CTLA-4 and Its Ligands on the Surface of T- and B-Lymphocyte Subsets in Chronic Hepatitis B Virus Infection

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Background: *During chronic hepatitis B virus (CHB) infection, a number of co-stimulatory, co-inhibitory molecules and theirs ligands play a prominent role in the immune-regulation.*
Objective: *To compare the number of peripheral-blood mononuclear cells expressing co-inhibitory marker, cytotoxic T lymphocyte associated antigen-4 (CTLA-4) and program cell death ligand-1 (PD-L1) between CHB infected patients and healthy controls.*
Material and Method: *Peripheral-blood mononuclear cells (PBMCs) from 19 CHB-infected patients and nine healthy controls were stained with specific combinations of the following monoclonal antibodies: CD3-PE/cy5, CD4-APC, CD8-APC, CD152-PE (CTLA-4), CD19PE/Cy5, CD80-FITC (B7-1), CD86-PE (B7-2) and CD274-FITC (B7-H1) according to standard protocol.*
Results: *The frequencies of B-lymphocyte expressing B7-1, B7-2 and B7-H1 of CHB-infected patients and healthy controls were not shown any statistical differences. The mean percentage of B-lymphocyte with B7-2 molecule was higher than those with B7-1 molecules in both infected- and non-infected groups. In contrast, the frequencies of T-lymphocyte subsets, CD3+, CD4+ and CD8+ expressing CTLA-4 molecules in CHB-infected patients were significantly higher than those in healthy controls with p = 0.04, 0.01 and 0.04 respectively.*
Conclusion: *An increase in percentage of circulating CD4+/CD152+ (T-cell) was observed in CHB-infected patients. A small but significant increase in percentage of CD8+/CD152+ T-cells raises the possibility that CTLA-4 are involved in the development of HBV-specific CD8+ T-cell exhaustion. Overall, CD4+ and CD8+ T-cells presenting CTLA-4 might contribute to the impaired immune response and likely to be a factor influencing in failure of immunological control of the persisting pathogens.*

Keywords: *Chronic hepatitis B virus, Co-stimulatory molecules, CTLA-4, Ligands, Immune-regulation, Lymphocyte subsets, Flow cytometer*

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Hepatitis B virus (HBV) infection continues to present a major public health problem in worldwide. Particularly, it is an important clinical issue in areas such as south-east asia where HBV infection is endemic[1]. Despite its clinical importance, it remains unknown whether the secondary molecules in immune-responsiveness are related to the chronic HBV infection. As the virus itself is non-cytopathic DNA virus, it is widely accepted that both viral clearance and disease pathogenesis are mediated by the host immune system[2]. Several studies focus on adaptive immune responses[3,4], which play a prominent role in controlling HBV infection[5].

Focusing on cell-mediated immune response, a lymphocyte requires two distinct signals in order for complete activation to occur[6]. The first signal is provided by the interaction of the T-cell receptor (TCR)

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on the lymphocyte with major histocompatibility complex (MHC) antigens on the antigen-presenting cell (APC). The second, co-stimulatory, signal is required to avoid an apoptotic or anergic response by the lymphocyte. Based on secondary signal, CD28 and CTLA-4 molecules are receptors on T-cells that play critical roles in the initial activation and subsequent control of cellular immunity[7]. CD28, which is expressed constitutively on T-cells, provides a major co-stimulatory signal upon binding to target ligands on antigen-presenting cells. In contrast, CTLA-4 is transiently expressed following T-cell activation. The signal delivered via CTLA-4 down-regulates T-cell function and inhibits excessive expansion of activated T-cells. Both CD28 and CTLA-4 bind two well-characterized ligands, B7-1 and B7-2 and newly identified B7 family members: B7-H1, B7-H2, B7-H3, PD-L2, ICOS and PD-1[8,9]. These B7 proteins are expressed in various types of antigen-presenting cells and differ from one another in their expression characteristics and in their affinities and kinetics of binding to CD28 and CTLA-4[6].

During chronic viral infection, there is diversity in the cell surface inhibitory pathways available to regulate T-cell on negative responses. One such co-inhibitory signal is CTLA-4, which plays a pivotal role in regulating T-cell activation. This signal is believably critical for the outcome of hepatitis B virus (HBV) infection[10,11]. The other one is PD-1, and its ligand, PD-L1, which has been shown to contribute to the failure of some T-cell specificities in CHB[12]. The programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) both negatively regulate the T-cell response in chronic hepatitis B virus (CHB) infection[13]. The precise inhibitory receptors of CD4+ or CD8+ T-lymphocytes are of interest as well as other factors such as their ligands. In defining the critical function of T-lymphocyte subset in CHB infection, the number of lymphocyte subsets expressing CD28 family receptors and their ligands in B7 family were investigated on peripheral-blood mononuclear cells (PBMCs) of HBV-infected patients.

