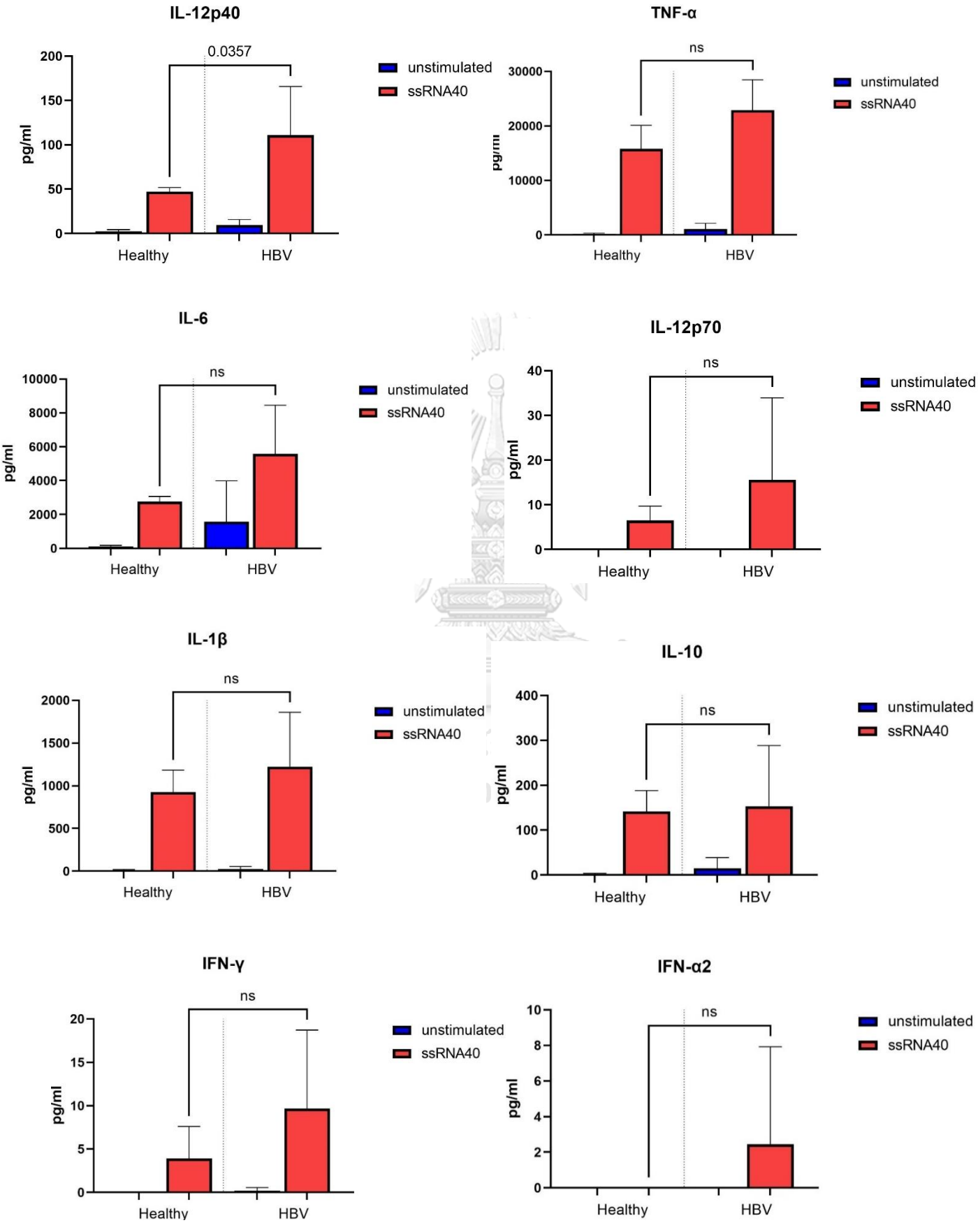

IL-12p40
0.0357
pg/ml
200
150
100
50
0
Healthy
HBV
unstimulated
ssRNA40
TNF-α
ns
30000
20000
10000
0
IL-6
IL-12p70
IL-1β
IL-10
IFN-γ
IFN-α2

Figure 9. Bar graphs demonstrated that the cytokines levels (IL-12p40, TNF-α, IL-6, IL-12p70, IL-1β, IL-10, IFN-γ and IFN-α2) of monocytes between HBV-infected and healthy cord blood monocytes after stimulation with ssRNA40 (TLR8 agonist) were determined by Luminex assay. Mann–Whitney test was used for statistical analysis and p-value<0.05 is significantly different.

