ผลของคิลเลอร์เซลล์ที่ถูกชักนำด้วยไซโตไคน์ต่อภาวะตับอักเสบที่ถูกชักนำด้วยคอนคานาวาลินเอใน
หนูเมาส์

นายวรากร ศรีสันติสุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสัตววิทยา ภาควิชาชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

EFFECTS OF CYTOKINE - INDUCED KILLER CELLS ON CONCANAVALIN-A INDUCED

HEPATITIS IN MOUSE

Mr. Warakorn Srisantisuk

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Zoology

Department of Biology

Faculty of Science

Chulalongkorn University

Academic Year 2017

Thesis Title	EFFECTS OF CYTOKINE - INDUCED KILLER CELLS ON CONCANAVALIN-A INDUCED HEPATITIS IN MOUSE
By	Mr. Warakorn Srisantisuk
Field of Study	Zoology
Thesis Advisor	Associate Professor Chanpen Chanchao, Ph.D.
Thesis Co-Advisor	Assistant Professor Kitipong Soontrapa, M.D., Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

....Dean of the Faculty of Science

(Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

....Chairman

(Assistant Professor Noppadon Kitana, Ph.D.)

....Thesis Advisor

(Associate Professor Chanpen Chanchao, Ph.D.)

....Thesis Co-Advisor

(Assistant Professor Kitipong Soontrapa, M.D., Ph.D.)

....Examiner

(Assistant Professor Pongchai Harnyuttanakorn, Ph.D.)

....Examiner

(Assistant Professor Amornpun Sereemaspun, M.D., Ph.D.)

....External Examiner

(Associate Professor Adisak Wongkajornsilp, M.D., Ph.D.)

วรากร ศรีสันติสุข : ผลของคิลเลอร์เซลล์ที่ถูกชักนำด้วยไซโตไคน์ต่อภาวะตับอักเสบที่ถูกชักนำ ด้วยคอนคานาวาลินเอในหนูเมาส์ (EFFECTS OF CYTOKINE - INDUCED KILLER CELLS ON CONCANAVALIN-A INDUCED HEPATITIS IN MOUSE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. นพ. กิติพงศ์ สุนทราภา, 70 หน้า.

โรคตับอักเสบที่ถูกทำลายโดยระบบภูมิคุ้มกันตัวเองมีสาเหตุเกิดจากภูมิต้านทานทำลายเซลล์ตับ โดยตรงปัจจุบันกลไกการเกิดโรคยังไม่ทราบแน่ชัด อาการของผู้ป่วยโรคตับอักเสบมักจะนำไปสู่โรคตับแข็ง และมะเร็งตับ การรักษาโรคนี้ทำโดยการใช้ยากดภูมิคุ้มกันและปลูกถ่ายตับซึ่งจะทำให้เกิดผลข้างเคียง ค่า รักษาโรคสูง และยังมีแนวโน้มที่จะสามารถกลับมาเป็นโรคได้อีก ดังนั้นการรักษาทางเลือกโดยวิธีภูมิคุ้มกัน บำบัดจึงถูกใช้โดยฉีดเซลล์เข้าสู่ผู้ป่วย การศึกษาที่ผ่านมาแสดงถึงคิลเลอร์เซลล์ที่ถูกชักนำด้วยไซโตไคน์ หรือ เซลล์ที่มีคุณสมบัติของเซลล์เพชรฆาต ซึ่งมีศักยภาพในการรักษาโรคเกี่ยวกับตับหลายชนิด งานวิจัยนี้มี วัตถุประสงค์เพื่อศึกษาผลในเชิงป้องกันของคิลเลอร์เซลล์ต่อโรคตับอักเสบที่ถูกชักนำด้วยคอนคานาวาลิน เอ ในหนูเมาส์ เลี้ยงคิลเลอร์เซลล์จากต่อมไทมัส ด้วยโพรโทคอลเลี้ยงโดยเติมไซโตไคน์ IFN-g, anti CD-3 ใน วันแรกของการเลี้ยงเซลล์ และเติม IL-2 ทุก 2 วัน จากนั้นนำคิลเลอร์เซลล์ที่ถูกชักนำด้วยไซโตไคน์มา ตรวจสอบด้วยเครื่องวิเคราะห์เซลล์อัตโนมัติ พบว่าคิลเลอร์เซลล์ที่ถูกชักนำด้วยไซโตไคน์จากต่อมไทมัสพบ ค่าชี้วัดต่างๆของคิลเลอร์เซลล์ดังนี้ NK1.1 41.60 %, IFN-g 8.56 %, Granzyme 53.40 % และ Perforin 8.31 % ทำการฉีดคิลเลอร์เซลล์จำนวน 10×10^6 ล้านเซลล์ใน PBS 200 ไมโครลิตรเข้าทางเส้นเลือดดำ บริเวณหาง หลังจากนั้น 24ชั่วโมงทำการชักนำโรคตับอักเสบโดยฉีด คอนคานาวาลิน เอ (25 mg/kg) เข้า ทางเส้นเลือดดำบริเวณหาง สุดท้ายทำการการุณยฆาตหนูเมาส์ จากนั้นเก็บตับ เพื่อทำการวิเคราะห์ผลทาง พยาธิสภาพ พบว่าในกลุ่มที่ได้รับคอนคานาวาลิน เอ และกลุ่มที่ได้รับ คอนคานาวาลิน เอ ควบคู่กับคิลเลอร์ เซลล์ มีการบวมของเซลล์ตับ, การแทรกตัวของเม็ดเลือดขาว, การตายของเซลล์หลายพื้นที่, ไม่พบความ เสียหายที่ central vein และไม่มีภาวะ fibrosis ซึ่งแตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ส่วน ผลของค่าซีรัมในเลือดที่นำไปวัดค่า AST และ ALT พบว่าในกลุ่มควบคุม กลุ่มที่ได้รับคอนคานาวาลิน เอ และกลุ่มที่ได้รับ คอนคานาวาลิน เอ ควบคู่กับคิลเลอร์เซลล์ มีค่า AST เฉลี่ยคือ 48.33, 398.75 และ 487.33 หน่วยต่อลิตร ในขณะที่ค่า ALT เฉลี่ยคือ 38.33, 113.75 และ 199 หน่วยต่อลิตร ผลการวิเคราะห์ ทางสถิติของคะแนนพยาธิสภาพตับและค่าซีรัมในเลือดพบว่า กลุ่มที่ได้รับคอนคานาวาลิน เอ และกลุ่มที่ ได้รับ คอนคานาวาลิน เอ ควบคู่กับคิลเลอร์เซลล์ ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (P<0.05) โดยสรุป คิลเลอร์เซลล์ไม่มีผลในเชิงป้องกันต่อโรคตับอักเสบที่ถูกชักนำด้วยคอนคานาวาลิน เอ ในหนูเมาส์

ภาควิชา ชีววิทยา	ลายมือชื่อนิสิต ....
สาขาวิชา สัตววิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
ปีการศึกษา 2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

5872049823 : MAJOR ZOOLOGY

WARAKORN SRISANTISUK: EFFECTS OF CYTOKINE - INDUCED KILLER CELLS ON CONCANAVALIN-A INDUCED HEPATITIS IN MOUSE. ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: ASST. PROF. KITIPONG SOONTRAPA, M.D., Ph.D., 70 pp.

Autoimmune hepatitis (AIH) is a chronic inflammatory disease of liver that the pathogenic mechanisms of AIH have not yet been clarified. All patients with AIH lead to cirrhosis and liver cancer. Presently the best feasible medicines of AIH need aid immunosuppressive medications and liver transplantation which have many side effects, high cost and sustained remission. Thus, immunotherapy is an alternative therapy in which cellular material is injected into a patient. Many previous studies showed cytokine induced killer (CIK) cells, T lymphocytes that have a phenotype of NK cells, have a potential to against several diseases associated with liver. The current research aims to examine protective effects of CIK cells on Concanavalin A (Con A) induced AIH in mice. In this study we use Con A because it is a generally used model for AIH in mice, the activation and recruitment of T cells to the liver. CIK cells cultured from thymus were then used to develope a protocol for generating CIK cells by adding interferon gamma (IFN-ɡ), monoclonal antibody (mAb) against CD3 and interleukin-2 (IL-2). In this study markers show NK1.1 41.60 %, IFN-ɡ 8.56 %, Granzyme 53.40 % and Perforin 8.31 %. Twenty-four hours before Con A injection mice were injected with CIK cells through IV (10×10^{6} cells per 200 µl). Mice were intravenously (IV) injected through lateral tail vein for AIH induction (25mg/kg). Lastly mice were euthanized and organs (blood and liver) were collected for biochemical test (AST, ALT) and histopathology study. In histopathology study show significant difference between control group and experimental group, hepatocellular swelling, scattered area of leukocytic infiltration, multiple necrotic area, central veins are grossly intact and no fibrotic tissue. For biochemical test we found that the average of AST level in three group are 48.33, 398.75 and 487.33 U/ml respectively while the average of AST level are 38.33, 113.75 and 199 U/ml respectively. Statistical analysis by SPSS program were used to compare between 2 groups (ConA and ConA & CIK cells group) in pathological study and biochemical test ($P < 0.05$). In this study concluded that CIK cells does not show protective effect on Concanavalin-A indued hepatitis in mouse.

Department:	Biology	Student's Signature	....
Field of Study:	Zoology	Advisor's Signature	....
Academic Year:	2017	Co-Advisor's Signature	....

ACKNOWLEDGEMENTS

CONTENTS

LIST OF FIGURES

Figure 8 CIK cells were phenotypically analyzed by flow cytometry. Thymocytes from C57BL/6 mice were cultured under the CIK culture condition. Fourteen days later, CIK cells were stained by using specific directly conjugated anti-mouse

LIST OF ABBREVIATIONS

$^{\circ}$C	degree Celsius
µl	microliter
µm	micrometer
AIH	Autoimmune hepatitis
ALT	Alanine aminotransferase
ANA	Autoantibodies
ANOVA	Analysis of Variance
Anti - LKM	liver kidney microsomal antibody
Anti - SMA	Anti-smooth muscle
AP	Alkaline phosphatase
ASI	Antigen-specific immunotherapy
AST	Aspartase aminotransferase
CD	Cluster of differentiation
ConA	Concanavalin A
CIK	Cytokine-induced killer cell
FBS	Fetal bovine serum
FITC	Fluorescein isothiocynate
HLA	Human leukocyte antigen

H & E	Hematoxylin and eosin
IFN - g	Interferon - gamma
iNOS	Inducible nitric oxide synthase
iNKT	Invariant natural killer T
IL-2	Interleukin - 2
KC	Kupffer cell
kg	Kilogram
mg	milligram
MHC	Major histocompatibility complex
ml	milliliter
NK	Natural killer cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
SE	Standard error of mean
SEC	Sinusoidal endothelial cell
TCR	T – cell receptor
Th 1	T helper 1
THIOPEN	Thiopental sodium

TNF - a	tumor necrosis factor - alpha
T_{reg}	Regulatory T-cell

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Autoimmune hepatitis or AIH is a chronic inflammatory disease of liver caused by the immune system which damages the liver. When the immune response from white blood cells (WBC) is stimulated, there is the secretion of various types of cytokines such as tumor necrotic factor (TNF) and interferon (IFN) gamma which leads to inflammation of the liver. If there is no treatment, this could eventually lead to death (Czaja, 2016). AIH is separated into 2 types, AIH-1 and AIH-2 which is characterized by types of antibody in organ (Vergani et al., 2002). Patients with AIH disease will progress to cirrhosis around 45 - 80% and 4% of this group will develop hepato-cellular carcinoma (Czaja and Manns, 1995; Longhi et al., 2010; Oo et al., 2010; Yuksel et al., 2014). Sex is the important factor involving in this disease because previous studies showed that women are more at risk than men (3.6: 1) and this is because the effect of estrogen (Czaja, 1998; Czaja et al., 2002; Al-Chalabi et al., 2008).

AIH could occur in all races and at all ages. (Fainboim et al., 1994; Gregorio et al., 1997; Scott and Garland, 2008). The only treatments available are the injection of immunosuppressant and liver transplantation. Excessive immunity can have high side

effects; such as, fever, weakness and pain while urinating and these might be life threatening (Ichai et al., 2007; Manns et al., 2010).

Currently there are many reports of the development of immunotherapy using cytokine induced killer (CIK) cells, T helper (Th) 1 cells are stimulated by many of cytokines: IFN - g, anti CD-3 and IL-2. Murine CIK cells in this experiment are supported and evaluated by CIK cells markers; CD3+, CD8+ and NK1.1. (Pluangnooch et al., 2017) The results is increased efficiency in the removal of pathogen by release many IFN - g, perforin and granzymes (Schmidt-Wolf et al., 1991; Verneris et al., 2005; Pluangnooch et al., 2017).

The main function of CIK cell-based immunotherapy is to treat hematology and solid cancer in humans. Moreover, researcher claimed that CIK is safe for clinical studies when using CIK cells for immunosuppressants (Jakel et al., 2012). Many previous studies showed the protective role of immune cells such as Treg, B lymphocytes, especially on invariant natural killer T (iNK) cells. NKT cells are group of T cells that have properties of both T cells and natural killer cells (Caligiuri et al., 2002; Liu et al., 2005).

In this research CIK cell have T cells and NK cells properties. However, AIH is a chronic inflammatory disease of liver which caused by their own white blood cells that damage normal liver cells. Therefore, CIK cells would play a role in both preventive and therapeutic treatment. Currently, there is a few researches about CIK cells with AIH in immunocompromised patients. The important point is AIH caused

by T helper 1 cells and CIK cell is a type of Th1 cells (Guo and Han, 2015). Thus, the interesting question of this study is how will CIK cells affect AIH disease. This study aims to investigate about CIK cells on AIH to be knowledge and scientific evidence to the actual treatment in the future.

1.2 Research Objectives

To investigate the protective effects of cytokine - induced killer cells on concanavalin - A induced autoimmune hepatitis

1.3 Research Hypothesis

Cytokine - induced killer cells have a protective effect on concanavalin - A induced autoimmune hepatitis.