Material and Method

Study subjects

In the present study; nine healthy controls and 19 CHB-infected patients whom have been treated at Mahachakri Sirindhorn Medical Center (MSMC) were recruited. The demographic data of HBV-infected patients were shown in Table 1. Nine healthy controls were blood donors from MSMC blood bank. Controls and HBV-infected subjects were similar with respect to gender and age. In the control group, the infectious markers, HBV, HCV, HIV, TPHA and malaria have been tested to exclude possible infection. The present study was approved by the Ethical Committee of Srinakharinwirot University (SWUEC/EX 2/2554).

Flow cytometry analysis

Peripheral blood specimens were obtained in EDTA tubes. After a maximum storage period of 3 h, whole blood samples were diluted 1:1 with phosphate-buffer saline (PBS). According to standard protocols, diluted whole blood was incubated with specific combinations of the following antibodies: CD3-PE/cy5, CD4-APC, CD8-APC, CD152-PE (CTLA-4), CD19PE/Cy5, CD80-FITC (B7-1), CD86-PE (B7-2) and CD274-FITC (B7-H1) (BioLegend, San Diego CA). After 30 minutes of incubation, the erythrocytes were lysed with 1 ml of FACS lysing solution (BD Bioscience). The PBMCs were washed twice in PBS. The pelleted were fixed in 0.5% paraformaldehyde and analyzed with a four-color FACS Calibur flow cytometer (FACScan; Becton Dickinson, San Jose, CA) using the CellQuest software (BD Biosciences). The electronic compensation was performed and a minimum of 10,000 PBMCs per sample was acquired. Lymphocytes were gated and defined as the lymphocyte region R1. Irrelevant FITC, PE, PE-Cy-5 and APC-conjugated isotype-matched

Table 1. Clinical and biological characteristics of patients and healthy controls

Characteristic	Patients (n = 19)	Healthy controls (n = 9)
Age, mean (range)	49.5 (25-80)	31 (21-48)
Sex; male/female	11/8	6/3
ALT (range)	51 (26-190)	ND
ART; treated/untreated	9/10	ND

ALT = alanine aminotransferase, ARV = antiretroviral therapy, ND = not done

antibodies of each sample were used as negative control.

Statistical analysis

The statistical analysis was performed with GraphPad Prism (GraphPad, San Diego, CA) Mann-Whitney U-test was used to compare the difference between two subject groups. A p-value < 0.05 was considered statistically significant.

Results

The frequencies of B-lymphocyte expressing B7-1 (CD80), B7-2 (CD86) and B7-H1 (CD 274)

The frequency of B-cells (CD19) expressing B7 family molecules was determined using a combination of CD19+/CD80+, CD19+/CD86+ and CD19+/CD274+ in the lymphocyte gate. As summarized in Fig. 1, the frequencies of lymphocyte expressing B7-1, B7-2 and B7-H1 in CHB- infected patients were 0.4 ± 0.1, 3.3 ± 0.6 and 0.3 ± 0.04, respectively. In normal controls, they were 0.3 ± 0.07, 1.8 ± 0.4 and 0.4 ± 0.07, respectively. In comparison between patients and controls, no significant difference in each marker of B7 family was observed. However, it is clearly demonstrated that the percentages of B-lymphocyte expressing B7-2 (CD86) ligands were higher than those with B7-1 ligands in both subject groups. In addition, increasing in the ratio of B-lymphocyte expressing B7.2 (CD86) to B-lymphocyte expressing B7.1 (CD80) from CHB infected patient was much more than the ratio in the normal controls as shown in Fig. 1.

The number of T-lymphocyte subsets expressing CTLA-4 inhibitory molecules

In the patients who had CHB infection, the percentages of CD3+, CD4+ and CD8+ expressing CD152 (CTLA-4 molecules) were 1.2 ± 0.08, 0.5 ± 0.04 and 0.6 ± 0.05, respectively. In normal controls, the double positive cells of CD3+/CD152+, CD4+/CD152+ and CD8+/CD152+ were 0.9 ± 0.08, 0.3 ± 0.05 and 0.4 ± 0.03, respectively. The frequencies of all subsets of T-lymphocyte, CD3+, CD4+ and CD8+ expressing CTLA-4 molecules in CHB-infected patients were markedly higher than those in healthy controls (p = 0.04, 0.01 and 0.04 respectively) as shown in Fig. 2.