4.2 Optimization of CUT&RUN from input step

Sonications were optimized by varying the number of cells and percentage of amplitude of the sonicator. Firstly, the number of cells is optimized at 400,000, 500,000, and 700,000 monocytes were used for sonication testing (input step), and the percentage of amplitude for sonication was set at 25 % with 20 seconds of pulse in and 30 seconds pulse off for 3 sonication cycles. The optimal DNA fragment size is between 100 to 600 bp. The suitable number of cell for CUT&RUN is at 400,000 cells and 500,000 monocytes (Figure 10).

Secondly, the amplitude of sonicator was optimized. Sonication at 400,000 and 500,000 cells were continued to test the amplitude. The amplitudes at 25 %, 27%, and 29% (20 seconds of pulse in and 30 seconds pulse off for 3 sonication cycles) were used for testing of 400,000 and 500,000 cells. The optimal conditions for sonication of CUT&RUN were 400,000 cells (25 % amplitude) and 500,000 cells (29 % amplitude) (Figure 11).

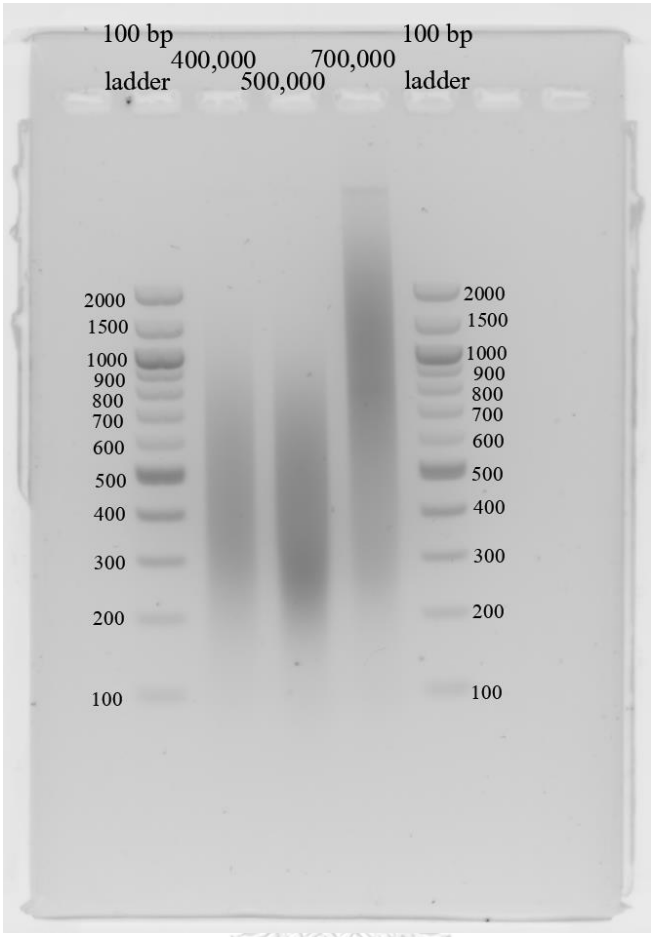


Figure 10. Sonication efficiency was shown by 2% agarose gel electrophoresis. From left to right, 100 bp ladder, 400,000, 500,000, and 700,000 cells for sonication, and 100 bp ladder.