1.4 Places of study

Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University and Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

1.5 Conceptual Framework

```
                              ┌─────────────┐
                              │  Th 1 cell  │ - - - + →  IFN –g, IL-2
                              └─────────────┘            Anti CD-3
                      -  ↗        │ +
                                  │ IFN -g
                                  │ TNF - a
                                  ↓
┌─────────────┐            ┌──────────────────┐    ?    ┌──────────────┐
│  Treg cell  │            │ Liver inflammation│ ←────── │   CIK cell   │
└─────────────┘            └──────────────────┘         │  (Th1 + NK)  │
┌─────────────┐                  │ +                    └──────────────┘
│  iNKT cell  │                  │                       IFN -g
└─────────────┘                  ↓                       TNF – a
         +  ↘                                            Granzyme B
                                                         Perforin
         ?        ┌───────────────────────┐    ?
                  │  Autoimmune hepatitis  │
                  └───────────────────────┘
```

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY
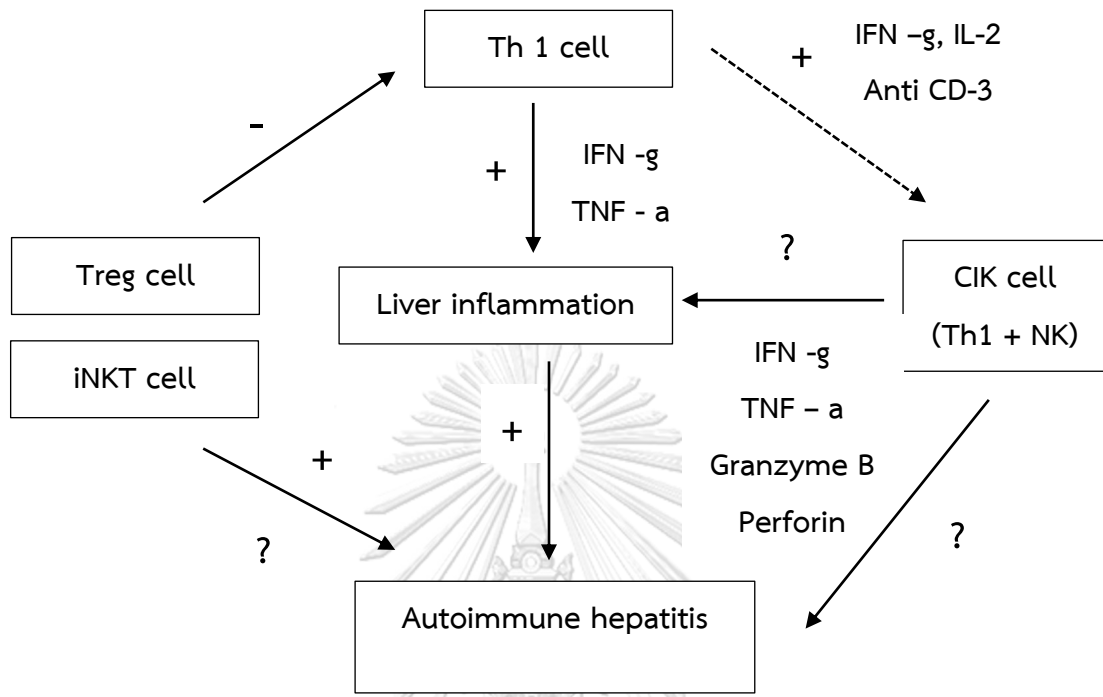
CHAPTER II

LITERATURE REVIEWS

2.1 Autoimmune hepatitis (AIH)

Autoimmune hepatitis is a chronic inflammatory liver disease of unknown aetiology in which the body's immune system attacks the normal components, or cells, of the liver and causes inflammation and liver damage. The immune system normally protects the body from infection by identifying and destroying bacteria, viruses, and other potentially harmful foreign substances (Yuksel et al., 2014; Czaja, 2016). AIH can be classified into 2 types, AIH-1 and AIH-2. AIH-1 is characterized by antibody in anti-smooth muscle or anti-nuclear (anti-SMA/ANA) autoantibodies. AIH-2 is characterized liver kidney microsomal antibody type 1 (anti-LKM-1) (Vergani and Mieli-Vergani, 2008).

Previous research showed that the genetic factors involved in the AIH disease. The pathogenesis is to induce T helper (Th) 1 cell of the immune system within the body. This leads to inflammation in the liver related with hepatitis interfering, plasma liver enzyme increasing, autoantibodies and regulatory T-cell (Tregs) dysfunction (Vergani et al., 2002; Czaja, 2007; Yuksel et al., 2014). Previous study showed the mechanism of the AIH disease in mice stimulated the destruction of the liver cells by the lectin concanavalin A (Con A). Con A is used in biological research and blood

analysis. T-cell lymphocytes have been using more than 40 years, which is obviously shown that this protein is the first lectin in crystalline form in Figure 1. Con A induceds red blood cells recruitment (haemagglutination) and binds between many receptors of the cell and Con A lead to inflammatory of cell stimulation (Heymann et al., 2015). Con A will stimulate and accumulate cells in the immune system in various areas of the body. T-cell lymphocytes and natural killer T (NKT)-cell. Although the result of *in vitro* studies showed Con A have a partial toxicity in liver cells. On the other hand, *in vivo* studies showed that lymphocytes (CD4$^+$ T helper) play an important role in inducing AIH.

However, the mechanism of action of the disease are not yet known and remain controversial. Researchers have investigated remarkable mechanisms of AIH which demonstrated that many components such as lymphocytes, Kupffer cells (KCs) and Stellate cells are related in the liver (Wang et al., 2012). After 15 minutes of Intravenous injection, Con A begins to bind with the mannose receptor on the surface of the sinusoid endothelial cells (SECs). Four hours later, Con A begins to bind with Kupffer cells (KCs). CD4$^+$ T helper can recognize major histocompatibility complex (MHC) class II through T-cell lymphocyte of Kupffer cell receptors that capture of Con A molecules and lead to inflammatory induction. Therefore, this could be concluded that both SECs and KCs play an important role in these mechanisms but at different intervals. The Inflammatory signaling within the liver stimulates white blood cell (lymphocytes and neutrophils) into the liver cells,

resulting in the release of various cytokines such as tumor necrotic factor (TNF) - a,

interferon (IFN)-g as shown in Figure 2. The increase of chemokines levels, adhesion

molecules, tissue factor, endothelin-1, and inducible nitric oxide synthase (iNOS) lead

to the destruction of liver cells, eventually causing hepatitis as shown in Figure 3

(Kusters et al., 1996; Wang et al., 2012; Heymann et al., 2015; Fujita et al., 2016).
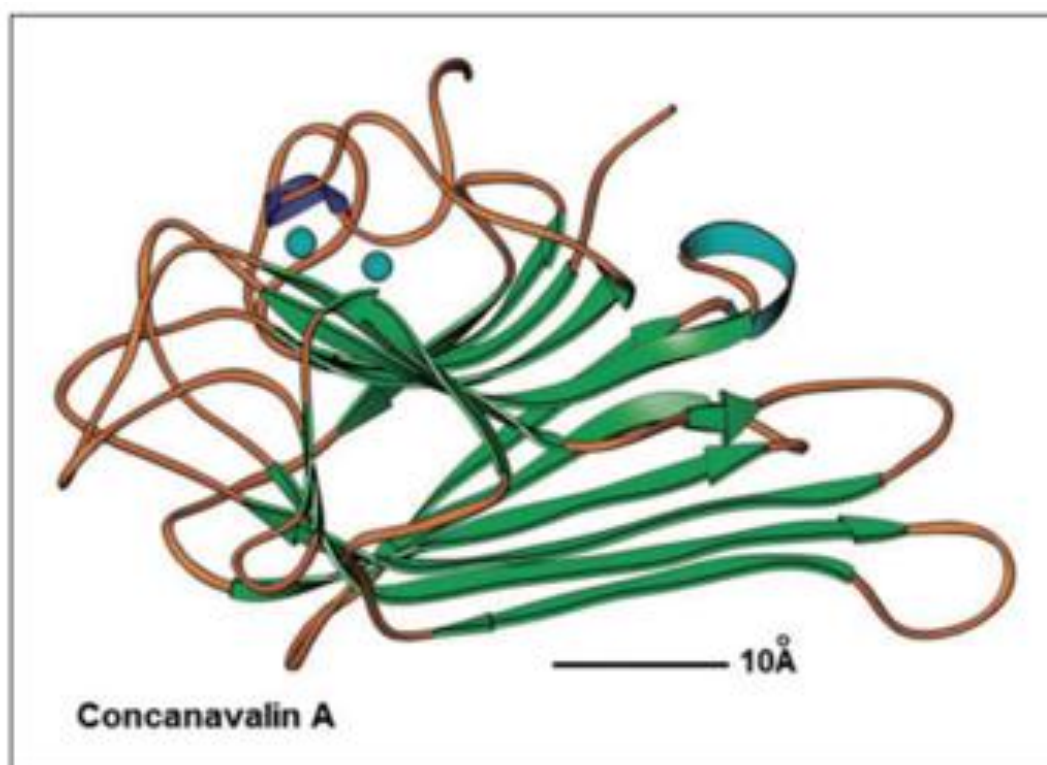
Figure 1 Three-dimensional structure of Concancavalin A (ConA) monomer by using Ribbons XP software (Version 3.0) showed a unique arrangement of molecule, the backbone and binding sites of molecules (Heymann et al., 2015).
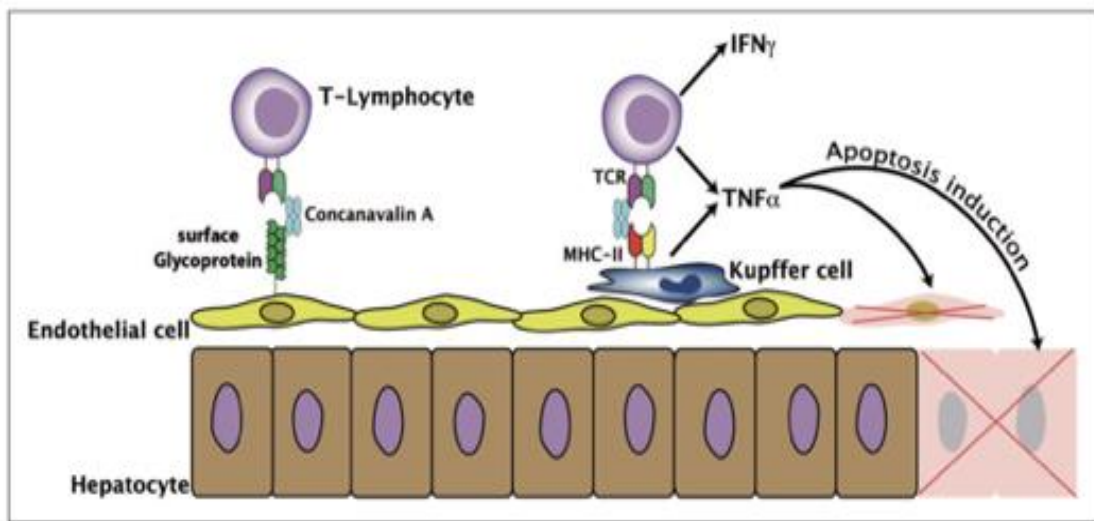
Figure 2 Mechanism of Concanavalin A (Con A), Apoptosis is induced by stimulation of T-cells and macrophages. Firstly, Con A will bind to surface glycoprotein on sinusoid endothelial cells and kupffer cells. In addition, T-cell becomes activated by cross linking their T-cell receptors (TCR) through major histocompatibility complex (MHC) class II. The singling between TCR and MHC class II lead to cytokine release from T-cell such as IFN-g, TNF- a which subsequently induce apoptosis and necrosis. (Heymann et al., 2015).
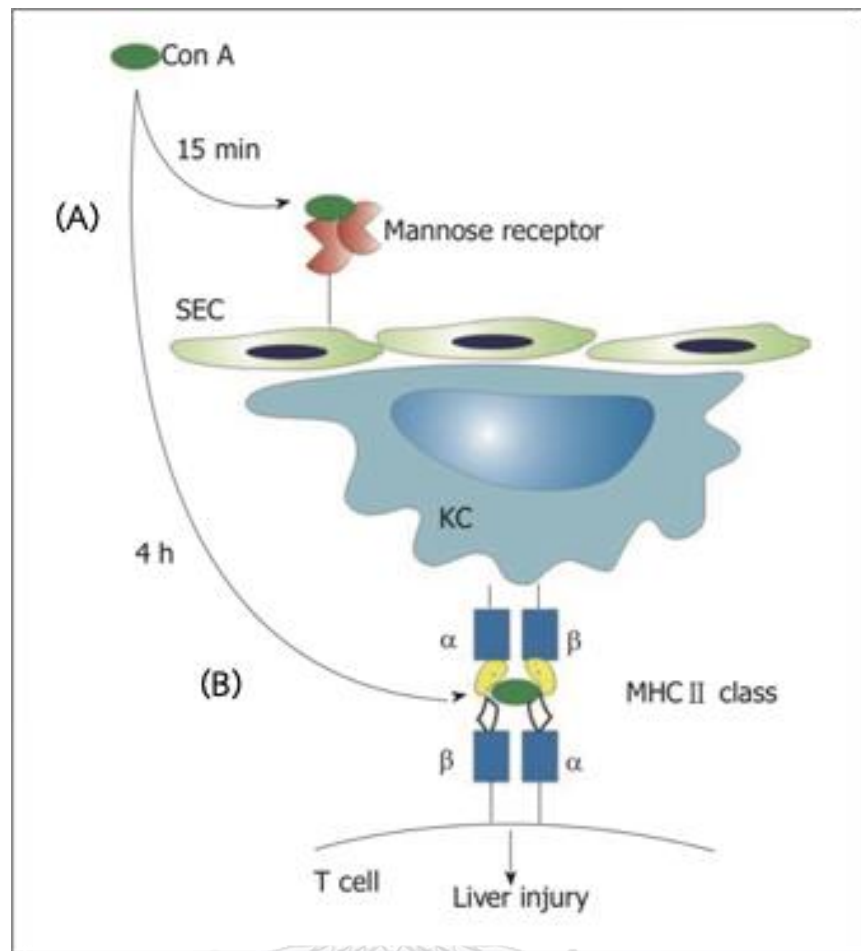
Figure 3 Mechanism and timing of concanavalin A (Con A) induced T cell activated liver injury. (A) First 15 minutes, Con A will bind with mannose receptor on sinusoid endothelial cells and then Con A molecules will infiltrate into Kupffer cells. (B) After 4 hours, Kupffer cells – Con A induce T-cell activation through major histocompatibility class II and liver injury has occurred. (Wang et al., 2012).

2.2 Immunotherapy on autoimmune disease

Immunotherapy or biologic therapy is one type of cancer treatments which boosts the body's natural defenses to treat cancer or autoimmunity. Immonotherapy involves in host defense and immunosurveillance. This therapy utilizes substances made by cell in human body or in a laboratory to improve cell's potential or restore immune system function. Immunotherapy might slow down the growth of cancer cells, inhibit cancer from spreading to other parts of the body and help immune system in order to work better to destroy cancer cells (Caspi, 2008b).

Autoimmune disease is the presence of T-cell and B-cell auto reactivity directed against self-proteins (autoantigens). The early symptoms of many autoimmune diseases are very similar, such as fatigue, achy muscles, swelling and redness, low-grade fever, trouble concentrating, numbness and tingling in the hands and feet, hair loss and skin rashes. Previous researches in animal and clinical studies present antigen-specific immunotherapy or ASI and antigen-non-specific therapies for autoimmune disease (Peakman and Dayan, 2001; Caspi, 2008a). Currently, the diagnosis of AIH by using many indicators such as Liver histopathology, which is observed from the interface of the liver and apoptotic cells compared with characteristics of normal liver. According to studies, it has been found that the liver in the hepatitis patients is noted to be a major abnormality on the liver surface, hepatitis is caused by the autoimmune system and no plasma cells are found. In

most diagnoses, there are eosinophils, lobular inflammation, necrosis, bridging necrosis and multiacinar necrosis, the rare presence of epitheloid histiocytes (granuloma), including bile ducts inflammation, fibrosis, and cirrhosis. The aminotransferase is composed of serum alanine (ALT) or serum aspartate (AST) aminotransferase and alkaline phosphatase (AP). The cytosol of the hepatocytes having plenty of ALT. So, if damage or injury occurs to the hepatocytes, ALT levels will increase in the serum. In addition, hepatocellular injury can detect by AST levels because AST is commonly found in liver and other organs but less than ALT. Thus, in this study AST/ALT were used as an indicator of liver disease evaluation. Previous study found that patients with hepatitis have a significantly higher serum value than normal (Manns et al., 2010).

Autoimmune hepatitis is characterized by at least of the following symptoms is fatigue, hepatosplenomegaly, yellow eyes, itching, skin rash, pain in the joints, the lines appear frayed skin, spider veins on the skin, nausea, vomiting, anorexia, black urine and fecal pale gray (Kogan et al., 2002; Kessler et al., 2004; Czaja, 2005; Feld et al., 2005). In patients with severe symptoms of AIH usually occurs as a result of chronic liver disease with ascites in the abdomen. In a female, many studies reported have no menstruation like normal. Symptoms of AIH are from mild to severe, and more than 70% occur in females (Manns et al., 2010). Studies show that over 40% of untreated will be dead within six months. Patients with a history of AIH have been

found to be more likely to develop cirrhosis and liver cancer (Mistilis et al., 1968; Soloway et al., 1972).