Discussion

The importance of membrane-bound co-stimulatory receptors and theirs ligands have been well documented. The reciprocal ligands of co-stimulatory receptors have been studied on several cell types, including B cells, which can serve as antigen presenting cells[14]. In the present study, B-lymphocytes expressing CD80 and CD86 from two subject groups were similar. However, it is clearly demonstrated that the percentages of B-lymphocyte with B7-2 (CD86) ligands were higher than those with B7-1 (CD80) ligands in both infected

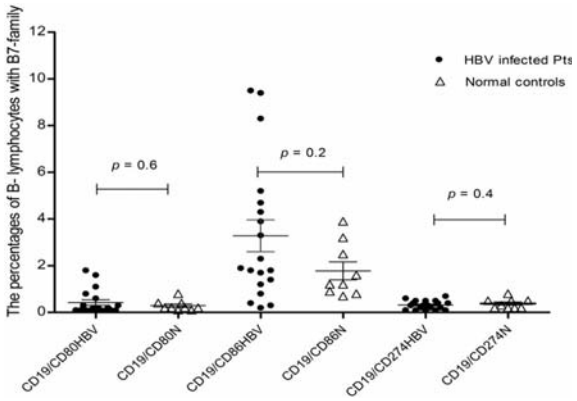

Fig. 1 Comparison of the frequencies of B- lymphocytes expressing CD80 (B7-1), CD86 (B7-2) and CD274 (B7-H1) molecules between CHB-infected patients and normal controls. Each dot represents an individual's value, which is expressed in percentage of positive cells in the lymphocyte gate. Data are expressed as the mean percentage ± standard error. The significant differences were not found as marked by their respective p-values

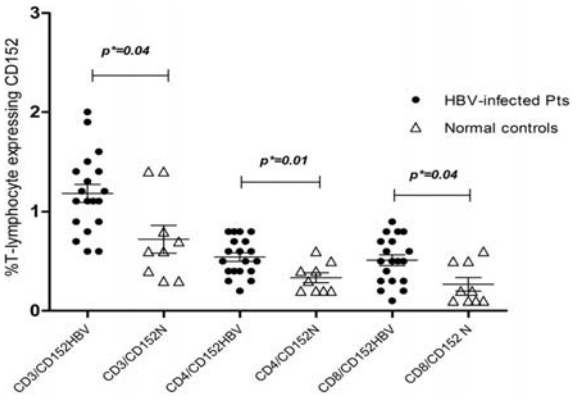

Fig. 2 Comparison of the percentage of T- lymphocyte sub sets expressing CLTA-4 (CD152) co-inhibitory molecules in HBV-infected patients and healthy controls. Each dot represents an individual's value, which is expressed in percentage of positive cells in the lymphocyte gate. Data were presented as the mean percentage ± standard error. The statistical significance between patient and normal control groups were analyzed and indicated by p* < 0.05

and uninfected groups. In the present study, the ratio of B-lymphocyte expressing CD86 to B-lymphocyte expressing CD80 from the infected patient was much more than the ratio in the normal control (as shown in Fig. 1). In this regard, the previous study suggested that B7.1 ligands and B7.2 ligands are comparable in their ability to co-stimulate responses in T-cells previously primed in vitro[15]. The other study provided evidence that B7.2 molecule is the major ligand for the CD28 receptor on vaccinia virus-specific CD8 T-cells[16]. Alternatively, in the context of CTLA-4, the expression CD80 and CD86 on dendritic cells (DCs) might be associated with the inability of DCs in presenting the viral antigens[17,18]. For B7-H1 (CD274), the earlier study demonstrated that expression of this ligand is closely associated with the suppression of T cell function and inability of antigen presentation[19,20]. However, the difference in B7-family ligand's expression may provide the distinct functional roles for B7-1 and B7-2 that are not yet completely defined.

Based on inhibitory receptors; CTLA-4 (CD152), is known as a marker for regulatory T-cells (T-regs) in the experimental and clinical analysis[21,22]. These cells present 2-4% of peripheral blood CD4+ T-lymphocytes[23]. Therefore, CD4+/CD152+ is categorized as T-regulatory cell. In the present study, the most striking difference between T-lymphocyte subsets was shown in CD4+/CD152+ (T-regs) population. This observation was supported by previous study in which an increase in percentage of circulating CD4+/CD25+Treg was observed in CHB infected patients[24]. Increasing in circulating CD4+/CD25+ might be associated with a negative immune response and poor viral clearance in HBV- infected patients[25]. In a similar approach, a small but significant increase in the percentage of CD8+/CD152+ T-lymphocytes from CHB-infected patients was found when compared with those in healthy controls. As earlier report, CD8+ T-lymphocytes in patients with CHB-infection have an increased propensity to express CTLA-4, and this correlates with viral load[11]. Increasing in frequency of CD8+/CD152+ raises the possibility that CTLA-4 might involve in the development of CD8+T cell exhaustion[26]. These features can contribute to weak HBV-specific T-cell responses and thus increase the chances of HBV to persist[17]. In concordance with another study, the functional impairment is present in the generalized T-cell population in patients with CHB infection, regardless of antigen specificity[27]. In addition, functional defects of CD8+T cells are also mediated by CD4+/CD25+T-regs. In conclusion, the inhibitory function of CD4+/CD152+ and CD8+/CD152+ might be a factor which involved in persisting pathogens. The interactions between the co-stimulatory/co-inhibitory molecules and their ligands are proving to expand the scope of our understanding of the immune response in CHB-infection.