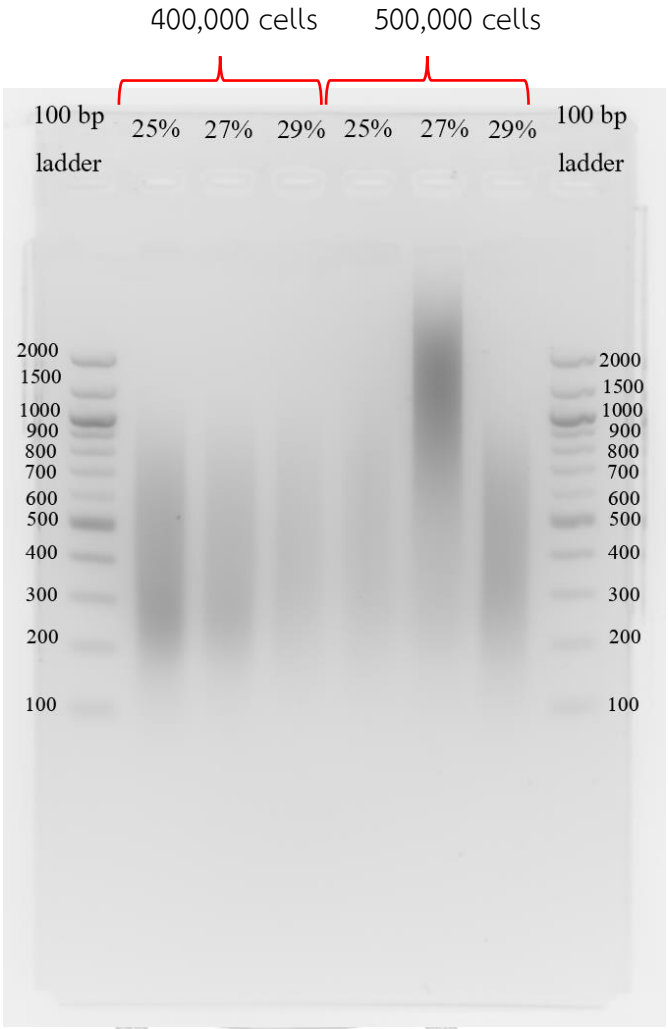
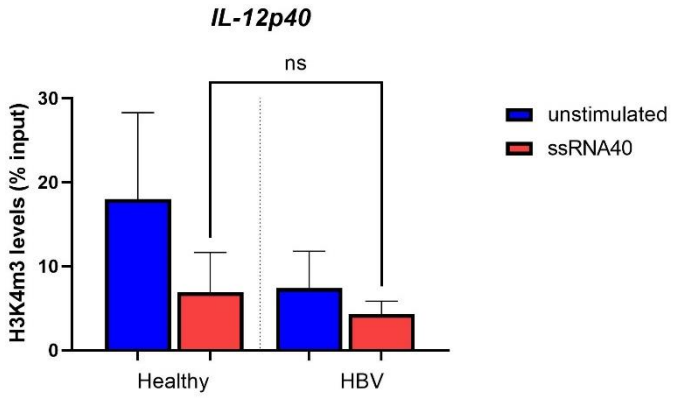
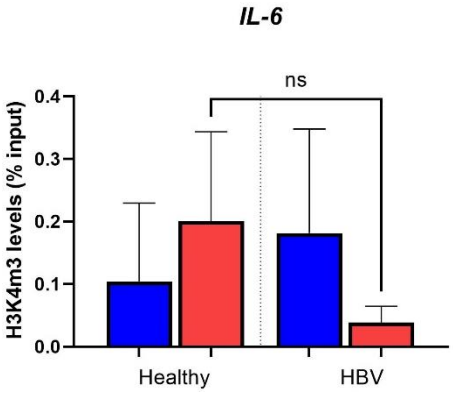


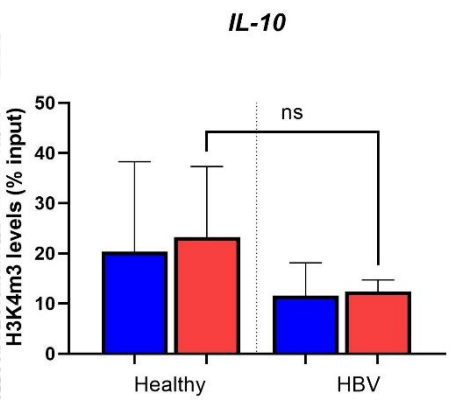
Figure 11. Sonication efficiency was shown by 2% agarose gel electrophoresis. Lane 1, and 8 were 100 bp ladder. Lane 2, 3, and 4 were 400,000 cells for sonication at 25 %, 27%, and 29% of amplitude (20 seconds of pulse in, 30 seconds pulse off, 3 sonication cycles), respectively. Lane 5, 6, and 7 were 500,000 cells for sonication at 25 %, 27%, and 29% of amplitude (20 seconds of pulse in, 30 seconds pulse off, 3 sonication cycles), respectively.