Nowadays, the treatment of AIH is only delaying the disease or preventing the progression of the disease to cirrhosis and liver cancer by giving the medicine corticosteroids, the anti-allergy (prednisone). More than 90% of patients treated with prednisone are required to be immunocompromised. (azathioprine, mycophenilate, mofetil, or methotrexate) to relieve symptoms after steroid therapy. However, long-term and long-term prednisone therapy often produces side effects from medications, such as Moon face, acne, back pain, fat and diabetes or occasionally inflammation has occured. However, studies and development of the same drug, budesonide, have been shown to have a direct effect on inflammation within the liver and have a lesser effect on other organs. The study was not able to evaluate in the long term. There are no alternative treatments that are effective in treating this disease (Ichai et al., 2007; Manns et al., 2010).

2.3 Cytokine-induced killer (CIK) cells

Cytokine-induced killer (CIK) cells is a Th 1 cell-mediated monoclonal antibody, anti-CD3 monoclonal antibody, IL-1 And interferon gamma (IFN-g). The destruction of CIK does not affect the major histocompatibility complex (MHC), group of genes that code for proteins found on the surfaces of cells that help the immune system recognize foreign substances. MHC proteins are found in all higher

vertebrates. MHC molecules in humans are called human leukocyte antigen (HLA). There are two major types of MHC protein molecules—class I and class II. Class I MHC molecules span the membrane of almost every cell in an organism, while class II molecules are restricted to cells of the immune system called macrophages and lymphocytes. MHC molecules are important components of the immune system because they allow T lymphocytes to detect cells, such as macrophages, that have ingested infectious microorganisms. When a macrophage engulfs a microorganism, it partially digests it and displays peptide fragments of the microbe on its surface, bound to MHC molecules. The T lymphocyte recognizes the foreign fragment attached to the MHC molecule and binds to it, stimulating an immune response. In uninfected healthy cells, the MHC molecule presents peptides from its own cell (self-peptides), to which T cells do not normally react. CIK cells can be basically expanded from peripheral blood mononuclear cells (PBMC). The standard culture conditions require three to four weeks with the timed addition of IFN-g, mAb-anti CD3 and interleukin (IL) - 2 (Schmidt-Wolf et al., 1991; Vergani and Mieli-Vergani, 2008). IFN-g is cytokine produced by Th 1 cells, CD8^{+} T cells, NK T cells, and NK cells. IFN-g is added in the first days of cell culture. The main function of IFN-g is stimulating T-cells through CD58 and IL-12 to increase number of CIK cells and act as a critical mediator of T cell recruitment and phagocyte-mediated clearance of pathogens. In macrophages, IFN-g triggers cytokine and chemokine synthesis, enhanced oxygen radical generation and nitric oxide (NO) production, and induction

of major histocompatibility complex class II expression (Schmidt-Wolf et al., 1994). Monoclonal antibody-anti CD3 or mAb-anti CD3 is added in the second days of cell culture. mAb-anti CD3 is antibody that binds to CD3 on the surface of T cells, it serves signal to T-cell and support IL-2 to expand the number of CIK cells (Schmidt-Wolf et al., 1994). Interleukin or IL-2 is added in every 2 days for 14 days in Figure 4 (Jiang et al., 2013; Cappuzzello et al., 2017; Pluangnooch et al., 2017; Gao et al., 2018). In cell culture, it is a cytokine that acts as an important regulator of the immune system and increases the accumulation of cells (Cappuzzello et al., 2017).

From this study shows the relationship between hepatitis disease and CIK cells, scientists found that CIK cells act by inhibiting viral replication in patients with chronic hepatitis (Sun et al., 2006). *In vitro* and *in vivo*, because CIK cells can damage irregular or cancer cells without MHC (Aravalli and Steer, 2017). Previous research shows that CIK cells exhibit CD4 expression (protein molecules on helper T cell surface or T_H) and CD8 (protein molecules on the surface of cytotoxic T cells or T_C). Moreover, CIK cells have an indicted molecule of CD3 (protein molecules on the surface of the T cell in general) and CD56 (protein molecules on the surface of the natural killer cell or NK) on the cell surface. This means that both the T-cell and the natural killer (NK) cells are expressed in the same cell. The study found that autologous CIK cells are capable of destroying and inhibiting the distribution of hepatitis in patients with liver cancer (Shi et al., 2009). It was also found that in certain patients treated with CIK cells in combination with dendritic cells, the genetic

material of the virus could be reduced. AIH has not yet been studied by using CIK cells. AIH is a disease caused by its own white blood cell, which is a normal cell. CIK cells are unlikely to destroy and may damage the liver cells as well. Cytokines released from the cytoplasm are responsible for cell damage such as IFN-g, granzyme B, and perforin. Granzyme B is an enzyme in the serine protease group released by cytotoxic T lymphocytes and natural killer cells, the mediator that leads to cell apoptosis and against viral infections of cells include cancer (Lieberman and Fan, 2003). The mechanism of granzyme B is related with perforin by secretory granules that contain both enzymes in cytotoxic T lymphocytes. Secretory granules will traffic into outside by microtubule and will fuse of granule with the presynaptic membrane and release both of granzyme B and perforin to target cell for cell death by pore construction around target cell in Figure 5 (Voskoboinik et al., 2015).

2.4 Protective effects of immune cells on autoimmune disease

Autoimmune is a condition in which immune system attacks our cells. In the body, regulatory T cell or Treg have an important role in the maintenance of self-tolerance. Previous study shows the relationship between iNKT cells and Treg in protective effects on autoimmune myasthenia gravis (EAMG) (Liu et al., 2005). Moreover, B-cells have protective effects on atherosclerosis and reduces disease progression of hypercholesterolemic mice (Caligiuri et al., 2002).

From previous study showed a relationship between immune cells and several types of autoimmune diseases. The important point is AIH caused by T helper 1 cells and CIK cell is a type of Th1 cells. Thus, the interesting question of this study is how will CIK cell affects AIH disease and how does CIK cells play a role on AIH? There are few studies about CIK cell studies of AIH patients in both animal and clinical trials. The hypothesis of this study is CIK cells does not affect on AIH. The aim of this study was to investigate and observe the protective effects of CIK on AIH.
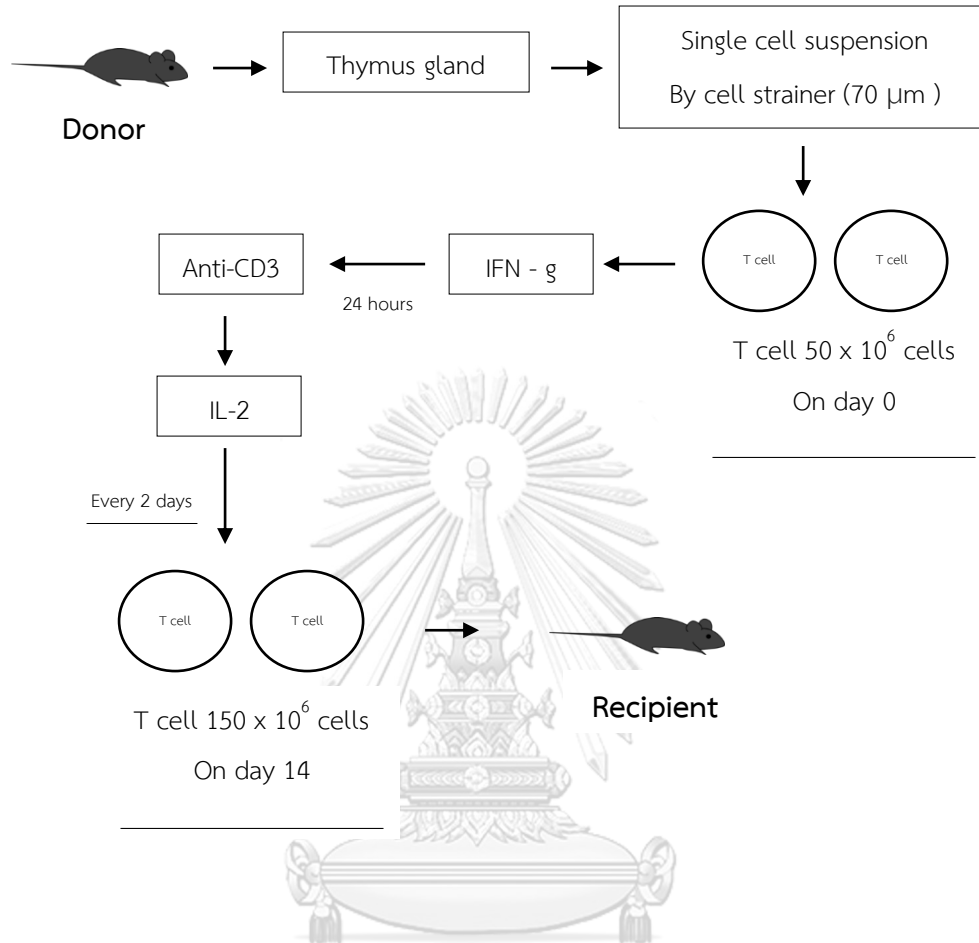
Figure 4 Overview of the procedure in CIK cells culture by using standard protocol

from peripheral blood mononuclear cells (PBMC) from donor.
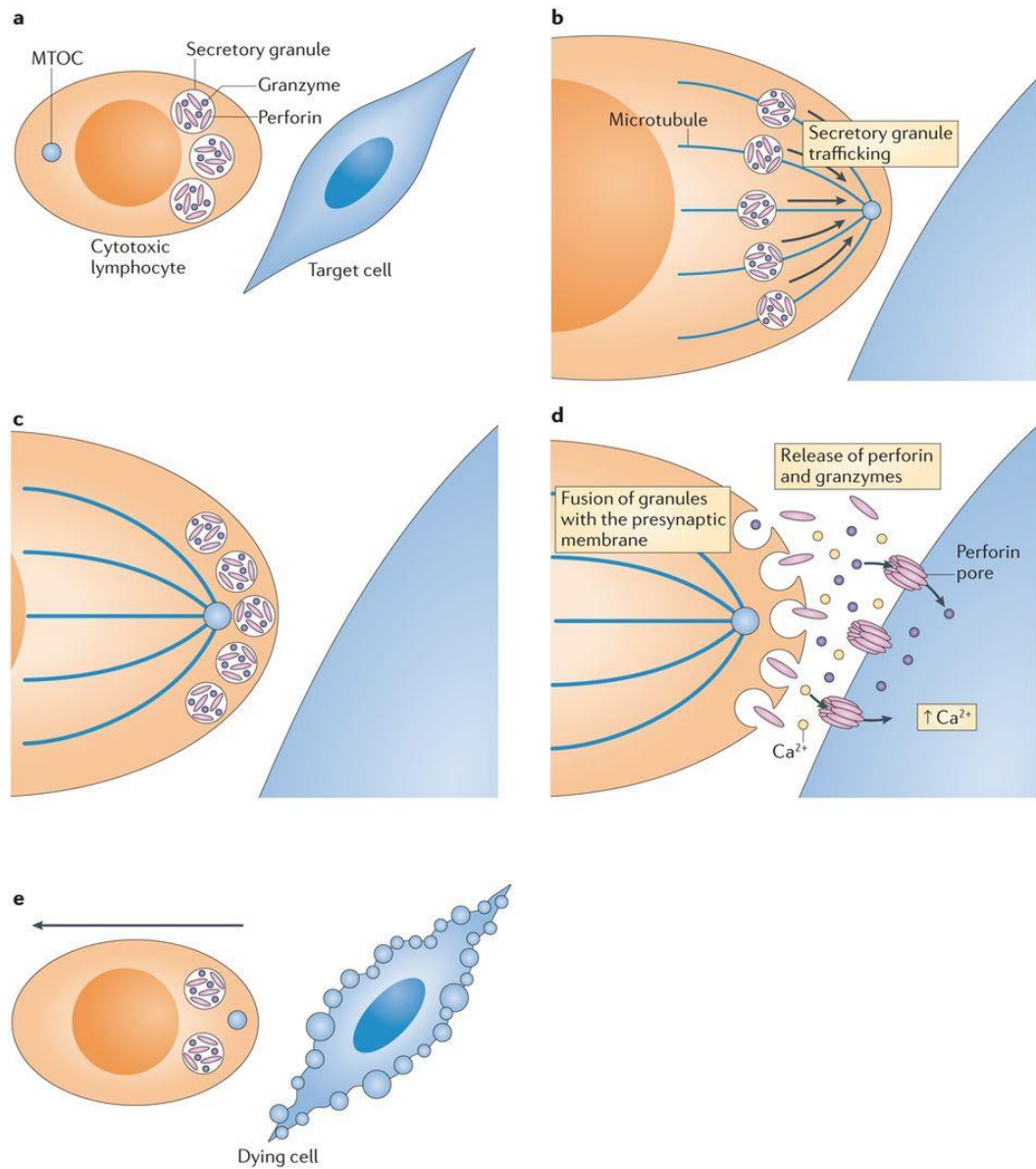
Figure 5 Overview of the procedure in cell death by using granzyme B - perforin that released from secretory granule in cytotoxic T lymphocytes (Voskoboinik et al., 2015).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Culture media consists of

- Roswell Park Memorial Institute Medium; RPMI-1640 (Invitrogen, USA)

- Fetal bovine serum (Biochrome, Germany)

- Penicillin-streptomycin (Invitrogen, USA)

- Trypan blue stain (Invitrogen, USA)

- Sodium hydrogen carbonate; $NaHCO_3$ (Merck, Germany)

- Sodium chloride; NaCl (Merck, Germany)

- Potassium chloride; KCl (Merck, Germany)

- Sodium hydrogen phosphate; Na_2HPO_4 (Merck, Germany)

- Potassium dihydrogen phosphate; KH_2PO_4 (Merck, Germany)

3.1.2 Recombinant cytokines and Antibody

- Recombinant mouse interferon gamma; IFN-g (eBioscience, USA)

- Recombinant mouse interleukin 2; IL-2 (eBioscience, USA)

- Anti-mouse CD3e (eBioscience, USA)

- Anti-mouse CD90.2 (Thy1.2) monoclonal antibody, Fluorescein

isothiocyanate (FITC) conjugated (eBioscience, USA)

- Anti-mouse NK1.1 monoclonal antibody, Phycoerythrin (PE)

conjugated (eBioscience, USA)

- Anti-mouse IFN-g monoclonal antibody, Phycoerythrin (PE)

conjugated (eBioscience, USA)

- Anti-mouse granzyme B monoclonal antibody, Allophycocyanin

(APC) conjugated (eBioscience, USA)

- Anti-mouse perforin monoclonal antibody, Phycoerythrin (PE)

conjugated (eBioscience, USA)

3.1.3 Chemicals

- Phorbol 12-myristate 13-acetate; PMA (Sigma-Aldrich, USA)

- Ionomycin calcium salt (Sigma-Aldrich, USA)

- GolgiPlug (BD biosciences, USA)

- Fixation/Permeabilization solution (BD biosciences, USA)

- Perm/Wash buffer (BD biosciences, USA)

- Cytoflex sheath fluid (Beckman Coulter, USA)

- Thiopental sodium (THIOPEN, India)

- Paraformaldehyde; $OH(CH_2O)_nH$ (Merck, Germany)

- L7647-25MG LECTIN FROM CONCANAVALIN A TYPE VI, CAS number:

011028-71-0 (Sigma-Aldrich, USA)

- Alanine Aminotransferase (ALT/GPT) (BioSystems, Spain)

- Aspartate Aminotransferase (AST/GOT) (BioSystems, Spain)

3.1.4 Instruments

- Biological safety cabinets (Thermo Scientific, USA)

- CO_2 incubator (Forma Scientific Inc, USA)

- Inverted microscope (Olympus, Japan)

- Fluorescence microscope (Nikon, USA)

- Flow cytometer (Beckman Coulter, USA)

- Centrifuge for 15, 50 ml centrifuge tubes (Eppendorf, Germany)

- Centrifuge for microcentrifuge tubes (Tomy, Japan)

- Random access analyser (A15) (BioSystems, Spain)

- Micropipette 10, 20, 100, 200, 1000 µl (Eppendorf, Germany)

- Multichannel pipettor (Eppendorf, Germany)

- Cell strainer; 70 uM (BD Falcon, USA)

- 15 and 50 ml centrifuge tube (Corning, USA)

- 6 and 10 cm tissue culture dish (Corning, USA)

- 1 ml Insulin syringe (BD, USA)

- 1, 3 ml syringes (TERUMO, Philippines)

3.2 Methods

3.2.1 Animals and ethic statement

Use C57BL/6Mlac mouse ages 6 to 8 weeks, body weight 20 - 25 grams from National Laboratory Animal Center (Mahidol University, Bangkok, Thailand). The mice were acclimatised for 1 week. Mouse were Isolated under an aseptic system and a 12-hour light-dark cycle (8.00 - 19.00 hrs). The mice were house in temperature-controlled (22 ± 1 °C), humidity 30-70% with water and food freely (Ad Libitum) and then divided into 6 mice per group; 1.donor group 2.recipients group, 2.1 PBS and Con A group (PBS + ConA) with 2.2. CIK and ConA received CIK + ConA for protection effect study. All experiments were performed in accordance with the guidelines of Chulalongkorn University Laboratory Animal Center (CULAC) and approved by the Committee on Animal Care and Use of CULAC No. 1673020 (27 October 2016).