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Potential conflicts of interest

None.

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CTLA-4 และ ligands บนผิวของประชากรย่อยของเซลล์ชนิดลิมโฟซัยท์ในการติดเชื้อไวรัสตับอักเสบ บี ชนิดเรื้อรัง

ชัชวาลย์ วงศ์จิตรัตน์, สุจิตรา สุขวิทย์, ทิพย์วรรณ ชื่นจิตร, พัชรินทร์ แสงจารึก, พรพรรณ โรจนแสง, พรสุข ร่มพุฒตาล, สุรางค์รัตน์ ศรีสุรภานนท์

ภูมิหลัง: *ขณะที่มีการติดเชื้อไวรัสตับอักเสบ บี ชนิดเรื้อรัง (CHB) การทำงานของระบบภูมิคุ้มกันจะถูกควบคุมโดยโมเลกุลของ co-stimulatory, co-inhibitory และ ligands*

วัตถุประสงค์: *ตรวจวิเคราะห์หา co-inhibitory โมเลกุล ชนิด CTLA-4 บนผิวของ ที-ลิมโฟซัยท์ และ ligands บนผิวของ บี-ลิมโฟซัยท์ ในผู้ติดเชื้อไวรัสตับอักเสบ บี ชนิดเรื้อรัง และกลุ่มควบคุมซึ่งมีสุขภาพแข็งแรง*

วัสดุและวิธีการ: *การย้อมสีเซลล์เม็ดเลือดขาว จากผู้ติดเชื้อไวรัสตับอักเสบ บี ชนิดเรื้อรัง และกลุ่มควบคุม โดยใช้ monoclonal antibodies ดังต่อไปนี้ CD3-PE/cy5, CD4-APC, CD8-APC, CD152-PE (CTLA-4), CD19PE/Cy5, CD80-FITC (B7-1), CD86-PE (B7-2) และ CD274-FITC (B7-H1)*

ผลการศึกษา: *ในคนที่ติดเชื้อไวรัสตับอักเสบ บี ชนิดเรื้อรัง พบว่าจำนวนของ บี-ลิมโฟซัยท์ที่มี B7-1, B7-2 and B7-H1 ไม่แตกต่างจากกลุ่มควบคุม เมื่อเปรียบเทียบในกลุ่ม บี-ลิมโฟซัยท์ ที่มี B7-1, B7-2 พบว่า บี-ลิมโฟซัยท์ ชนิดที่มี B7-2 มีจำนวนมากกว่า บี-ลิมโฟซัยท์ ชนิดที่มี B7-1 ทั้งในกลุ่มที่ติดเชื้อและไม่ติดเชื้อ ในทางตรงข้าม ที-ลิมโฟซัยท์ ชนิด CD3+, CD4+ and CD8+ ที่มี CTLA-4 โมเลกุลบนผิวเซลล์ ในคนที่ติดเชื้อ มีมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ คือ มีค่า p = 0.04, 0.01 and 0.04 ตามลำดับ*

สรุป: *ในผู้ติดเชื้อไวรัสตับอักเสบ บี ชนิดเรื้อรังพบว่า ที-ลิมโฟซัยท์ ทั้ง 2 ชนิด คือ CD4+ ที่มีโมเลกุลของ CTLA-4 บนผิวเซลล์และ CD8+ ที่มีโมเลกุลของ CTLA-4 มีจำนวนเพิ่มมากขึ้นอย่างมีนัยสำคัญการพบ CTLA-4 บนผิวของ CD8+ ที-ลิมโฟซัยท์ น่าจะส่งผลให้ประสิทธิภาพการทำงานของ CD8+ ที-ลิมโฟซัยท์ลดลง ในภาพรวมการทำงานของ CD4+ และ CD8+ ที-ลิมโฟซัยท์ที่มี CTLA-4 อยู่บนผิวเซลล์ อาจเป็นสาเหตุหนึ่ง ที่ทำให้ระบบภูมิคุ้มกันไม่สามารถควบคุมการติดเชื้อจึงเกิดการติดเชื้อไวรัสตับอักเสบ บี ชนิดเรื้อรัง.*