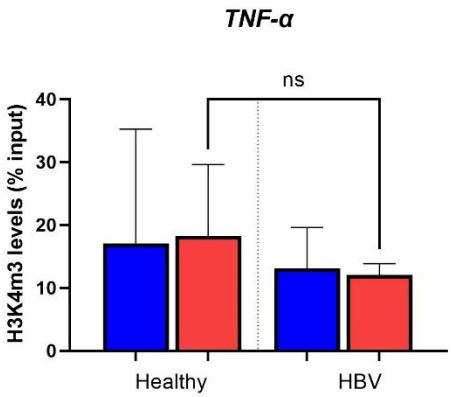
4.3 ChIP-qPCR H3K4me3 levels of interesting gene promoters in cord blood monocytes

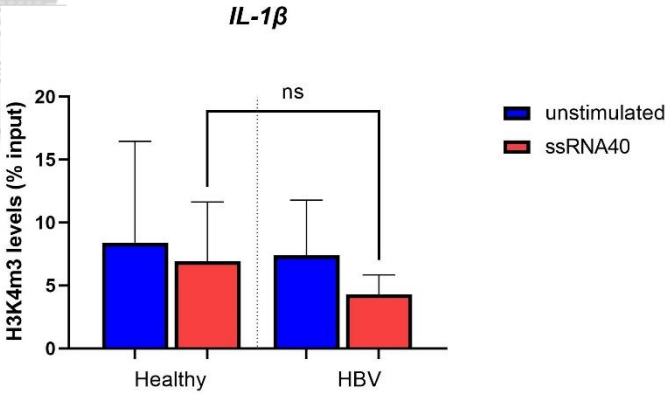
Because the cytokine production of IL-12p40 and others were higher in HBV-exposed cord blood monocytes than healthy controls, we further investigated the H3K4me3 levels of interesting genes (*IL-12p40, IL-6, IL-10, TNF-α,* and *IL-1β*) at the promoter region under ssRNA40 stimulation. The sort of cord blood monocytes of HBV-infected mothers and healthy mothers were stimulated with ssRNA40 for 18 hours and harvested for ChIP with H3K4m3 antibody by CUT&RUN technique. The purified DNA after ChIP assay was determined the H3K4me3 levels by qPCR. The result demonstrated that the H3K4me3 levels of *IL-12p40, IL-6, IL-10, TNF-α,* and *IL-1β* gene promoters of HBV-exposed cord blood monocytes stimulated with ssRNA40 were not significantly different from healthy cord blood monocytes (Figure 12). However, the trend of H3K4me3 level of *IL-6* promoter in unstimulated HBV-exposed cord blood monocytes that was higher than unstimulated healthy cord blood monocytes that show the role of H3K4me3 at *IL-6* promoter of cord blood ssRNA40 stimulated cord blood monocytes of HBV-infected mothers during trained immunity (Figure 12).

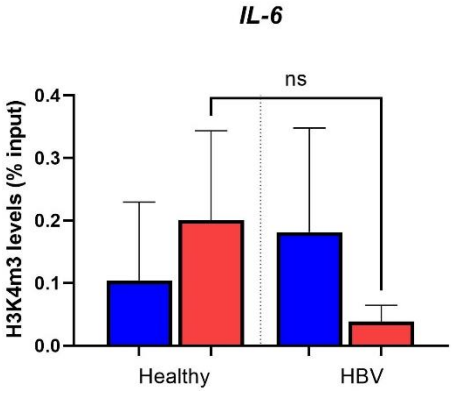

IL-12p40
ns
H3K4m3 levels (% input)
30
20
10
0
Healthy
HBV
unstimulated
ssRNA40


IL-6
ns
H3K4m3 levels (% input)
0.4
0.3
0.2
0.1
0.0
Healthy
HBV
unstimulated
ssRNA40


IL-10
ns
H3K4m3 levels (% input)
50
40
30
20
10
0
Healthy
HBV
unstimulated
ssRNA40


TNF-α
ns
H3K4m3 levels (% input)
40
30
20
10
0
Healthy
HBV
unstimulated
ssRNA40


IL-1β
ns
H3K4m3 levels (% input)
20
15
10
5
0
Healthy
HBV
unstimulated
ssRNA40


IL-6
ns
H3K4m3 levels (% input)
0.4
0.3
0.2
0.1
0.0
Healthy
HBV
unstimulated
ssRNA40