3.2.2 Generation of CIK cells

C57BL/6Mlac mouse were euthanised and thymus gland were removed from C57BL/6Mlac mice and single cell suspenend through a cell strainer. Thymocytes were suspended at a 3×10^6 cells per ml in a RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS; Biochrome, Germany), 100 U/ml penicillin-streptomycin (Gibco, USA). The cell suspensions were transferred to 10 cm cell culture dish (10mL/dish) with 2000 U/mL recombinant mouse IFN-g (eBioscience, USA) on the first day of cell culture and incubated at 37°C in a 5% CO_2 incubator.

Twenty-four hours later, cells were transferred to 100 ng/mL of anti-CD3 mAb (eBioscience, USA) coated dish and 300 IU/mL recombinant mouse IL-2 (eBioscience, USA) was added. Every 2 days, complete RPMI was added supplemented with IL-2 (50 IU/mL). On day 14, cells were collected and evaluated by flow cytometry as shown in Figure6.
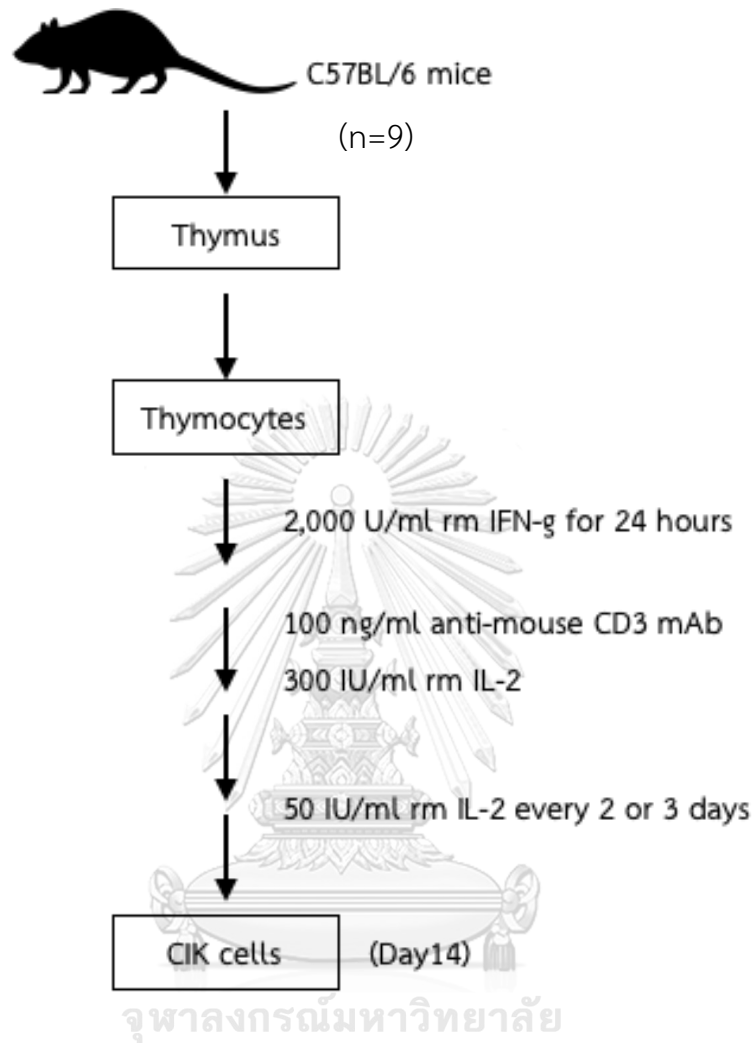
Figure 6 Schematic protocol for generation of CIK cells

3.2.3 Flow cytometry analysis

The mAbs were used to evaluate CIK cell markers: CD3 mAbs (eBioscience, USA) conjugated to fluorescein isothiocynate (FITC), NK1.1 mAbs (BioLegend, CA) conjugated to phycoerythrin (PE). Approximately 10^6 cells were incubated with specific antibodies for 30 minutes at 4°C. Excess antibody was removed by washing, and cells were analyzed using a CytoFLEX (Beckman Coulter, USA). For intracellular cytokine staining, CIK cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, USA) and 1ug/mL ionomycin calcium salt (Sigma-Aldrich, USA) and incubated overnight. Three hours later, Brefeldin A (BD biosciences, USA) was added to prevent the secretion of cytokines. CIK cells were first stained for cell surface markers, then fixed with Fixation/Permeabilization solution (BD biosciences, USA), washed with Perm/Wash buffer (BD biosciences, USA) and incubated with PE-labeled anti-IFN-g, perforin and APC-labeled anti-granzyme B (eBioscience,USA) for 30 minutes on 4°C or on ice. Stained cells were analyzed by CytoFLEX (Beckman Coulter, USA).

3.2.4 Concanavalin A model of AIH

Induction of AIH by injection of Concanavalin A (Con A) solution (Sigma-Aldrich, USA) at 25 mg/kg of mouse in 200 μl PBS solution, through the lateral tail vein, the mouse was injected with Con A solution in single dose.

3.2.5 Cytokine-induced killer (CIK) cells administration in animal

To observe the role of CIK cells in prevention effects. Ten million of CIK cells were injected intravenously (200 µl PBS) before induction of AIH with Con A solution (25 mg / kg) (Kim et al., 2007). After 24 hours by using 1 ml Insulin syringe (BD, USA). Mice were euthanized with 270mg/kg thiopental sodium and the liver and serum in blood were collected as shown in Figure 7.

3.2.6 Histopathology of liver

After euthanasia, remove the liver carefully and wash with normal saline (0.9%). Liver were fixed in Davidson solution (37% formalin, alcohol, glacial acetic acid, tap water) or formalin. Hisopathology studies were performed on the liver with fixed solution for at least 24 hours, followed by tissue preparation with paraffin method, and then cut to 4-6 µm. Hematoxylin and eosin (H & E) were examined before imaging using a light microscope by staining the liver tissue with H & E.

Formula of Hematoxylin (Delafield)

Harris's Hematoxylin

Hematoxylin crystals	5 g
Ammonium alum [$(NH_4)_2SO_4.Al_2(SO_4)_3.24(H_2O)$]	100 g
100% ethanol	50 ml

Mercuric oxide (red)	2.5 g
Distilled water	100 ml

Dissolve the hematoxylin in the alcohol, the alum in water by the aid of heat. Remove from heat and mix the two solutions. Bring to boil rapidly possible. Remove from heat and add mercuric oxide slowly. Reheat to simmer until it become dark purple, remove from heat immediately and plunge the vessel into basin of cold water until cool. Finally add 2-4 ml of glacial acetic acid per 100 ml of solution. Before using, mixed solution was keep away from light for 2-4 hour.

Differentiator

Conc. HCl	4 drops
70% ethanol	100 ml

Eosin + Phlosine B

Eosin stock solution	100 ml
Eosin Y	1 g
Distilled water	100 ml
Phloxine stock solution	10 ml
Phloxine B	1 g
Distilled water	100 ml

Working solution

Eosin stock solution	100 ml
Phloxine stock solution	5 ml

95% ethanol	780 ml
Glacial acetic acid	4 ml

Harris hematoxylin and eosin staining procedure

Harris hematoxylin and eosin were performed by deparaffinize and hydrate with xylene 30 second for 2 times. Then the slides were dipped in absolute ethanol 30 second for 2 times. The slides were dipped in 95% ethanol 30 second for 2 times and tap water. The slides were transfered to Harris hematoxylin for 15 minutes, rinsed in tap water, dipped in 1% acid alcohol for 2 second and then washed in tap water. The slides were dipped in saturated lithium carbonate solution, washed in running tap water. The slides were stained with eosin for 1 minute and dehydrated and clearing with 95% ethanol 30 second for 2 times, absolute ethanol 30 second for 2 times and xylene 30 second for 2 times. Finally, all slides were mounted with DePex mounting media.

The histopathology and pathology were evaluated using the criteria with the following details:

- Characteristics of hepatocellular, central veins

- Leukocytic infiltration

- Necrotic foci or area

- Fibrosis

Then use Image J program version 2.0.0 to measure the necrotic area from 40x histopathological image of liver that represents in mean ± standard deviation (SD) and compare with normal group for histopathology evaluation by using ratio between average of necrotic area in experimental group; average of normal area then percentage were calculated.

3.2.7 Blood chemistry test

Blood samples were drawn from retro-orbital vein or cardiac puncture and incubated at room temperature for 30 min. Then centrifuge at 4 °C, 1,000 xg for 20 min and the serum was collected about 150-200 µl per test. All serums were stored at -80 °C and then serums were measured aspartase aminotransferase (AST) and alanine aminotransferase (ALT) levels in the laboratory by using Analyzer A15.

3.2.8 Statistical analysis

The data in each group is represented as mean ± standard error of mean (SEM). SPSS version 22.0 (SPSS Inc., USA) was used to compare data between experimental groups. The blood chemistry (AST and ALT levels) test between control group and experimental group were performed by unpaired student's t test to analyze significant difference (P-value <0.05).

In the case of compare more than two groups, the data are parametric. Using one-way analysis of variance (ANOVA) to test significant difference and Tukey HSD tests were used for data analysis. On the other hand, using statistics called Kruskal-

Wallis test for non-parametric data. All statistical analysis was set 95% confidence (P-value <0.05).

3.3 Study design
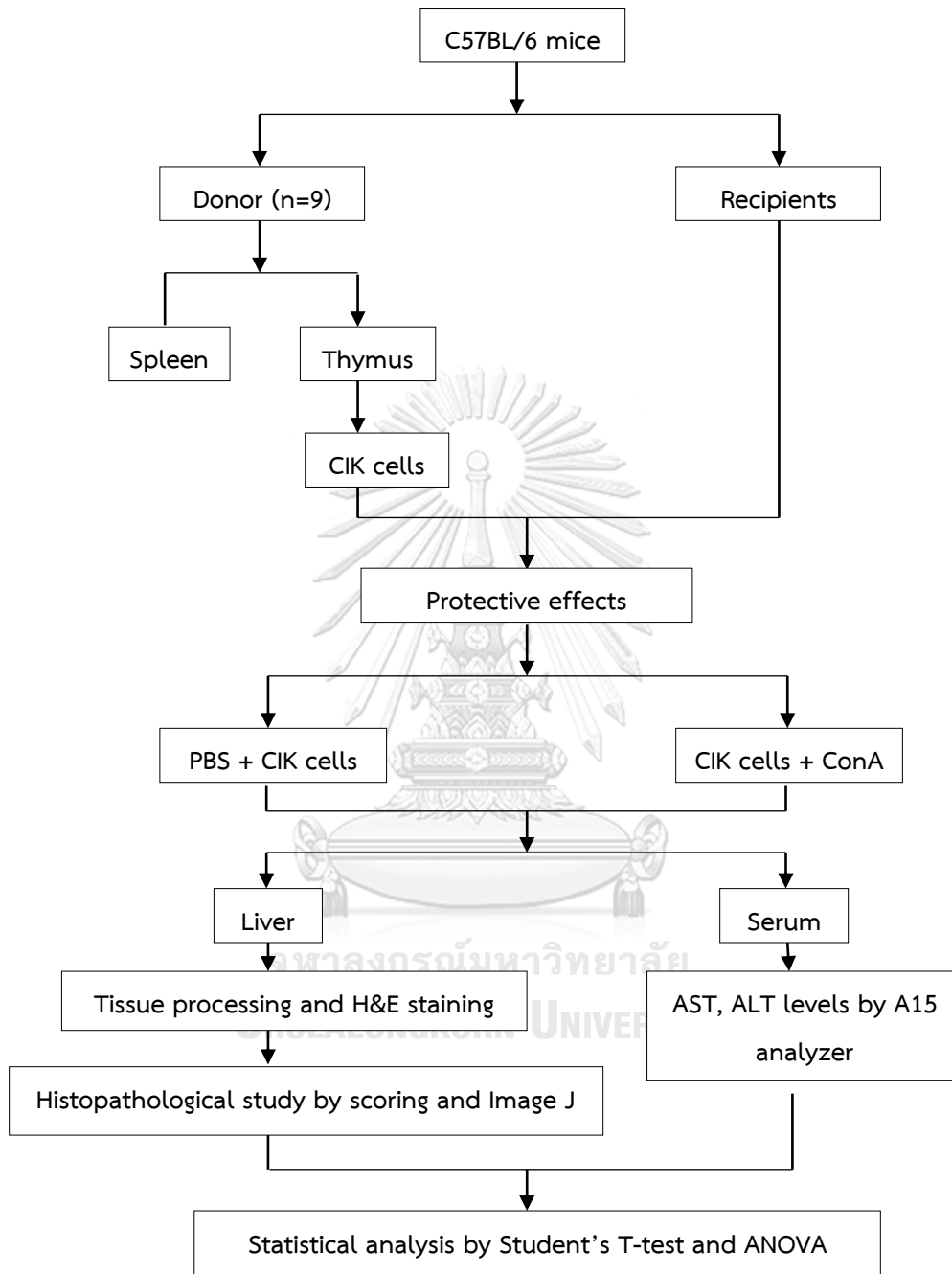
Figure 7 Study plan of concanavalin - A induced autoimmune hepatitis in C57BL/6

mice using CIK cells.

CHAPTER IV

RESULTS

4.1 CIK cells confirmation by T cells and NK cells properties

Confirmation T-cell from thymus of C57BL/6 mice were really CIK cells, CIK cells are T-cell and Natural killer (NK) cells phenotypic combination so flow cytometry was used to examine phenotypic and functional properties of the cells. In this study under the cultured condition show the increase surface marker up to 41.60% and the cytokines that release from CIK cells compared with previous study showed IFN-g (CD3 IFN-g: 5.43% versus 1.24%) and cytolytic granules granzyme B (58.96% versus 0.14%) and perforin (9.14% versus 0.14%) (Pluangnooch et al., 2017). From this result confirms that T-cell from cell culture were CIK cells as shown in figure 8.
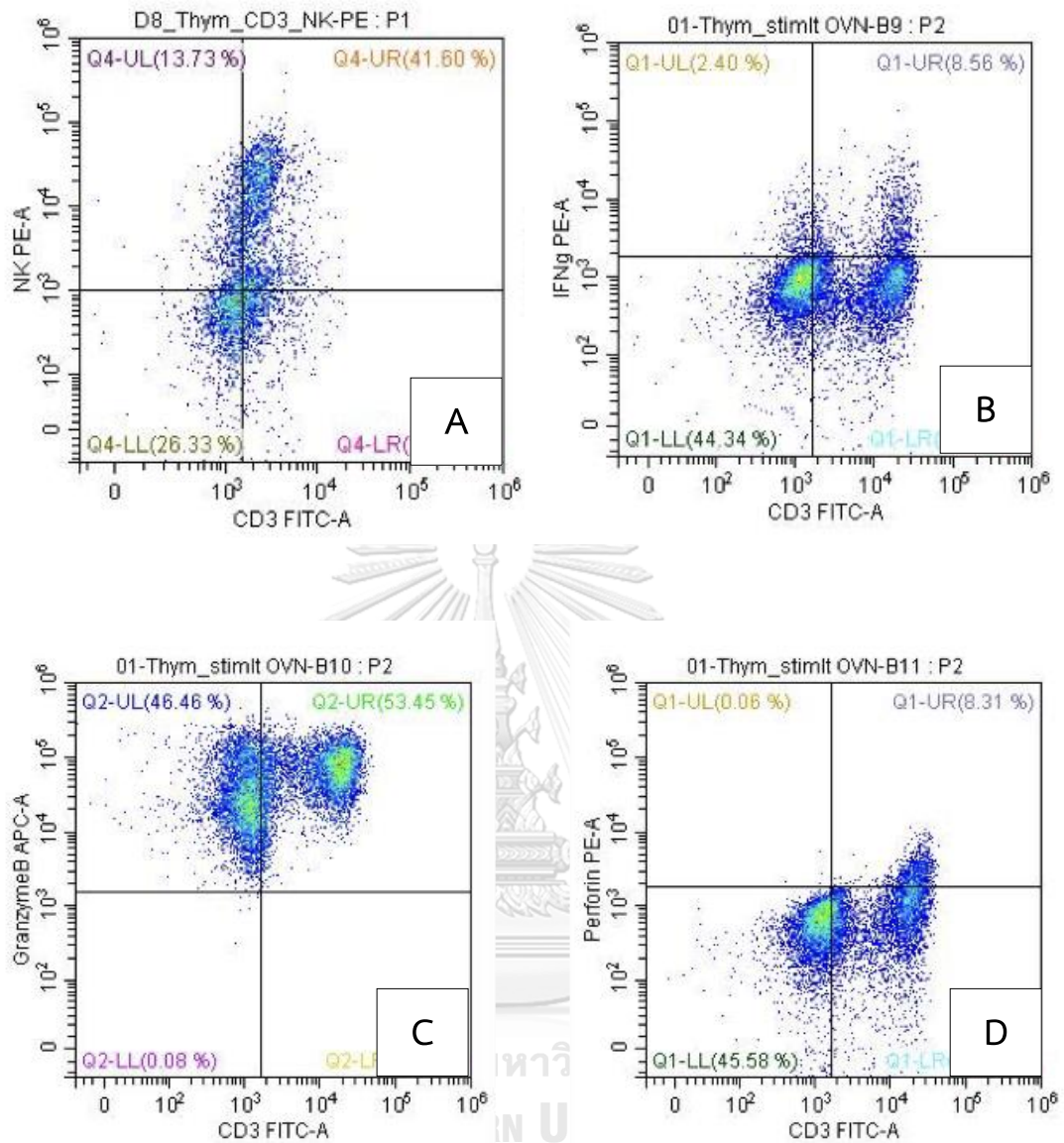
Figure 8 CIK cells were phenotypically analyzed by flow cytometry. Thymocytes from C57BL/6 mice were cultured under the CIK culture condition. Fourteen days later, CIK cells were stained by using specific directly conjugated anti-mouse surface or cytokine mAbs. A. CD3$^+$NK1.1 41.60%, B. CD3$^+$IFN-g 8.56%, C. CD3$^+$GranzymeB 53.45% and D. CD3$^+$Perforin 8.31%.

4.2 Pathological examination of mouse liver

Liver from C57BL/6 mice after euthanasia with thiopental sodium (THIOPEN, India) 270 mg/kg or overdose and rinse with PBS for examination. In control group, normal liver found a clear and smooth surface, a yellow gallbladder and no sign of cell apoptosis/necrosis. In experiment group; PBS + Con A found white spots or patches spread in liver and the color of gallbladder is green, inflammatory gallbladder as shown in Figure 9. In experiment group; PBS + Con A found symptoms similar to the CIK + Con A group as shown in Figure 10.
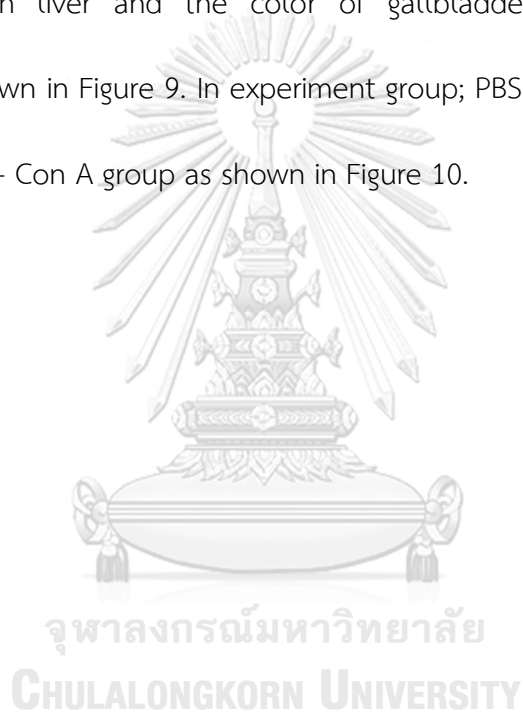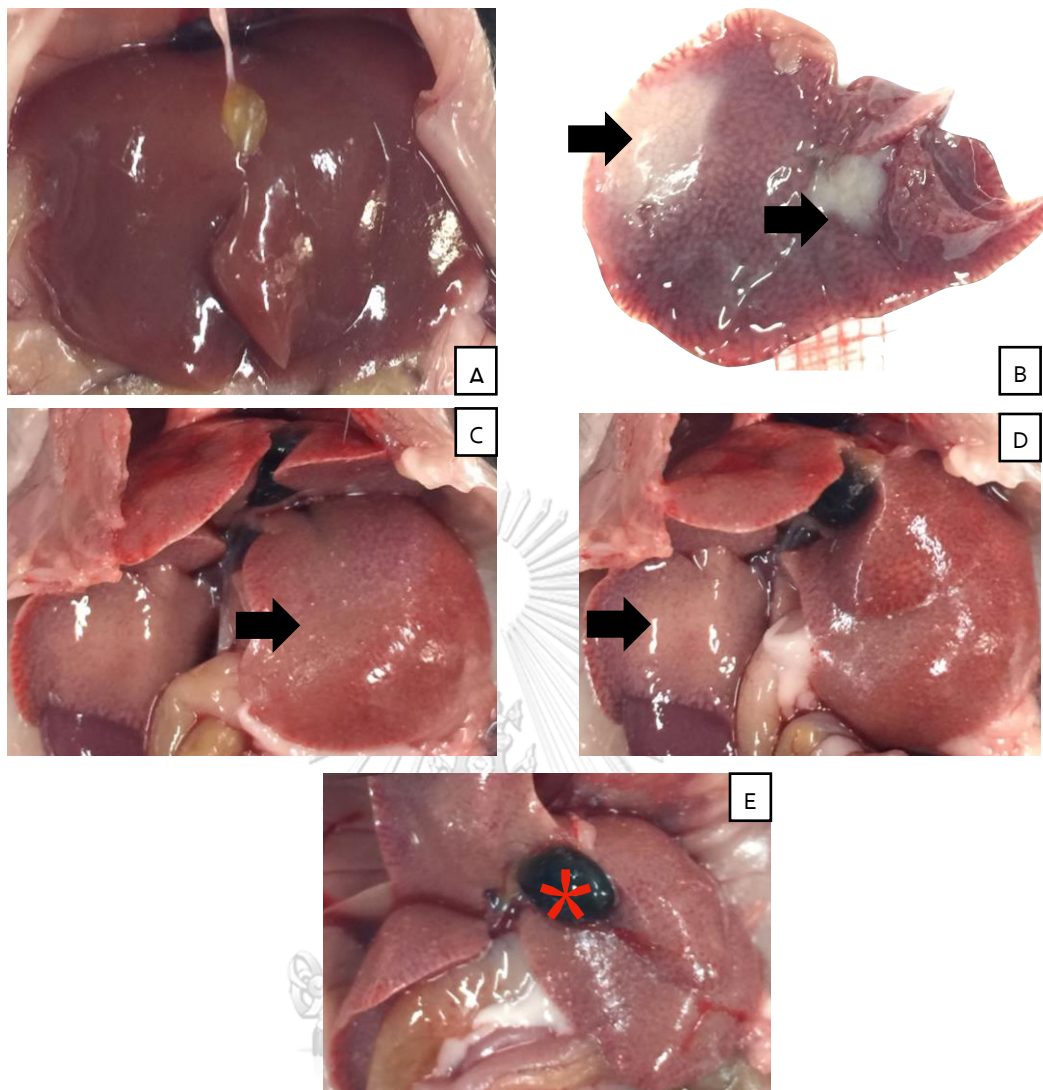
Figure 9 Pathology of mouse liver. A: Control group showing normal liver have a clear surface area and have a yellow color of gallbladder. B-D: PBS + Con A group showing a white spots or patches spread the liver (red arrow). E: PBS + Con A group showing inflammatory gallbladder in green color (red star).
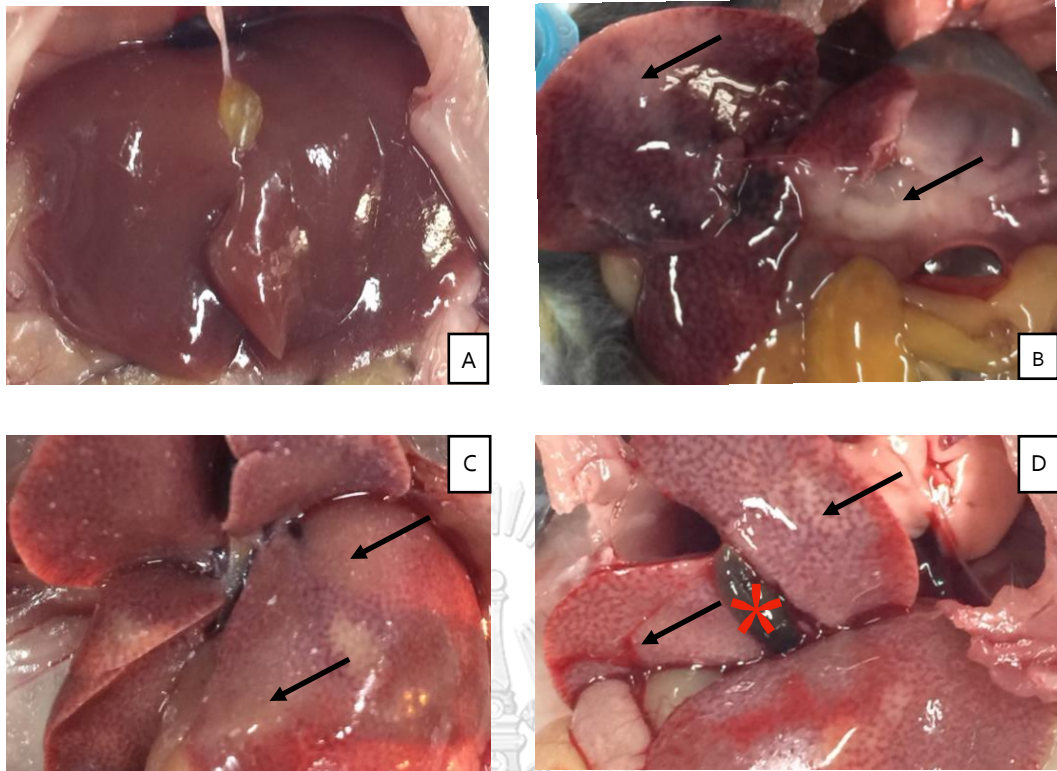
Figure 10 Pathology of mouse liver. A: Control group showing normal liver have a clear surface area and have a yellow color of gallbladder. B-C: CIK + Con A group showing a white spots or patches spread the liver (arrow). D: CIK + Con A group showing inflammatory gallbladder in green color (red star).

4.3 Histopathological examination of mouse liver

Sections of mouse liver stained with a Harris hematoxylin and eosin (H&E) procedure reveal acute inflammation of liver by using characteristics of hepatocellular, central veins, leukocytic infiltration, necrotic foci or area and fibrosis. Liver section composes of 3 zones, based on oxygen supply. Zone 1 (periportal lobule) encircles the portal tracts where the oxygenated blood from hepatic arteries enters. Zone 3 (Centrilobular area) is located around central veins, where oxygenation is poor. Zone 2 (Midzonal region) is located in between (Figure 11.)

The results found that in control group showed normal hepatocellular, no infiltration of leukocytes, no apoptotic or necrotic area. Central veins were grossly intact and no fibrotic tissue was observed (Figure 12.) But both of experimental groups (PBS + Con A and CIK + Con A) showed generalized hepatocellular swelling with scattered area of leukocytic infiltration. Multiple necrotic foci at the periportal zone (zone 1) occupy approximately 4.00 and 5.80 percentage of total liver parenchyma respectively. Central veins are grossly intact. No fibrotic tissue was observed as shown in Figures 13 and 14.
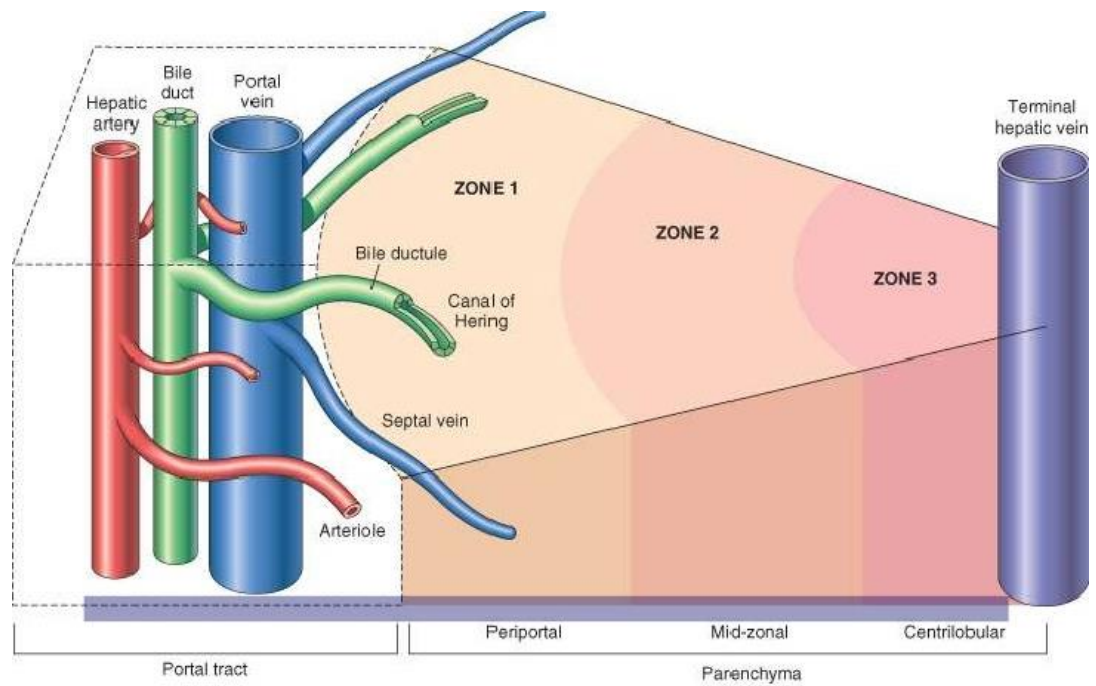
Figure 11 Diagram shows 3 zones of hepatic lobule, zone1 (periportal lobule),

zone 2 (Midzonal region) and zone 3 (Centrilobular area).