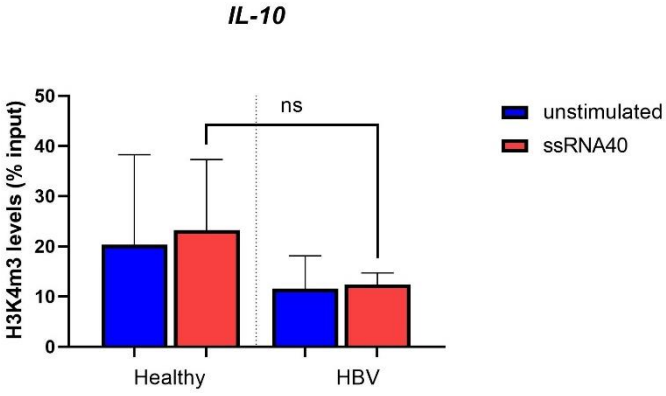

IL-10
ns
H3K4m3 levels (% input)
50
40
30
20
10
0
Healthy
HBV
unstimulated
ssRNA40

Figure 12. ChIP analysis of H3K4me3 levels at the promoter of IL-12p40, IL-6, IL-10, TNF-α, and IL-1β by qPCR in cord blood monocytes of HBV-infected mothers and healthy controls stimulated with or without ssRNA40 for 18 hours. Coimmunoprecipitated DNA was shown as percent of the total input. Mann–Whitney test was used for statistical analysis and p-value<0.05 is significantly different.

CHAPTER V
DISCUSSION AND CONCLUSION

The concept of innate immune memory or trained immunity is determined by epigenetic reprogramming at the level of histone modifications such as H3K4 and H3K27 trimethylation (82). Currently, trained immunity has been studied in many pathogens or antigens for example BCG-induced trained immunity (83), trained monocytes with β-glucan (65), trained monocytes with oxLDL (Oxidized low-density lipoprotein) or β-glucan, and others (74). In trained immunity of HBV, there was only one study in HBV-exposed cord blood monocytes of newborns who get a natural viral exposed from their mothers during the perinatal period (5). It showed that HBV can induce trained immunity after exposed cord blood monocytes and re-stimulation with TLR8 agonist (ssRNA40) (5). However, no evidence has been elucidated the mechanisms that regulate the trained immunity in HBV infection. In this study, we demonstrated the role of histone modification on HBV-induced trained immunity.

We showed the function of monocyte that is one of innate immune cells in newborns and confirmed the training HBV-exposed umbilical cord blood monocytes. Monocytes which are the most prevalent population of innate immune cells in the umbilical cord blood were isolated for investigating the trained immunity. TLR8 agonist (ssRNA40)-stimulated cord blood monocytes from HBV-infected mothers produced significantly IL-12p40 higher than healthy cord blood monocytes. Moreover, we investigated the other anti-viral and proinflammatory cytokines; IFN-α2, IL-12p70, TNF-α, IL-6 and IL-1β, which are mainly secreted from monocyte upon stimulation (ref). Although, these cytokines was not significantly different, it showed a trend of these cytokine levels higher in cord blood monocytes of HBV-infected mothers than in healthy controls. Our results can confirm previous report that IL-12p40 was upregulated higher in HBV-exposed cord blood monocytes than controls upon TLR8 agonist stimulation, while other cytokines from stimulated CB monocytes

were not investigated (87). In the ex vivo cytokine production, IL-12p40 in the supernatant of ex vivo-isolated CB monocytes was undetectable, whereas the levels of other cytokines and chemokines were not different between HBV patient and controls (87). In addition, we found the higher levels of IL-6 in supernatant of cord blood monocytes of HBV-infected mothers than in healthy controls upon unstimulated condition (direct ex vivo; overnight culturing) that likely results from induction of trained immunity in newborn infants of HBV-infected mothers. It indicated that HBV can trigger trained immunity on infant monocytes of HBV-infected pregnant women in our patients.