Reference: http://geoface.info/ef8714/microscopic-anatomy-of-liver.
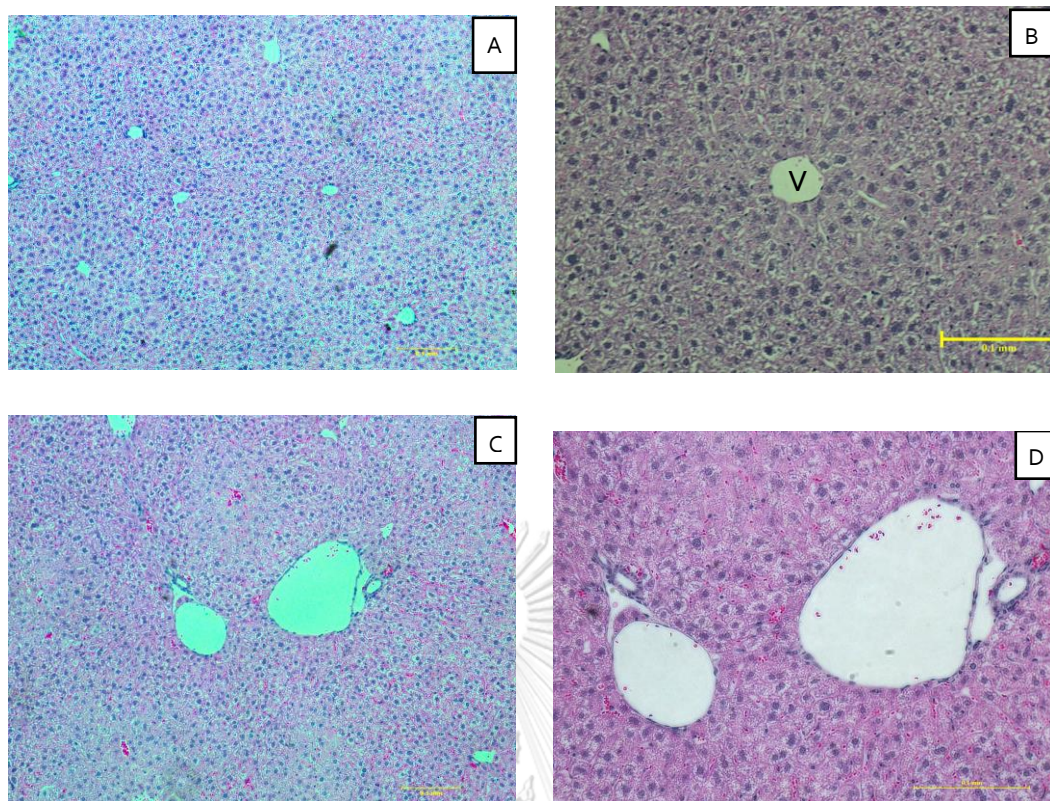
Figure 12 Light photomicrography of liver of control mices stained with H&E. A-B showing normal hepatic lobule has a thin walled central vein (V), hepatic cords radiating toward the periphery alternating with hepatic sinusoids lined by Kupffer cells and endothelial cells. No apoptotic or necrosis area in liver surface. A - X,40, B - X,400. C-D showing normal portal triads, contain a bile duct, a small hepatic artery and portal vein branch, surround by type I & III collagen. No fibrotic tissue was observed. C - X,200, D - X,400.
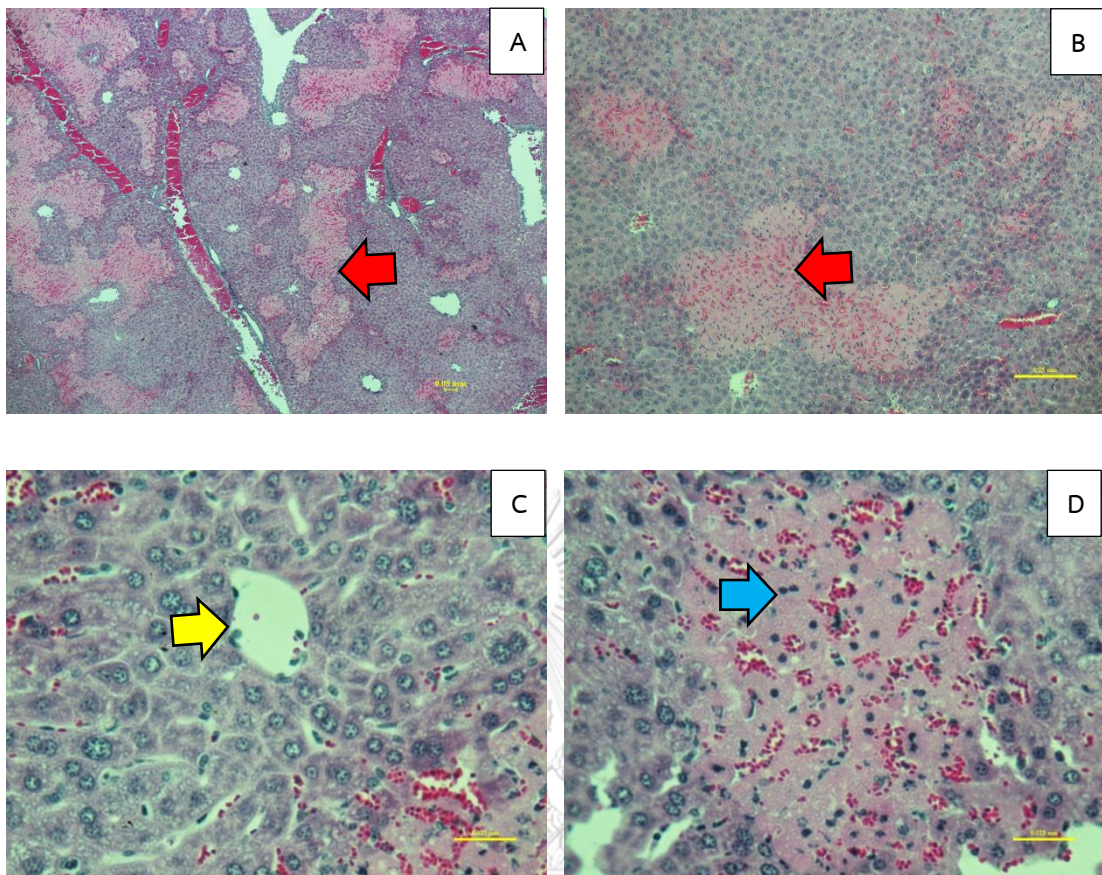
Figure 13 Light photomicrography of liver of experimental (PBS + ConA) mices stained with H&E. A-B showing generalized hepatocellular swelling with scattered area of leukocytic infiltration (blue arrow). Multiple necrotic foci at the periportal zone (zone 1) (red arrow) occupy approximately 4.00 percentage of total liver parenchyma. Central veins are grossly intact (yellow arrow). No fibrotic tissue was observed. A - X,40, B - X,100, C - X,400, D - X,400.
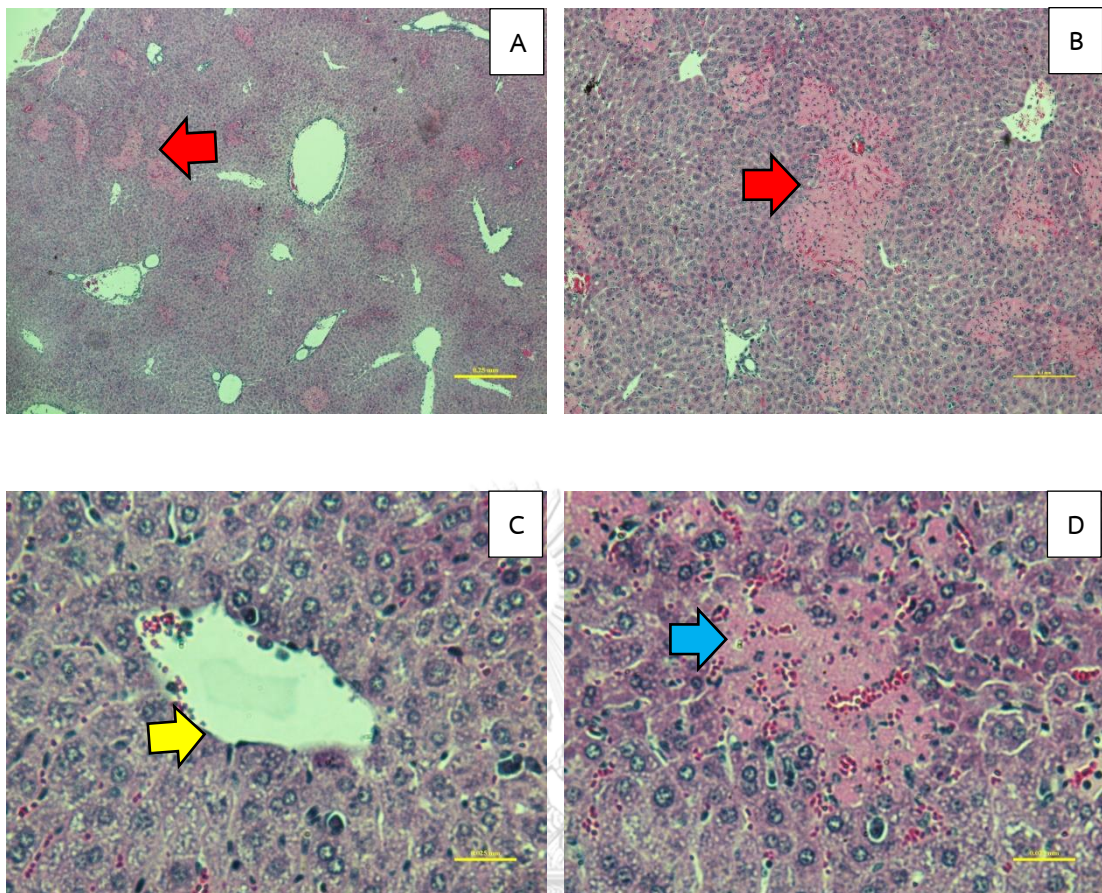
Figure 14 Light photomicrography of liver of experimental (CIK + ConA) mices stained with H&E. A-D showing generalized hepatocellular swelling with scattered area of leukocytic infiltration (blue arrow). Multiple necrotic foci at the periportal zone (zone 1) (red arrow) occupy approximately 5.8 percentage of total liver parenchyma. Central veins are grossly intact (yellow arrow). No fibrotic tissue was observed A - X,40, B - X,100, C - X,400, D - X,400.

4.4 Biochemistry examination of mouse serum

Biochemical blood serum levels for AST measurements were presented as mean ± standard error (SE). The mean values of control, Con A + PBS and Con A + CIK were 48.33 ± 0.88 U L, 398.75 ± 87.86 U / L and 487.33 ± 67.90 U / L, respectively. There was no statistically significant differences (p <0.05) between the control and experimental groups (p <0.05). But the trend of AST was higher in the experimental group CIK + Con A as shown in Figure 15. In addition, Biochemical blood serum levels for ALT measurements were presented the mean values of control, Con A + PBS and Con A + CIK 38.33 ± 2.03 U / L, 113.75 ± 22.44 U / L and 199.00 ± 44.18 U / L, respectively. The control group and the PBS + Con A group showed no statistically significant difference (P> 0.05) while the control group and the CIK + Con A group showed significant difference (P <0.05). There was no statistically significant difference (P> 0.05) among the experimental groups. However, the trend of elevated ALT was higher in the CIK + Con A as shown in Figure 16.
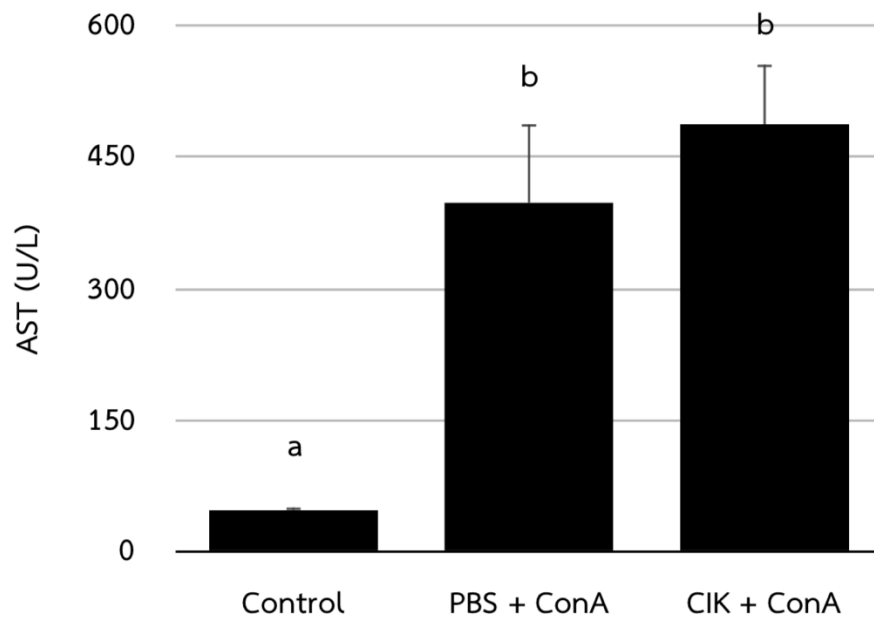
Figure 15 The levels of AST were measured in control group and experimental group

(PBS + ConA, CIK + ConA). The results shown are from 2 independent experiments

and each bar represents mean ± SEM of 6-9 mice per group.
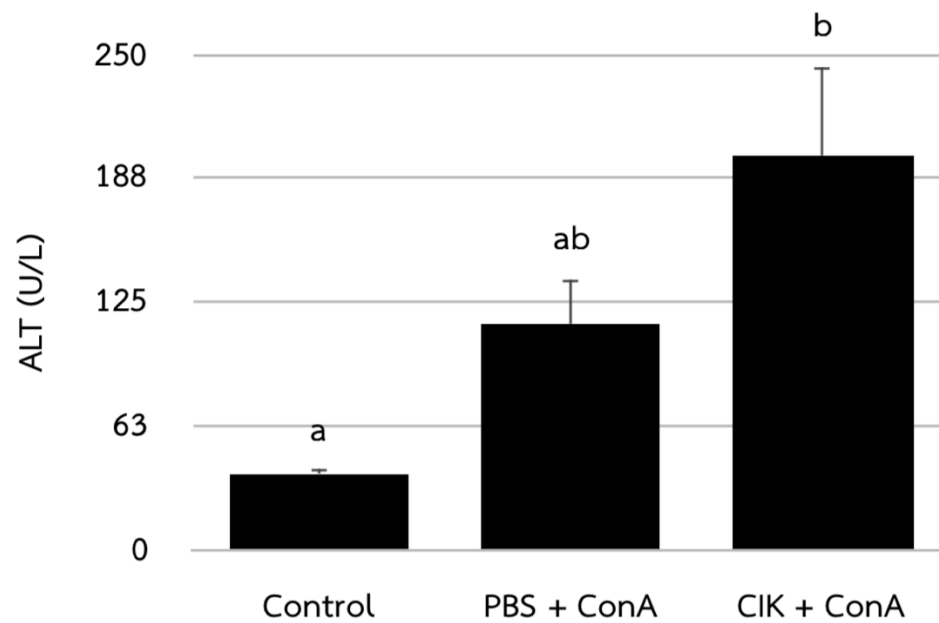
Figure 16 The levels of ALT were measured in control group and experimental group (PBS + ConA, CIK + ConA). The results shown are from 2 independent experiments and each bar represents mean ± SEM of 6-9 mice per group.

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATION

CIK cells were culture from thymocytes in thymus gland of naive C57BL/6 Mlac mice by using developing protocol for 14 days. After that CIK cells were evaluated surface marker (T and NK cells) and cytokines (IFN-g, cytolytic granzyme B and Perforin) by using flow cytometry. In this study, CIK cells presented double positive T and NK cells (CD3$^+$NK1.1$^+$) up to 41.60% and the cytokines that release from CIK cells compared with naive thymocytes of previous study showed IFN-g (CD3 IFN-g: 5.43% versus 1.24%) and cytolytic granules granzyme B (58.96% versus 0.14%) and perforin (9.14% versus 0.14%) (Pluangnooch et al., 2017). This result confirms that CIK cells from developing culture can use in this experiment. Previous study reports many cytokines that release from CIK cells related with autoimmune disease, IFN-g play an important role in cell regulatory T-cell (T$_{reg}$) development such as exogenous IFN-g in animal model plays a critical role to increase disease severity (Sabine K. et al., 1996). On the other hand, IFN-g appears to be downregulating role in some autoimmune disease (Matthys et al., 2000). Normally granzyme B and perforin co-express for induce apoptosis and necrosis by secreting granules to target cell for cell death, pore construction around target cell and then granzyme B will activate apoptotic enzymes to leading cell death (Metkar et al., 2008).

In autoimmune hepatitis condition, immune system mistakes liver cells for foreign aggressors and create antibodies to attack hepatocytes. There is increase in total number of inflammatory or apoptotic cells. So, this research aims to study protective effects of CIK cells on autoimmune hepatitis by injecting CIK cells first at the dose $10X10^6$ cells (Kim et al., 2007) and Con A in next 24 hours. Liver were biopsy from mice after experiments. Liver is the largest parenchymal organ and lying just below the diaphragm. The external surface of a normal liver is clear or smooth and the colour is brown. Pathological study of liver in control group show clear surface, a yellow gallbladder and no sign of cell apoptosis or necrosis compared to both of experimental group (PBS + Con A and CIK + Con A). Pathological study of liver in experimental groups found that white spots or patches spread in liver and the colour of gallbladder is green, inflammatory gallbladder comply with histopathological study in liver tissue reveal acute inflammation of liver because of characteristics of hepatocellular, necrotic area or fibrosis. From the analysis by image J showed that in CIK cells have not significant different between experimental group, In PBS + Con A and CIK + Con A were 4.00 % and 5.80% of necrotic foci respectively. In fact, AIH will have a chronic hepatitis but in this study the mice were observed 24 hours after Concanavalin A injection. This result is agreed with other one which done in C57BL/6 or C3H mice after intravenous injection of 25 mg/kg Con A (Heymann et al., 2015).

In this study of blood chemical test using AST and ALT level to evaluate severity of liver, the results showed that AST and ALT levels in PBS + Con A were 398.75 ± 87.86 U/L and 113.75 ± 22.44 U/L respectively while in CIK + Con A group were 487.33 ± 67.90 U/L and 199.00 ± 44.18 U/L respectively. Previous studies reported that in Con A-indued AIH was more than 1,000 U/L both of AST and ALT levels (Heymann et al., 2015; Fujita et al., 2016). While some studies observed AST levels higher than ALT levels in acute inflammation of liver (0 to 24 hours), because of its longer plasma half-life. This results agreed with previous studies that found the relationship between serum albumin levels and stage of hepatocellular severity (Williams and Hoofnagle, 1988; Sheth et al., 1998). In this study, histopathological observation showed no significantly difference between experimental groups and blood chemical tests represented higher level of AST and ALT in CIK + ConA than in PBS + ConA. These results can be explained by many previous researches. Noreldin and others (2015) studied about correlation between liver function tests and polymerase chain reaction in chronic hepatitis C patients and that characteristics of the liver are not correlated with serum albumin tests (Noreldin A. et al., 2015). Calabrese and others (2000) study the relationship between liver cell apoptosis in chronic hepatitis C showing liver damage can explain by using histological study but not biochemical activity of serum. Thus, many studies suggested that severity of liver injury should be histological indicator more blood chemical tests (Calabrese F. et al., 2000). Moreover, The signs of apoptotic cells are dense mass (pyknotic nuclei),

cytoplasmic condensation, loss of cell–cell contact and cell shrinkage (Garrity et al., 2003). These characters could be obviously detected from morphology. Therefore, TUNEL staining is not necessary in this study.

Clinical evidences showed that hepatitis is the causes of liver's disease such as cirrhosis and hepatocellular carcinoma (Degos et al., 2000). The hepatitis patients caused by B and C virus infection without continuous treatment lead to high risk of cirrhosis and liver cancer (Chen and Morgan, 2006; Vescovo et al., 2016; Ringelhan et al., 2017). Previous studies show mechanism of Th 1 cells released IFN-g and TNF-a to liver inflammation. CIK cells is Th 1 cells that have NK property (Guo and Han, 2015). Thus, in this study CIK cells does not affect to hepatocytes so this results can use CIK cells based on animal's model.

Further study, the variation amount of CIK cells intravenous injection in mice will be $3X10^6$ cells, $10X10^6$ cells and $30X10^6$ cells for study the effects of cells number on AIH. TUNEL staining will use for apoptosis confirmation. The study about relationship between AST and ALT in liver injury caused from AIH should be observed more. Moreover, CIK cell might be study in other autoimmune diseases for base information in the medical treatment and CIK cells might be try this protocol in human for clinical use. In this study, all experiments about Con A and CIK cells injection in mice were injected by intravenous through lateral tail vein. Difficulty of this step was similarity of color between tail vein and tail texture, small blood vessel and mice handle. So these problems were solved by using restrainer for stabilize

mice's body, applying 70% alcohol at tail for 2-3 times and dipping a tail into warm water (40-45 °C) for 30 second.

In conclusion, current treatment of AIH is immunosuppressants applications or liver transplants that have many side effects and this research aim to study immunotherapy by using CIK cells, the results show no significantly difference between experimental groups in histopathological studies and blood chemical test. Histopathological studies demonstrate that CIK cells have not the effects on liver Con A - induced inflammation and no statistically significant difference in aminotransferase levels. Finally, this research is a first study of CIK cells on AIH treatment so in the future of this field hope to have more information of AIH by using immunotherapy treatments.

REFERENCES

Al-Chalabi T, Underhill JA, Portmann BC, McFarlane IG and Heneghan MA. 2008. Impact of gender on the long-term outcome and survival of patients with autoimmune hepatitis. J Hepatol. 48(1): 140-147.

Aravalli RN and Steer CJ. 2017. Immune-mediated therapies for liver cancer. Genes (Basel). 8(2).

Caligiuri G, Nicoletti A, Poirier B and Hansson GK. 2002. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. J Clin Invest. 109(6): 745-753.

Cappuzzello E, Sommaggio R, Zanovello P and Rosato A. 2017. Cytokines for the induction of antitumor effectors: The paradigm of cytokine-Induced killer (CIK) cells. Cytokine Growth Factor Rev. 36: 99-105.

Caspi R. 2008a. Autoimmunity in the immune privileged eye: pathogenic and regulatory T cells. Immunol Res. 42(1-3): 41-50.

Caspi RR. 2008b. Immunotherapy of autoimmunity and cancer: the penalty for success. Nat Rev Immunol. 8(12): 970-976.

Chen SL and Morgan TR. 2006. The natural history of hepatitis C virus (HCV) infection. Int J Med Sci. 3(2): 47-52.

Czaja AJ. 1998. Frequency and nature of the variant syndromes of autoimmune liver disease. Hepatology. 28(2): 360-365.

Czaja AJ. 2005. Diverse manifestations and evolving treatments of autoimmune hepatitis. Minerva Gastroenterol Dietol. 51(4): 313-333.

Czaja AJ. 2007. Autoimmune hepatitis. Part A: pathogenesis. Expert Rev Gastroenterol Hepatol. 1(1): 113-128.

Czaja AJ. 2016. Diagnosis and management of autoimmune hepatitis: Current Status and Future Directions. Gut Liver. 10(2): 177-203.

Czaja AJ, Freese DK and American association for the study of liver D. 2002. Diagnosis and treatment of autoimmune hepatitis. Hepatology. 36(2): 479-497.

Czaja AJ and Manns MP. 1995. The validity and importance of subtypes in autoimmune hepatitis: a point of view. Am J Gastroenterol. 90(8): 1206-1211.

Degos F, Christidis C, Ganne-Carrie N, Farmachidi JP, Degott C, Guettier C, Trinchet JC, Beaugrand M and Chevret S. 2000. Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. Gut. 47(1): 131-136.

Fainboim L, Marcos Y, Pando M, Capucchio M, Reyes GB, Galoppo C, Badia I, Remondino G, Ciocca M, Ramonet M and et al. 1994. Chronic active autoimmune hepatitis in children. Strong association with a particular HLA-DR6 (DRB1*1301) haplotype. Hum Immunol. 41(2): 146-150.

Feld JJ, Dinh H, Arenovich T, Marcus VA, Wanless IR and Heathcote EJ. 2005. Autoimmune hepatitis: effect of symptoms and cirrhosis on natural history and outcome. Hepatology. 42(1): 53-62.

Fujita T, Soontrapa K, Ito Y, Iwaisako K, Moniaga CS, Asagiri M, Majima M and Narumiya S. 2016. Hepatic stellate cells relay inflammation signaling from sinusoids to parenchyma in mouse models of immune-mediated hepatitis. Hepatology. 63(4): 1325-1339.

Gao D, Cai Y, Chen Y, Li W, Wei CC, Luo X and Wang Y. 2018. Novel TLR7 agonist stimulates activity of CIK/NK immunological effector cells to enhance antitumor cytotoxicity. Oncol Lett. 15(4): 5105-5110.

Garrity MM, Burgart LJ, Riehle DL, Hill EM, Sebo TJ and Witzig T. 2003. Identifying and quantifying apoptosis: navigating technical pitfalls. Mod Pathol. 16(4): 389-394.

Gregorio GV, Portmann B, Reid F, Donaldson PT, Doherty DG, McCartney M, Mowat AP, Vergani D and Mieli-Vergani G. 1997. Autoimmune hepatitis in childhood: a 20-year experience. Hepatology. 25(3): 541-547.

Guo Y and Han W. 2015. Cytokine-induced killer (CIK) cells: from basic research to clinical translation. Chin J Cancer. 34(3): 99-107.

Heymann F, Hamesch K, Weiskirchen R and Tacke F. 2015. The concanavalin A model of acute hepatitis in mice. Lab Anim. 49(1 Suppl): 12-20.

Ichai P, Duclos-Vallee JC, Guettier C, Hamida SB, Antonini T, Delvart V, Saliba F, Azoulay D, Castaing D and Samuel D. 2007. Usefulness of corticosteroids for the treatment of severe and fulminant forms of autoimmune hepatitis. Liver Transpl. 13(7): 996-1003.

Jakel CE, Hauser S, Rogenhofer S, Muller SC, Brossart P and Schmidt-Wolf IG. 2012. Clinical studies applying cytokine-induced killer cells for the treatment of renal cell carcinoma. Clin Dev Immunol. 2012: 473245.

Jiang J, Wu C and Lu B. 2013. Cytokine-induced killer cells promote antitumor immunity. J Transl Med. 11: 83.

Kessler WR, Cummings OW, Eckert G, Chalasani N, Lumeng L and Kwo PY. 2004. Fulminant hepatic failure as the initial presentation of acute autoimmune hepatitis. Clin Gastroenterol Hepatol. 2(7): 625-631.

Kim HM, Lim J, Park SK, Kang JS, Lee K, Lee CW, Lee KH, Yun MJ, Yang KH, Han G, Kwon SW, Kim Y and Han SB. 2007. Antitumor activity of cytokine-induced

killer cells against human lung cancer. Int Immunopharmacol. 7(13): 1802-1807.

Kogan J, Safadi R, Ashur Y, Shouval D and Ilan Y. 2002. Prognosis of symptomatic versus asymptomatic autoimmune hepatitis: a study of 68 patients. J Clin Gastroenterol. 35(1): 75-81.

Kusters S, Gantner F, Kunstle G and Tiegs G. 1996. Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A. Gastroenterology. 111(2): 462-471.

Lieberman J and Fan Z. 2003. Nuclear war: the granzyme A-bomb. Curr Opin Immunol. 15(5): 553-559.

Liu R, La Cava A, Bai XF, Jee Y, Price M, Campagnolo DI, Christadoss P, Vollmer TL, Van Kaer L and Shi FD. 2005. Cooperation of invariant NKT cells and CD4+CD25+ T regulatory cells in the prevention of autoimmune myasthenia. J Immunol. 175(12): 7898-7904.

Longhi MS, Ma Y, Mieli-Vergani G and Vergani D. 2010. Aetiopathogenesis of autoimmune hepatitis. J Autoimmun. 34(1): 7-14.

Manns MP, Czaja AJ, Gorham JD, Krawitt EL, Mieli-Vergani G, Vergani D, Vierling JM and American Association for the Study of Liver D. 2010. Diagnosis and management of autoimmune hepatitis. Hepatology. 51(6): 2193-2213.

Matthys P, Vermeire K, Heremans H and Billiau A. 2000. The protective effect of IFN-gamma in experimental autoimmune diseases: a central role of mycobacterial adjuvant-induced myelopoiesis. J Leukoc Biol. 68(4): 447-454.

Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, Kim S, Raja SM, Shi L, Simon MM and Froelich CJ. 2008. Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity. 29(5): 720-733.

Mistilis SP, Skyring AP and Blackburn CR. 1968. Natural history of active chronic hepatitis. I. Clinical features, course, diagnostic criteria, morbidity, mortality and survival. Australas Ann Med. 17(3): 214-223.

Oo YH, Hubscher SG and Adams DH. 2010. Autoimmune hepatitis: new paradigms in the pathogenesis, diagnosis, and management. Hepatol Int. 4(2): 475-493.

Peakman M and Dayan CM. 2001. Antigen-specific immunotherapy for autoimmune disease: fighting fire with fire? Immunology. 104(4): 361-366.

Pluangnooch P, Timalsena S, Wongkajornsilp A and Soontrapa K. 2017. Cytokine-induced killer cells: A novel treatment for allergic airway inflammation. PLoS One. 12(10): e0186971.

Ringelhan M, McKeating JA and Protzer U. 2017. Viral hepatitis and liver cancer. Philos Trans R Soc Lond B Biol Sci. 372(1732).