To study the mechanism mediating trained immunity in HBV-exposed infants, epigenetic reprogramming at the level of histone modifications were observed in HBV-exposed cord blood monocytes. H3K4me3 and H3K27me3 marks of histone modification are enriched at promoter or transcription start sites (TSSs) (84). The active promoters had a high level of H3K4me3 marks, whereas the heterochromatin lacked these marks (84). In contrast, H3K27me3 is a repressive mark associated with heterochromatin or repressive transcription (84, 85). We found that the expression levels histone modification enzymes KDM6B (JMJD3) having a role in removing methyl groups from H3K27 (81) in HBV-exposed cord blood monocytes were higher than the expression levels of healthy cord blood monocytes upon TLR8 agonist activation. The levels of EZH1 and EZH2 having a role in transferring methyl groups to H3K27 (81) were lower in HBV-exposed cord blood monocytes than healthy controls. Moreover, in unstimulated condition, the trend of KDM6A (removing methyl groups from H3K27) and KMT2A (transferring methyl groups from H3K4) was higher in HBV-exposed cord blood monocytes than healthy controls. In contrast, KDM6A and KMT2A in HBV-exposed cord blood monocytes were lower in HBV-exposed cord blood monocytes than healthy controls that do not support and associate with HBV-trained immunity. Altogether, it indicated that histone modification is likely to be mediate trained immunity in cord blood neonate monocytes of HBV-infected

mothers. Even though it did not showed statistically different, the expression levels of some histone modification enzyme genes still be correlate to cytokine production of cord blood monocytes of HBV-infected pregnant women. We have small sample size in the experiment that we need more samples for get consistent and reliable result. Previous studies demonstrated that proinflammatory cytokine was upregulated by KDB6A and KDM6B expression in respiratory syncytial virus (RSV)-infected dendritic cells (DC) (86), KMT2A regulated the expression of inflammatory cytokines in coronavirus-infected monocytes (87), and others.

The validation whether such histone methylation involves in the mechanism mediating trained immunity in HBV-exposed infants using inhibitor of histone demethylases or histone methyltransferases requires further investigation. The epigenetic changes at the level of H3K4me3 in innate immune genes on HBV-exposed cord blood monocytes were assessed after stimulation with TLR8 agonist. We did not find difference in the H3K4me3 levels on any genes between HBV-exposed cord blood monocytes and healthy controls. There are many factors that is likely to be that the cause of this finding. One factor is incubation time of stimulation. We stimulated sorted monocytes with TLR8 agonist for 18 hours before harvesting cells for ChIP assay and collecting supernatant for cytokine detection. This timing is optimal for cytokine evaluation but not suitable for determining H3K4me3 levels by ChIP-qPCR assay. The epigenetic changes at the level of H3K4me3 possibly occurred earlier the cytokine production, and this change may appear for a moment. Quintin J., *et. al.* showed that timing at 24 hr was suitable for study histone modification (H3K4me3) in trained immunity in beta-glucan by ChIP-seq (71). Thus, we need to vary time points of stimulation for evaluating H3K4me3 levels. Another factor is the positions on genes of interest which is determined the levels of H3K4me3. We investigated the levels of H3K4me3 at the promoter of candidate genes such as IL-12p40 that IL-12p40 cytokine levels are higher in HBV-exposed cord blood monocytes than controls. We chose the location on promoter of genes that

was validated in previous publications and based on H3H4me3 rich region on monocyte. However, these regions may not the right position that changes H3H4me3 levels during HBV-induced trained immunity in neonates. We should perform ChIP sequencing to give us more information in this epigenetic change. In addition, trained immunity is mediated not only by histone methylation (H3K4me3, H3K36me3, and H3K79me3) but also histone acetylation (H3K9ac and H3K27ac), and both methylation and acethylation are marks of active promoters or enhancers (89)(88). Our results that showed HBV-trained immunity may be controlled by acetylation instead of by methylation. Thus, studying the role of histone acetylation on HBV-trained immunity in neonates is interesting to further investigation. Lastly, a small sample size in each group is the limitation in our experiments, and increasing the sample size is necessary.

In conclusion, HBV exposure in utero can induce trained immunity in HBV-exposed cord blood monocytes of HBV-infected mothers which enhances cord blood monocyte activation after stimulation with TLR8 agonist by producing IL-12p40 significantly higher in patients than controls. We also observed epigenetic changes of histone methylation. It was shown the significant difference of the expression levels of histone modification enzyme genes; even though, the levels of H3K4me3 on innate immune genes were no significant difference between HBV and control group. More investigation is needed in further study.

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