Schmidt-Wolf IG, Lefterova P, Johnston V, Huhn D, Blume KG and Negrin RS. 1994. Propagation of large numbers of T cells with natural killer cell markers. Br J Haematol. 87(3): 453-458.

Schmidt-Wolf IG, Negrin RS, Kiem HP, Blume KG and Weissman IL. 1991. Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. J Exp Med. 174(1): 139-149.

Scott JD and Garland N. 2008. Chronic liver disease in aboriginal north americans. World J Gastroenterol. 14(29): 4607-4615.

Sheth SG, Flamm SL, Gordon FD and Chopra S. 1998. AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. Am J Gastroenterol. 93(1): 44-48.

Shi M, Fu J, Shi F, Zhang B, Tang Z, Jin L, Fan Z, Zhang Z, Chen L, Wang H, Lau GK and Wang FS. 2009. Transfusion of autologous cytokine-induced killer cells inhibits viral replication in patients with chronic hepatitis B virus infection. Clin Immunol. 132(1): 43-54.

Soloway RD, Summerskill WH, Baggenstoss AH, Geall MG, Gitnick GL, Elveback IR and Schoenfield LJ. 1972. Clinical, biochemical, and histological remission of severe chronic active liver disease: a controlled study of treatments and early prognosis. Gastroenterology. 63(5): 820-833.

Sun J, Gao Y, Chen HS, Wang SX, Li RB, Jiang D, Wei L and Wang Y. 2006. Transfusion of multi-factors activated immune cells as a novel treatment for patients with chronic hepatitis B. J Clin Virol. 35(1): 26-32.

Vergani D, Choudhuri K, Bogdanos DP and Mieli-Vergani G. 2002. Pathogenesis of autoimmune hepatitis. Clin Liver Dis. 6(3): 727-737.

Vergani D and Mieli-Vergani G. 2008. Aetiopathogenesis of autoimmune hepatitis. World J Gastroenterol. 14(21): 3306-3312.

Verneris MR, Arshi A, Edinger M, Kornacker M, Natkunam Y, Karimi M, Cao YA, Marina N, Contag CH and Negrin RS. 2005. Low levels of Her2/neu expressed by Ewing's family tumor cell lines can redirect cytokine-induced killer cells. Clin Cancer Res. 11(12): 4561-4570.

Vescovo T, Refolo G, Vitagliano G, Fimia GM and Piacentini M. 2016. Molecular mechanisms of hepatitis C virus-induced hepatocellular carcinoma. Clin Microbiol Infect. 22(10): 853-861.

Voskoboinik I, Whisstock JC and Trapani JA. 2015. Perforin and granzymes: function, dysfunction and human pathology. Nat Rev Immunol. 15(6): 388-400.

Wang HX, Liu M, Weng SY, Li JJ, Xie C, He HL, Guan W, Yuan YS and Gao J. 2012. Immune mechanisms of concanavalin A model of autoimmune hepatitis. World J Gastroenterol. 18(2): 119-125.

Williams AL and Hoofnagle JH. 1988. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. Gastroenterology. 95(3): 734-739.

Yuksel M, Laukens D, Heindryckx F, Van Vlierberghe H, Geerts A, Wong FS, Wen L and Colle I. 2014. Hepatitis mouse models: from acute-to-chronic autoimmune hepatitis. Int J Exp Pathol. 95(5): 309-320.

REFERENCES

Appendices

APPENDIX A.

Descriptive data (Mean ± SD) of serum albumin levels (AST and ALT) and necrotic areas were analyzed by A15 analyzer and Image J program in control (n = 3) and experimental groups; PBS + Con A and CIK + Con A (n = 8 or 9 per group).

Table A.1 Descriptive data (Mean ± SD) of serum albumin levels of control group (n = 3)

Mouse	Serum albumin levels (U/ml)	
	AST (U/ml)	ALT (U/ml)
No.1	48	35
No.2	50	38
No.3	47	42
Mean ± SD	48.33 ± 1.52	38.33 ± 3.51

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table A.2 Descriptive data (Mean ± SE) of serum albumin levels of PBS + Con A

group (n = 8)

Mouse	Serum albumin levels (U/ml)	
	AST (U/ml)	ALT (U/ml)
No.1	350	70
No.2	460	80
No.3	120	50
No.4	60	120
No.5	640	70
No.6	280	110
No.7	490	170
No.8	790	240
Mean ± SE	398.75 ± 248.50	113.75 ± 63.45

Table A.3 Descriptive data (Mean ± SE) of serum albumin levels of CIK + Con A group

(n = 9)

Mouse	Serum albumin levels (U/ml)	
	AST (U/ml)	ALT (U/ml)
1	371	331
2	185	50
3	540	200
4	360	120
5	350	160
6	430	140
7	780	490
8	580	160
9	790	140
Mean ± SE	487.33 ± 203.69	199 ± 132.54

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY

Tabe A.4 Descriptive data (Mean ± SD) of necrotic area of experimental groups

(n = 9 per group)

Mouse	Necrotic area (mm)	Normal area (mm)	Ratio of necrotic area / normal area	Percentage of necrotic area / normal area
PBS + ConA 1	0.049	2.815	0.017	
PBS + ConA 2	0.043	2.835	0.015	
PBS + ConA 3	0.027	0.575	0.046	
PBS + ConA 4	0.005	0.469	0.010	
PBS + ConA 5	0.055	0.558	0.098	
PBS + ConA 6	0.025	0.575	0.043	
PBS + ConA 7	0.019	0.574	0.033	
PBS + ConA 8	0.009	0.560	0.016	
PBS + ConA 9	0.038	0.482	0.078	
Mean ± SD	0.030 ± 0.017	1.049 ± 1.007	0.040 ± 0.031	4.00
CIK + ConA 1	0.011	2.717	0.004	
CIK + ConA 2	0.041	1.805	0.023	
CIK + ConA 3	0.011	2.717	0.004	
CIK + ConA 4	0.041	1.805	0.022	
CIK + ConA 5	0.004	0.557	0.007	
CIK + ConA 6	0.185	0.521	0.355	
CIK + ConA 7	0.013	0.527	0.024	
CIK + ConA 8	0.015	0.575	0.026	
CIK + ConA 9	0.013	0.27	0.048	
Mean ± SD	0.033 ± 0.058	0.902 ± 0.808	0.058 ± 0.112	5.80

Developing a protocol for CIK cells culture and flow cytometry

CIK cells were culture from thymus and spleen under conventional protocol and developed protocol and the results showed that thymus under developed protocol have a great percentage both of surface marker double positive (CD3+NK1.1) and intracellular marker double positive (CD3+IFN-g, granzyme B and perforin)

**Key steps to developing protocol for CIK cells culture**

Firstly, selection only floating cells on Day1 of CIK cells culture After T-cells were incubated with IFN-g in incubator for 24 hours then transfer only floating cells to anti-CD3 coated plate. Secondly, Amount of cytokines of CIK cells culture were used twice as conventional protocol. (CD3+ 1 μl, NK1.1 2 μl, IFN-g 1 μl, granzyme B 6 μl and perforin 2 μl per 1 well). Finally, Amount of times for CIK cells culture Normally, T cells from thymus used 21 days for transform to CIK cells but developed protocol used 14 days for CIK cells culture.

Key steps to developing protocol for CIK cells detection by flow cytometry

Firstly, surface marker staining was separated from intracellular staining. Secondly, incubating with PMA 50 ng/ml and Ionomycin (1 μg/ml) mixture for 3 hours. Finally, amount of antibodies was used twice as old protocol.
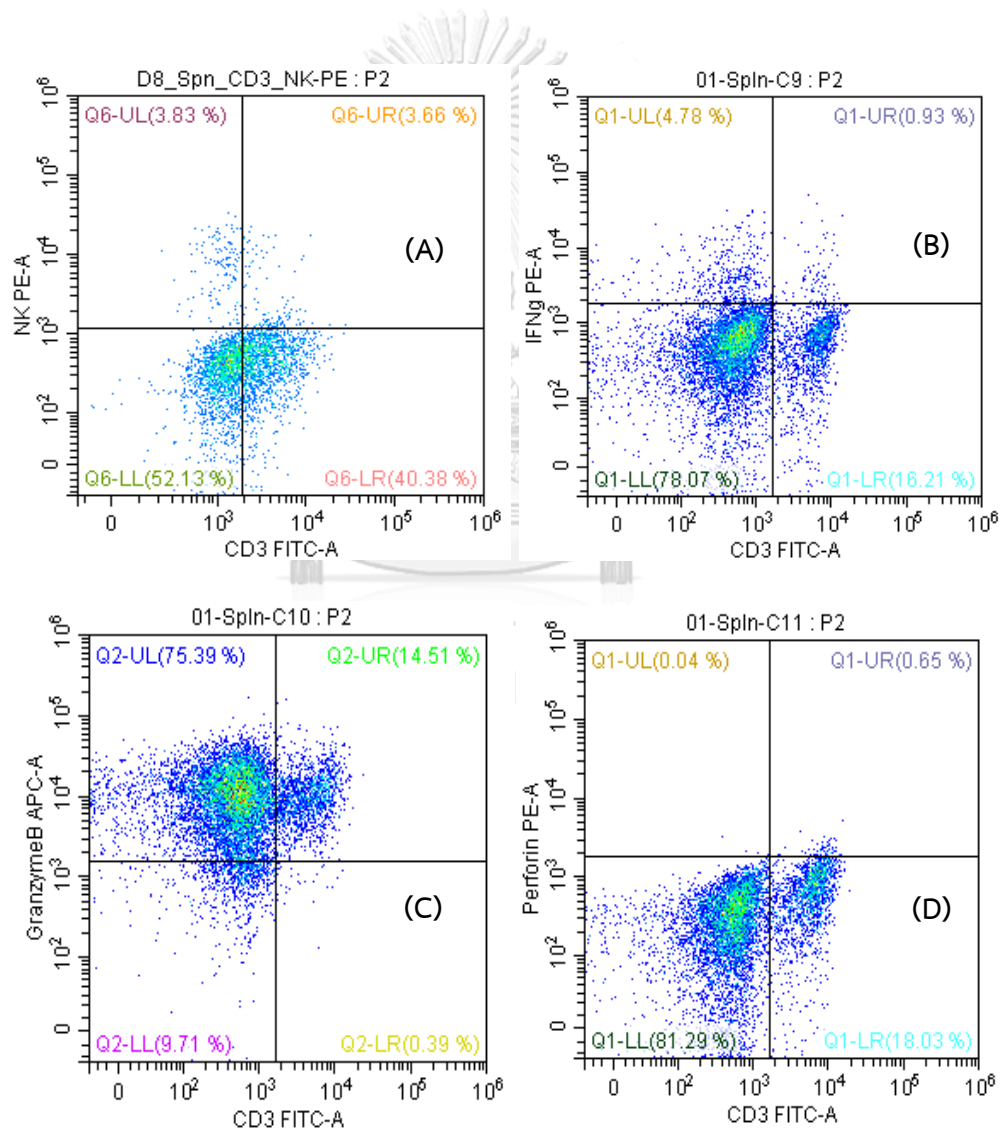
The results of CIK cells markers from thymus after 14 days were the following:

(A) CD3$^+$NK1.1 was 41.60%,

(B) CD3$^+$IFN-g was 5.43%,

(C) CD3$^+$GranzymeB was 58.96%

(D) CD3$^+$Perforin was 9.14%
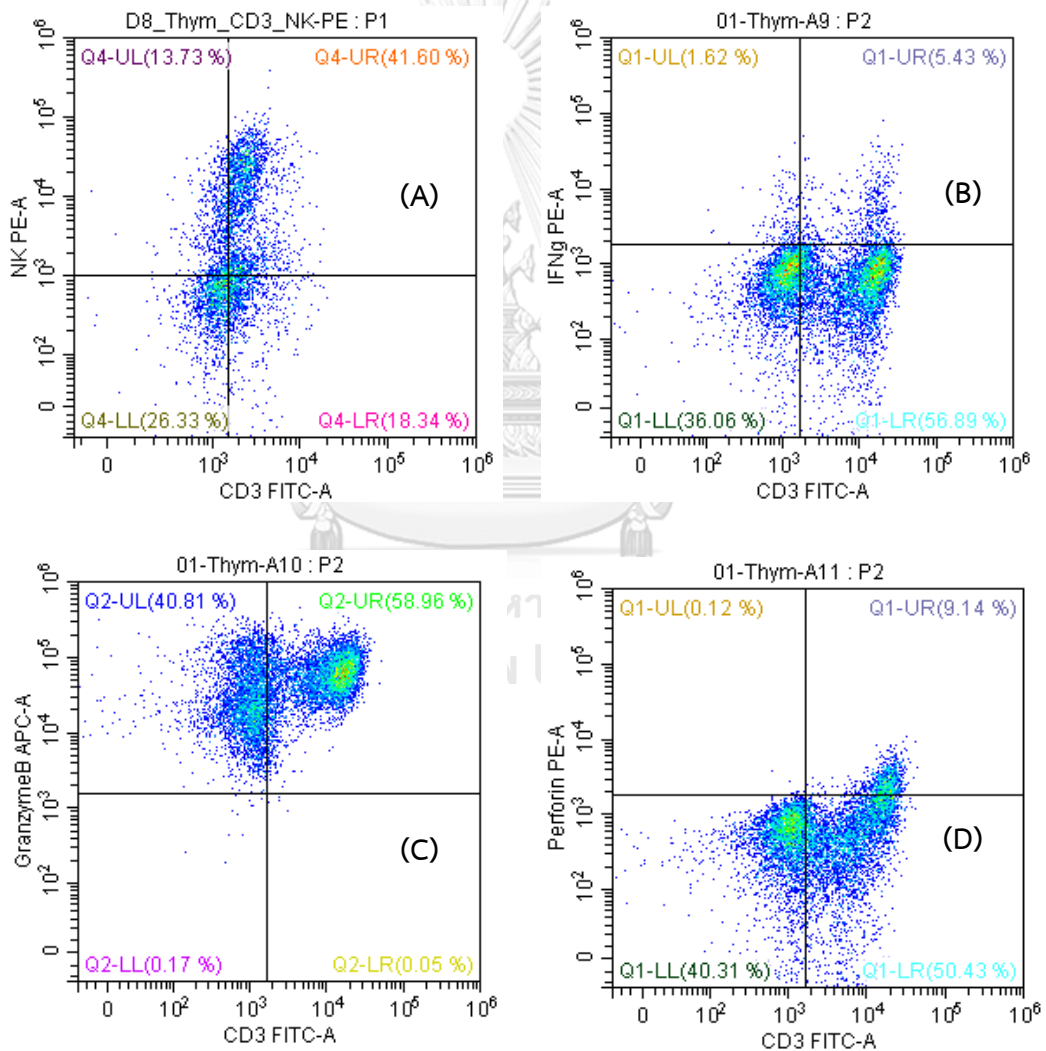
The results of CIK cells markers from spleen after 14 days were the following:

(A) CD3^{+}NK1.1 was 3.66%

(B) CD3^{+}IFN-g was 0.93%

(C) CD3^{+}GranzymeB was 14.51%

(D) CD3^{+}Perforin was 0.65%

VITA

Mr. Warakorn Srisantisuk was born on November 21 1992 in Bangkok, Thailand. He graduated Bachelor of Science (B.Sc.) in Biology from Faculty of Science, Chulalongkorn University, Bangkok, Thailand in 2015. He got a scholarship from 72 nd anniverssary of his majesty king bhumibol adulyadej for support me in graduate study and research and Japan Student Services Organization (JASSO) for internship study in Osaka University, Japan. He is currently pursuing a Master's degree in zoology at Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. He is interested in immunology and cell therapy in autoimmune disease and cancer.

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY