IMMUNOMODULATORY EVALUATIONS OF *THUNBERGIA LAURIFOLIA* CRUDE WATER EXTRACT ON HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

The immune system has been developed to defend the host against invading pathogens and/or tumor cells. Immune responses are the results of an efficient cooperation between innate and adaptive components of the immune system. To ensure immune homeostasis, immune system needs a delicate control of the balance of immune reactions through the production of a network of immunomodulators. Currently, there are only a few actual immunomodulators used in clinics and most of these did not originate from the plants. Various plants have been reported on concerning immune response. Therefore, investigation of plant-derived immunomodulators have become of great interest. *Thunbergia laurifolia* Lindl. (Acanthaceae) has been used in Thai traditional medicine as an antidote for poisons. Several constituents in *T. laurifolia* water extract (TLL) have been reported to display immunomodulatory activities. Nevertheless, there are no previous studies focusing on the effects of TLL on its immunomodulatory potential. Therefore, promising properties of TLL encourage us to evaluate its immunomodulatory activities.

In the present study, we evaluated the *in vitro* immunomodulatory effects of TLL at various concentrations (0-100 µg/ml) on peripheral blood mononuclear cells (PBMCs). Our study on immunotoxicity measurement by MTT assay suggested that TLL up to 250 µg/ml was not toxic to PBMCs compared to untreated cells. TLL did not show any modulations on T, B or NK cells distribution and expression of T cell subsets ($CD4^+$ and $CD8^+$) evaluating by immunophenotyping following by flow cytometry. Furthermore, TLL did not promote NK cells-mediated cytotoxicity against SK-N-SH among all evaluated effector:target (E:T) ratios. In contrast, our study showed that T cells proliferative capacity interfered with the suppressive effects of TLL as a dose dependent tendency, especially at 50 and 100 µg/ml ($p<0.01$). In addition, the cytokine profiles exploited the fact that TLL extract selectively induced Th1-mediated responses by insignificant enhancing the production of IL-2, a Th1-type cytokine, in a proportional manner to the concentrations of TLL. In contrary, IL-10, a Th2 cytokine, was significantly reduced the release at dose 100 µg/ml ($p<0.05$). Collectively, these results showed that TLL acted as an immunosuppressive agent and may directly polarize Th cells into Th1 sub-lineages and may provide benefit as immunosuppressant used in Th2 mediated pathologies in immunological related diseases like asthma and allergy.

KEY WORDS: *THUNBERGIA LAURIFOLIA* / IMMUNOMODULATION / FLOW CYTOMETRY / CFSE / HELPER T CELL

121 pages

การประเมินฤทธิ์ปรับภูมิคุ้นกันของสารสกัดชั้นน้ำของรางจืดที่มีต่อเซลล์โมโนนิวเคลียส

IMMUNOMODULATORY EVALUATIONS OF *THUNBERGIA LAURIFOLIA* CRUDE WATER EXTRACT ON HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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บทคัดย่อ

ระบบภูมิคุ้มกันได้พัฒนาขึ้นมาเพื่อป้องกันร่างกายจากสิ่งแปลกปลอมและเซลล์มะเร็ง โดยอาศัยการทำงานร่วมกันระหว่างระบบภูมิคุ้มกันชนิด innate และ adaptive ระบบภูมิคุ้มกันรักษาสมดุลโดยอาศัยการควบคุมสมดุลของปฏิกิริยาทางภูมิคุ้มกัน ด้วยการสร้าง immunomodulator ปัจจุบันมี immunomodulator ที่ใช้ในทางคลินิกไม่มากนัก และเกือบทั้งหมดไม่ได้มีแหล่งที่มาจากพืช อย่างไรก็ตามมีรายงานว่าพืชหลายชนิดออกฤทธิ์ต่อระบบภูมิคุ้มกัน ดังนั้นการค้นหาสาร immunomodulator จากพืชจึงได้รับความสนใจมาก

การแพทย์พื้นบ้านของไทยนำรางจืด (*Thunbergia laurifolia* Lindl. (Acanthaceae)) มาใช้เป็นยาต้านพิษ สารสำคัญบางชนิดที่พบในสารสกัดชั้นน้ำของรางจืด (TLL) เคยมีรายงานว่ามีฤทธิ์ปรับภูมิคุ้มกัน (immunomodulatory activities) แต่ยังไม่มีการศึกษาวิจัยเกี่ยวกับฤทธิ์ปรับภูมิคุ้มกันของรางจืดโดยตรง การศึกษาวิจัยในครั้งนี้จึงต้องการตรวจสอบฤทธิ์ปรับภูมิคุ้มกันของสารสกัดชั้นน้ำของรางจืด การศึกษาในหลอดทดลอง (*in vitro*) เพื่อประเมินฤทธิ์ปรับภูมิคุ้มกันพบว่าสารสกัดชั้นน้ำของรางจืดที่ความเข้มข้น 0-100 มคก,/มล. โดยใช้เซลล์โมโนนิวเคลียส (peripheral blood mononuclear cells (PBMCs)) พบว่าสารสกัดชั้นน้ำของรางจืดจนถึงความเข้มข้น 250 มคก,/มล. ไม่เป็นพิษต่อ PBMCs เปรียบเทียบกับกลุ่มควบคุมเมื่อวัดด้วยการใช้ MTT assay จากการทดลองยังพบว่าสารสกัดชั้นน้ำของรางจืดไม่ส่งผลให้เกิดเปลี่ยนแปลงการกระจายประชากรของเซลล์ลิมโฟซัยต์ชนิดที บี และ NK และไม่มีผลต่อการเปลี่ยนแปลงของประชากรย่อยของทีลิมโฟซัยต์ชนิด $CD4^+$ และ $CD8^+$ เมื่อวัดด้วยวิธี immunophenotyping และ flow cytometry รวมทั้งไม่มีผลต่อการทำลายเซลล์มะเร็ง SK-N-SH ของเซลล์ NK ในทุกอัตราส่วนของ Effector : target cells ที่ทดสอบ อย่างไรก็ตามพบว่าสารสกัดชั้นน้ำของรางจืดยับยั้งการแบ่งตัวของเซลล์ทีลิมโฟซัยต์ตามความเข้มข้นที่เพิ่มขึ้นโดยเฉพาะที่ความเข้มข้น 50 และ 100 มคก./มล. ($p<0.01$) และเหนี่ยวนำให้เกิดการตอบสนองแบบ Th1 โดยกระตุ้นให้เกิดการสร้างไซโตไคน์ชนิด IL-2 อย่างไม่มีนัยสำคัญและยับยั้งการสร้าง IL-10 ตามความเข้มข้นที่เพิ่มขึ้นโดยเฉพาะที่ 100 มคก,/มล. รางจืดยับยั้ง IL-10 อย่างมีนัยสำคัญ ($p<0.05$) ดังนั้นสารสกัดชั้นน้ำของรางจืดอาจมีฤทธิ์เป็นยับยั้งระบบภูมิคุ้มกันและอาจมีฤทธิ์เหนี่ยวนำให้เกิดการตอบสนองของภูมิคุ้มกันชนิด Th1 ซึ่งอาจนำไปใช้ประโยชน์ในการรักษาโรคที่เกิดจากการเหนี่ยวนำของภูมิคุ้มกันชนิด Th2 เช่นภูมิแพ้และหอบหืด

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ADCC	Antibody-dependent cellular cytotoxicity
AP	Activator protein
APCs	Antigen presenting cells
BCG	Bacillus Calmette-Guérin
CCR	Chemokine receptor
CD	Cluster of Differntiation
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
Con A	Concanavalin A
COX	Cyclooxygenase
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
ERK	Extracellular signal-regulated kinase
E : T	Effector : target
FCS	Fetal calf serum
G-CSF	Granulocyte colony-stimulating factors
GM-CFS	Granulocyte macrophage colony-stimulating factors
ICAM	Intercellular adhesion molecule
JNK	C-Jun N-terminal kinase
IL	Interleukin
INF	Interferon
iNOS	Inducible nitric oxide synthase
ITIMs	Immunoreceptor tyrosine based inhibitory motifs
KIR	Killer cell immunoglobulin-like receptors

LIST OF ABBREVIATIONS (cont.)

LFA	Leukocyte function-associate antigen
LOX	Lipooxygenase
LTB	Leukotriene B
MAPK	Mitogen-activated protein kinases
MEM	Minimum essential medium
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-AT	Nuclear factor for activated T cells
NF-kB	Nuclear factor-kappa B
NK cells	Natural killer cells
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OBX	Olfactory bulbectomized
ORT	Objective recognition test
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PGE	Prostaglandin E
PHA	Phytohemagglutinin
PLA	Phospholipase A
PRRs	Pattern-recognition receptors
PWM	Pokeweed mitogen
RPMI 1640	Roswell Park Memorial Institute1640 medium
SOD	Superoxide dismutase
TCR	T cell antigen receptor
TDAR	T-dependent antibody response
TGF	Transforming growth factor

LIST OF ABBREVIATIONS (cont.)

Th	Helper T lymphocytes
TIMP	Tissue inhibitors of metalloproteinase
TLL	*Thunbergia laurifolia* crude water extract
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TPC	Total phenolic content
Tregs	Regulatory T cells
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

CHAPTER I
INTRODUCTION

The immune system constitutes a natural defense that has developed to preserve life by protecting our body against invading pathogens or tumor cells. The immune responses are complex, dynamic and tightly regulated by organized mechanisms. The immune responses compose of two arms which are the innate and adaptive immune responses. After pathogens have breached external physical barriers, the innate immune system is the first line of host defense which rapidly combats with challenging pathogens without primary pathogen-recognition and does not provide long lasting immunity. In addition, several cellular components of the innate immune system, the antigen presenting cells (APC) (i.e., macrophages and dendritic cells), link the innate and adaptive immune system by processing presenting pathogenic protein fragments to adaptive immune system. The adaptive immune response provides later line of defense which is characterized by the generation of specific responses to foreign antigens and ability to maintain long lasting memory. The prominent cellular components of adaptive immune system are specialized effector cells, T lymphocytes. Appropriate signals provided by APC and cytokine environment lead naïve antigen specific T-lymphocytes to differentiate into their sub-lineages including T-helper, T-suppressor and cytotoxic T lymphocytes.

Foreign antigens are able to trigger the highly regulated networks of immune response however in healthy immune status, proper responses are ensured by an immunoregulatory mechanism containing various humoral and cellular factors. Moreover, the healthy immune system can produce a wide range of immunomodulators to maintain homeostasis and ensure proper defense reaction. A network of immunomodulators composes of cells, soluble mediators (e.g. antibodies, cytokines and chemokines) and membrane-associated molecules (e.g. receptors and co-stimulatory molecules). The imbalance of immunomodulators within the body has currently considered as the one of the major causes for several disorders such as

autoimmune disorders, cancer development, autoimmunity, chronic inflammatory diseases, hypersensitivity, allergic asthma. Therefore, an altered level of the activity of immunomodulators may be either beneficial or harmful to the body, depending on the overall degree of modulation and disordered conditions.

Immunomodulators are biological, natural or synthetic compounds that originated from endogeneous or exogenous sources that can act on complex network of mechanisms of the immune system to stimulate, suppress or modulate any aspects of the immune system including both adaptive and innate arms of the immune system . In clinical pharmacology, immunomodulators can be classified depending on their functions into the following category (1) immunostimulants, (2) immunoadjuvants and (3) immunosuppressants. Based on the increasing knowledge of the immune based-disordered, the strategies of using immunotherapy in combination with standard approaches are evoked to improve the results of treatment. To treat and prevent such these immune disorders, the immunomodulators have gained much interest from their ability to restore weak immune status and to moderate overactive immune system. Unfortunately, the classical immunomodulators exhibit undesirable side effects that often limit their use. Therefore, the study of novel natural compounds using as non-toxic immunomodulatory agents to manipulate the immune system has gained much interest.

Many experimental data suggested that immune responses, for examples following vaccination or infection, can be affected by plants-derived compounds, namely plant-derived immunomodulators. For examples, many plant-derived small organic compounds such as flavonoids, alkaloids, terpenoids, saponins and phenolics have shown beneficial effects on immune system. Investigation and identification of such plant-derived compounds will be important in discovery of novel immunomodulators that augment existing immunotherapy or vaccination or suppress overactive chronic immune responses.

Thunbergia laurifolia Lindl (Acanthaceae) is grown in tropics as ornamental vine and being a medicinal plant in some countries. In Thailand, *T. laurifolia* is well known as “Rang Chuet”. Thai traditional medicine used their leaves as an antidote for poisons and drugs including anti-inflammatory, anti-diabetic, and antipyretic properties. In addition, the ethno-pharmacological activities of *T. laurifolia*

in preparation of aqueous extract have been reported to exert antioxidant, hepatoprotective, detoxifying, anti-dementia and anti-inflammatory properties. The phenolic compounds contained in *T. laurifolia* Lindl aqueous extract (TLL) may contribute these effects. These results support the medicinal use of TLL for detoxification and antidote. However, with respect to these studies, there are no other studies focusing the effects of TLL on its immunomodulatory properties. Many previous studies have been reported that TLL contains several promising constituents displayed immunomodulatory activities including apigenin, apigenin glucosides and iridoid glucosides. Notably, apigenin has been demonstrated to inhibit Th cytokine production.

Since T lymphocytes play an important role in the pathogenesis of chronic inflammatory diseases, the promising immunomodulatory properties of TLL associated with its flavonoids and other phenolic constituents and the increasing interest in natural plant-derived compounds for possible T lymphocytes modulating properties, prompted us to evaluate its immunomodulatory activities.

1.1 Objectives

The purpose of this study is to evaluate the *in vitro* immunomodulatory properties of *Thunbergia laurifolia* Lindl crude water extract (TLL) on normal human peripheral blood mononuclear cells (PBMCs) including characterization of lymphocyte and T lymphocyte sub-lineages distribution, lymphocyte proliferation, Th cells-cytokine production and NK-mediated cytotoxicity.

CHAPTER II
LITERATURE REVIEW

The purpose of this chapter is to provide a general overview of the immune responses and to emphasis on the immunological mechanisms that response toward foreign molecules including plant constituents. Our major question is to ensure that TLL extract either specifically stimulates or suppresses one of the targeted functional networks of immune responses. Therefore, it is crucial to discuss current knowledge of immunological mechanisms. In addition, this Chapter II introduces some of the basic immunological mechanisms that may be involved when a plant-derived compound induces immunogenicity. The scope of this Chapter II is a brief review on the basic founding principles of immunogenicity against plant-derived compounds.

2.1 The human immune responses

The immune responses are complex, dynamic and tightly regulated by organized mechanisms. These responses have evolved to preserve life by protecting body against invading pathogens or tumor cells. The immune responses compose of two arms which are the innate and adaptive immune response. After pathogens have breached external physical barriers, the innate immune system is the first line of host defense which rapidly combats with challenging pathogens. The adaptive immune response provides later line of defense which is characterized by the generation of specific responses to foreign antigens and ability to maintain long lasting memory. These complex responses comprise a variety of components identified to date and are able to efficiently eliminate any known pathogens (1-3).

2.1.1 The innate immune response

The innate immune response represents the first line of host defense against invading pathogens that have entered into the human body (2). This innate response is characterized by lacking of memory and rapidly non-specific recognition to a pathogen invasion. The innate defense mechanism mostly recognizes on a limited set of microbial structures shared by groups of related pathogens. However, the innate immune response lacks of learning process. The innate immune response provides almost immediate protection against invading pathogens.

2.1.1.1 Cells of the innate immunity

Cells of the innate immune response are mainly composed of tissue-residing cells and moving cells. The tissue-residing cells include phagocytic cells such as macrophages and dendritic cells. These cells destruct invading microorganism by producing a wide range of enzymes, chemotactic proteins, regulatory and inflammatory cytokines. Additionally, these cells act as antigen-presenting cells that have the capacity to link the innate and adaptive immune response (2).

The moving cells migrate throughout the body via the blood and lymph circulation. These cells are composed of neutrophils, eosinophils, monocytes and natural killer (NK) cells. During the acute infection, circulating neutrophils can be rapidly recruited at the site of infection. Upon arrival, these cells will release a number of factors (such as chemokines and cytokines) that attract immature dendritic cells to the site of infection.

2.1.1.1.1 Natural killer (NK) cells

Natural killer (NK) cells are a minor subset of lymphocytes and an important effector cell of the innate immune system (4-6). It constitutes approximately 10% of human peripheral blood. NK cells can recognize without primarily stimulation and simultaneously mediate natural cytotoxicity against invading pathogens and abnormal cells like virus-infected or tumor cells. In addition, NK cells provide a major source of numerous cytokines and chemokines. NK cells can initiate a consequence of immune responses by secreting cytokine (6). Secretion of these cytokines, especially type I INF-γ as well as TNF-α and chemokines by NK cells in an early stage of infection induces their functions and recruits other immune cells.

For instance, during viral infection, NK cells induce antigen-specific T cell responses as similar as a virus does while also help in contributing both cytokines and cytolysis and limiting excessive T cells responses. Furthermore, NK cells play an important role in a regulatory crosstalk network with other cells such as dendritic cells and neutrophils to enhance or restrict immune responses. Overall, these abilities of NK cells help in controlling pathology and mortality.

At present, NK cells are formally classified as the prototype of the group I innate lymphoid cells (ILCs). These members are determined by their capability to produce INF-γ whereas not to secret type 2 cytokines (e.g. IL-4, IL-13 and IL-17). Human NK cells are determined as $CD56^{+}CD3^{-}$, discriminating them from $CD56^{+}CD3^{+}$ cells, which composed of a mixed member of antigen-exposed T cells and NK-like T cells that expressed several NK cell markers. Another marker virtually expressed on all human NK cells is the activating receptor NKp46 (natural cytotoxicity receptor [NCR] 1). NK cells are located in the blood, spleen, liver, lung and bone marrow in however a restricted numbers are found in lymph nodes (4-6). Interestingly, a few populations of NK cells that arrive at the lymph nodes play an important role in enhancing INF-γ responses by T cells through interaction with dendritic cells there. Two major members of NK cells are found in human individuals. These subsets can be divided by their levels of CD56 expression known as $CD56^{dim}$ and $CD56^{bright}$. $CD56^{dim}$ NK cells are completely mature and constituted approximately 90% of the peripheral blood NK cells. These subsets also prominently mediate cytotoxicity responses (4-6). Another subset, the $CD56^{bright}$ NK cells that constitute around 5-15% of whole NK cells population, are more immature but play a crucial role in production of cytokines while limit their cytotoxicity responses in contrary to $CD56^{dim}$ NK cells. Collectively, $CD56^{bright}$ NK cells are more productive at secreting cytokines however $CD56^{dim}$ NK cells are able to provide markedly cytokine production at an early stage because of a large portion in total NK cells pool and a more rapidly cytokine production. On the other hand, $CD56^{bright}$ NK cells are more efficient to leave the vasculature.

NK cells are rapidly stimulated to a certain non-self cell that spontaneously invade towards the body, especially tumor and virus-infected cells. The prominent targets of NK cells mediated cytotoxicity are abnormal

cells that have down-regulated expression of MHC class I molecule (MHC-I) which is presented on almost every healthy normal cells in the body (4-6). MHC class I down-regulation is a usual mechanism by which tumors and virus-infected cells use to escape from a recognition by T cell receptors of cytolytic T cells. Therefore, NK cells are considered to defeat this potential immunologic weak point. NK cells-mediated cytotoxicity is triggered by the directed exocytosis of cytolytic granules to release perforins and granzymes which can lead to porous plasma cell membrane on target cells and initiate apoptosis, respectively.

In the early 1990s, the discovery of ability of NK cells that killed the decreased MHC-I expressed target cells perplexed researchers at that time. This paradigm shift at that time had established that NK cells can recognize non-self antigens nevertheless detection mechanisms was unclear. The riddle was solved when it was encountered that mature NK cells tolerated that response towards normal cells by their expression of germline-encoded inhibitory receptors that recognize MHC-I (4-6). When these inhibitory receptors interact with MHC-I expressed on normal cells, they initiate the recruitment of Src homology domain-2 containing protein tyrosine phosphatases 1 and 2 at immunoreceptor tyrosine based inhibitory motifs (ITIMs) that subsequently arrest the activation. A mature NK cell therefore can initiate targeted attack since its inhibitory receptors are not engaged while face with a rare abnormal cell lacking MHC-I, thus the unsuppressed activating signals do not arrest. The foremost inhibitory receptors on human NK cells are killer cell immunoglobulin-like receptors (KIR) (4-6). Moreover, human exerts the CD94/NKG2A inhibitory receptor which can recognize a non-classical MHC-I (HLA-E in human subjects) whereas KIR family member recognize a series of classical MHC-I molecules that are HLA-A, HLA-B and HLA-C. NK cells within a human individuals, distinctly express various KIR family member, leading to a repertoire that exerts diverse MHC-I recognition capacities. Moreover, a mixed repertoire of cells inheritable expresses some inhibitory receptors that do not the self MHC-I and cause by polymorphism of KIR genes. This diversity of inhibitory receptors increases the capability in ligand recognition within human populations.

Different types of activating receptors are expressed on NK cells including, for examples, FcγRIIIA, activating forms of KIRs

(KIR2DS and KIR3DS), NKG2D and the NCRs. FcγRIIIA (CD16) is able to initiate antibody-dependent cellular cytotoxicity (ADCC) on confronting target cells opsonized with IgG (4-6). Furthermore, the most pertinent receptors that activate responses towards tumor cells seem to be NKG2D and NCRs. The ligands for these receptors are still unidentified however several ligands are exploited since they had up-regulated on stressed cells such as MICA and MICB ligands for NKG2D. Focus on adhesion on target cells, integrins is important in mediating adhesion and integrin mediated signaling is crucial for triggering the NK cell stimulation that results in targeted degranulation.

2.1.1.2 Humoral components of the innate immunity

The major humoral component of the innate immune response is complement (2). The binding of complement to carbohydrate determinants on pathogenic micro-organisms or antibodies that have bound (attached) to those pathogens activates cascade responses. The result of these activations is rapid killing of the micro-organisms and attraction of other immune cells by peptides production. Complement serves as humoral effector molecules of the innate immune response.

2.1.1.3 Effector mechanisms of the innate immune system

1. Phagocytes

The most importance effector mechanism of the innate immune response is phagocytosis (7). Indeed, all cells of the innate immune response are practically potential phagocytes. When micro-organisms contact with these phagocytes, pathogens are engulfed and destroyed by complex substances within the cells. These products are digestive enzymes or reactive oxygen species. Effective destruction of pathogens through phagocytosis requires a process referred to as inflammatory responses. These processes provide rapid recruitment of effector cells to the infection sites. These inflammatory responses are initiated by recognition of pathogenic determinants via innate receptors. These receptors are presented on the surface of macrophages or endothelial cells. After pathogenic determinants have been recognized, these cells secret a wide range of chemokines that attract phagocytic effector cells from blood circulation to the site of infection. Phagocytes and activated resident cells also secret soluble proteins called cytokines. These proteins such as tumor necrosis factor (TNF-α) and interleukins further mediate the phagocytic

capacities of innate immune cells. Because increased (elevated) level of cytokines and chemokines lead to increasing of vessel permeability that permits phagocytes and plasma proteins recruitment at the infection site. Not only lead these inflammatory responses to effective phagocytosis but also play an important role in the healing process of the damaged tissue. These phagocytic tissue-residing and circulating cells pay a crucial role in the innate immune response to prevent or eliminate infection of the host.

2.1.2 The adaptive immune response

The adaptive immunity constitutes a second line of defense. Adaptive response is characterized by the generation of specific immune responses that are able to efficiently recognize and eliminate any known pathogen. Although this immune response is slowly activated, it provides a state of "memory" which is characterized by the ability to promptly reactivate upon a same pathogenic challenge. The adaptive immune response has to combat pathogenic micro-organisms at a later stage of infection (2, 3).

2.1.2.1 Cells of the adaptive immunity

T and B lymphocytes are the major type of cells of the adaptive immunity (2, 3). These important cells protect the body against pathogenic challenges by patrolling throughout bloodstream and the lymph. Each diverse repertoire of lymphocytes carries a very specific surface receptor that is highly specific to a pathogenic protein structure. Therefore, T and B lymphocytes are capable to recognize and distinguish between self and non-self antigens. Engagement of the receptor with the pathogenic determinant activates T and B lymphocytes to proliferate and differentiate into effector cells. The cells that constitute the main population of adaptive immune response are antigen-specific lymphocytes.

2.1.2.2 Humoral components of the adaptive immunity

Antibodies can be considered as the major humoral component of the adaptive immune response. Antibodies are secreted by antigen-stimulating B lymphocytes as circulating proteins that can bind and render the targeted pathogen harmless or neutralize toxin. However, antibodies can exist on surface of B lymphocytes as receptor for specific antigens (broadly defined as a molecular

structure, from pathogenic origin or not, able to be recognized by an antibody). Antibodies that circulate around the body in the blood are main humoral component of the adaptive immunity (2, 3).

2.1.2.3 Effector mechanisms of the adaptive immune system

1. Antibodies

Antibodies are molecule considered to have bi-functional properties (3). Two functions of antibodies are both recognition and elimination of a challenged pathogen or antigen. The structure of an antibody displays roughly Y-shaped molecules. They are made up of two heavy chains and two light chains linked together with disulfide bonds. Both types of chains are consisted in functional determined-part referred as constant (C) region and antigen binding sites, namely variable region. Moreover, their light chains compose of K and L types that can join together with any of the five variant heavy chains (α, β, gamma E and μ). The five different heavy chains define the class, or isotype, of the antibodies molecule (i.e. IgA, IgG, IgD, IgE and IgM). The class of immunoglobulin is significant since they are associated with the ability of a given antibody to arrive at the site of infection and assemble the sufficient effector mechanism.

B cells secret antibodies to circulate around the body fluids and in the blood. Antibodies are able to bind with its target or bacterial toxin cellular targets that are adequate to render the antigen harmless or neutralize toxic effects, respectively (3). With respect to viral invasion, antibodies will similarly block the interaction between viral particles and their specific cellular receptors. Moreover, pathogens are eliminated after construction of antigen-antibody complexes leading to recruit of further effector mechanisms helping in pathogen elimination. For example, a procedure referred to opsonization help in destruction of pathogens by making the pathogens more sensitive to phargocytosis by innate immune cells following complexing of antibodies and surface antigens. The complement family of proteins can be stimulated to destroy and activate cell lysis by antigen-antibody complexes, depending on the antibody isotypes.

2. Effector T cells

T cells are activated by antigen-specific stimulation through their TCR. Activated T cells are able to present their anti-pathogenic effects by the production of various soluble factors responded to specific stimulation (3, 7).

2.1 $CD8^+$ expressing effector T cells ($CD8^+$ T cells)

$CD8^+$ T cells known as cytotoxic T lymphocytes (CTLs) or killer cells are defined as cells able to induce the death of infected or otherwise damaged/dysfunctional (e.g. tumor) cells (3, 7). As a consequence after recognition of a unique MHC class I/antigen complex, $CD8^+$ T cells sequentially secret perforin (a pore-forming protein) which is able to destruct the target cell through permitting the intracellular delivery of a series of protease to reach into target cell cytoplasm. These proteases known as granzymes lead to initiation of an apoptotic process causing the rapid cell death of the antigen bearing cells. CTLs can also impede the spread of pathogens through those cell-death inducing program since CTLs can destroy target cell expressing pathogenic peptide at the cell surface while the pathogen's replication is incomplete. However, recent data has also been suggested that CTLs are able to block viral replication without affecting the integrity of target cells, for example neuronal cells. Finally, pathogen specific T cells also produce several soluble molecules like cytokines (e.g. TNF) and interferons (INF) that adhere to infected cells and impede intracellular replication of pathogens (3, 7).

In conclusion CTLs are capable to impede intracellular pathogen replication by production of soluble mediators interfering with replication process and/or to induce the apoptosis of infected target cells through the production of secreted perforin and grnazymes.

2.2 $CD4^+$ expressing effector T cells ($CD4^+$T cells)

$CD4^+$ T cells bind with a specific antigen/MHC class II complex strictly presented by immune cells. $CD4^+$ T cells are known as helper T lymphocytes (Th cells). These sub-lineages display prominent effects in regulation of immune response. They are able to secret a wide range of cytokines and act themselves as effector cells (3, 7). As previously discuss, cytokines secreted at the

infection sites play an important role in pathogen survival like TNFs and IFNs. Furthermore, several cytokines have capability to mediate both stimulatory and inhibitory effects (profound effects) on the other immune cells activity including innate immune cells, B cells and cytotoxic T cell (3, 7). The complicated role of $CD4^+$ T cells on immune responses will be discussed in more details in the following chapter.

2.2 Generation of immune reactions

2.2.1 Pathogen recognition by the immune systems

2.2.1.1 Pathogen recognition by the innate immune system

Cells of the innate immune response express a limited set of germ-line encoded receptors known as pattern-recognition receptors, PRRs (7). These receptors can detect an invading pathogen that contains a set (series) of conserved molecular structures. These complex pathogenic structures are referred as pathogen-derived molecules or pathogen-associated molecular patterns, PAMPs. The hallmark component of PRRs is Toll-like receptors (TLRs). Nowadays, TLRs are classified into different type based on their localization and the recognition of PAMPs. These molecules constitute capacities to sense a wide range of organisms from viruses to parasites. TLRs 1, 2, 4, 5 and 6 recognize mostly bacterial products. On the other hands, TLRs 3, 7, 8, 9 recognize mostly viral products and nucleic acids. The first group is expressed on the surface of innate immune cells while another group is localized to intracellular compartments. Among PRRs, TLRs rapidly recognize pathogen-associated molecular patterns as soon as an infection occurs (2, 3).

Recently, another group of pattern-recognition receptors, the NOD-related family, has been identified. This NOD-related family expresses in the cytoplasm, thus can react to intracellular pathogenic structures in cytoplasm (2, 3). Not only senses pathogen-derived molecules but also recognizes cellular damages that are the consequences of an infection. Ultimately, these sensing lead to the processing and release of inflammatory cytokines. This type of pattern-recognition receptors expands the sensing capacity of the innate immune system to virtually all cellular

compartments and it is noteworthy that cellular damage or dying signals, also referred to as DAMPs, for “danger associated molecular patterns” are able to represent by NOD-related family.

2.2.1.2 Pathogen recognition by the adaptive immune system

Because of limitation of diversity of PRRs render pathogenic micro-organisms detection by innate immune cells. Pathogens displaying highly mutation rate and viruses that replicate intracellularly can escape recognition from innate immune cells. Adaptive immune response promptly recognizes and eliminates these challenging. The adaptive immunity expresses complicate recognition system including antibodies and T cell receptors (2, 3). Lymphocytes, the key cell population in the adaptive immune response express these antigen-specific receptors, thus have evolved in response to complex pathogenic challenging.

1. Pathogen recognition by antibodies

Antibodies are circulating proteins secreted by B lymphocytes, a subpopulation of lymphocytes. These molecules are highly diverse because of gene rearrangement mechanisms. These uncovered mechanisms have generated an infinite number of distinctive binding sites for antigen through a limited set of genes. This highly diverse character of antibodies principally recognizes virtually all known molecular structures including biological (such as proteins, lipids or nucleic acids), or synthetic (small organic compound) origin (2, 3).

Additionally, numerous copies of a unique antibody produced by each B lymphocyte can express on their surface as receptor (B cell receptor, BCR) for a specific antigen. This expression occurs during B cell development in the bone marrow. As a result of this phenomenon, each B lymphocyte is believed to be monospecific that each antibody can specifically react to a single antigenic structure. After B cells have encountered with a specific antigen, with the adequate auxiliary cells and signals, B cells (expressing) bearing a given antibody are activated to divide and differentiate into plasma cells and memory B cells. Most plasma cells then recirculate to the bone marrow. They will generate large amounts of soluble antibodies of a given specificity secreting in the blood and other body fluids (previously referred to as “humors”, hence the humoral response). Soluble antibodies

produced by stimulated B lymphocytes can fight infection without presentation at the infection site.

2. Pathogen recognition by T lymphocytes

Although antibodies produced by B cells have a potential to recognize with all known molecular antigens, these are not able to cross the plasma membrane because of their large molecules (2, 3). Thus, they cannot react and destroy intracellular replicate pathogens like virus. Another lymphocytes subset, T lymphocytes allow the adaptive immune response to interact and destroy intracellular pathogens. T lymphocytes achieve these difficult tasks by expressing their cellular surface peptide fragments derived from intracellular proteins. First of all, those intracellular proteins are subjected to a complicate cycle of degradation however intracellular proteins undergo limited degradation and re-synthesis. A sample of intracellular proteins is subjected to incomplete proteolysis in cytoplasm. This intracellular proteins rise to a set of small peptides, rather than amino acid. Then, these small peptides are transferred in to the endoplasmic reticulum

However, intracellular proteins undergo a limited degradation that is incomplete proteolysis in cytoplasm. These intracellular proteins rise to a set of small peptides, rather than amino acids. They are then subjected to endoplasmic reticulum and bind with transmembrane MHC or HLA presenting molecules. The peptide-MHC binding molecules are further transferred to the plasma membrane where these peptides will be expressed on the cell surface.

T cells also express an antigen specific receptor on their surface called T cell antigen receptor (TCR). TCR is highly diverse like B cell receptor and is presented on a repertoire of T cells. In contrast to antibodies, TCRs are expressed only on their cell surfaces therefore they are unable to react with soluble antigens. TCRs are designed to recognize a complex molecular structure of peptide/MHC binding molecule. TCRs are also able to adapt their structures to fit with that given peptide molecule. The diversity of TCRs makes them to be specialized receptor that specifically reacts to a given peptide/MHC-combined molecule. The antigen presentation and MHC restriction allow the immune system to detect and scan cytoplasmic originated peptide fragment while do not interfere cell integrity.

T cells can be classified base on the expression of cell surface marker known as the Cluster of Differntiation (CD) molecules (8). As well as MHC molecules, CD molecules can be classified into a subset. T cells presented antigen specific receptors that developed to react to peptide fragments from intracellular origin are $CD8^+$ expressing cells. $CD8^+$ T cells can interact with intracellular peptide fragments presented by all nucleated cells of organism.

Another subset of T cells expresses distinct marker known as $CD4^+$. $CD4^+$ T cells can recognize another MHC/peptide combination pattern formed in endocytic vesicle. The MHC encoded proteins presented the peptide produces in endosomal origin are known as MHC class II molecules. In contrast to MHC class I, MHC class II molecules are expressed only by immune cells. Furthermore, a peptide originated from extracellular compartment that can interact to $CD4^+$ T cells undergoes limited digestion after it has been internalized through endocytosis or phargocytosis by a specialized set of cells, namely antigen presenting cells (APC). Therefore, $CD4^+$ T cells seem to react to antigenic proteins from extracellular environment through presentation of extracellular degraded peptides (larger than intracellular originated peptides) by APC.

On the other hand, the MHC restriction does not seem to be a strict requirement since an alternative presentation, namely “cross presentation” has been demonstrated. The cross presentation refers to the phenomenon that the endocytosed protein fragments are presented by the MHC class I molecules rather than MHC class I. This phenomenon depends on the capability of endocytosed material to escape the endosomal compartment and reach the cytoplasm. The cross presentation in particular restrict to special antigen presenting cells known as dendritic cell family. Therefore, $CD8^+$ T cells restricted to MHC class I molecules can be activated in response to extracellular antigens. Moreover, another presentation referred to “autophargy” has been currently evoked to describe the attribute of intracellular antigens to be presented in association with MHC class II molecules however the immune consequence pathway are not firmly established.

In conclusion, antigenic protein fragments can be targeted to adaptive immune cells by a restricted mode of antigen recognition and presentation. The intracellular peptides can be presented by MHC class I molecules

and detected by $CD8^+$ expressing T cells whereas extracellular peptides loaded on MHC class II molecules can be reacted to $CD4^+$ expressing T cells.

2.2.2 Common traits of antigen recognition

2.2.2.1. Generation of antigen specific receptor diversity

The diversity of a large set of lymphocytes is generated by a mechanism known as gene rearrangement (2, 3). The goal of diversity development and clonal selection of a set of lymphocytes expressing antigen specific receptors is to recognize and remember each different pathogen, sometimes very closely related. Since the adaptive immune response must be able to discriminate a vast number of various antigens, the receptors that distinguish each specific antigen must be created in a large variation of conformations as one receptor for each different antigen that might ever be encountered. A whole set of T or B cells entirely represented a same conformation of receptor is called the repertoire of the immune system.

The huge diversity of T cell receptors and B cell antibodies is generated from a gene rearrangement called V(D)J recombination. V, D and J segment is a relative small set of genes that randomly rearrange to generate an infinite number of combinations during lymphocytes development (2, 3). These mechanisms are similarly involved in both T and B cells diversity generation. In addition, the diversity of antibodies is more complex than T cell receptors caused by a mechanism known as the process of somatic hypermutation.

2.2.2.2 Clonal selection and immune memory

Indeed, the generation of a vast set of immune repertoire can cause a serious harm to the host because of expression of autoreactive receptors during the process of somatic diversification. Thus, both T and B cells containing autoreactive receptors subsequently undergo an important process during differentiation to be taken out from repertoire of the immune system. That consequent process is referred to “negative selection” involving selectively generate apoptotic cell death on autoreactive receptor-bearing cells (2, 3). Another cell type expressing a non-self antigen receptor is reserved by this selection process. The spare cell types are permitted to circulate to the blood and migrate to peripheral organs. These mature lymphocytes therefore contain a unique receptor however they will be a few chances

to probably mount an effective response on their own. When an antigen invades the body, it reacts to cells expressing a specific receptor responsible to that antigen and that cells are activated and proliferated. This lymphocyte multiplication known as "clonal selection" induces the overrepresentation of a responsible lymphocyte during and after antigen recognition leading to a unique biological reinforcement learning process. As a consequence of the permanent modification of the immune repertoire, immune memory is indeed an event that a part of lymphocytes following the first stimulation is preserve alive during the life of the host, allowing a more active and faster response during a secondary confront with the same pathogen (2, 3).

2.2.3 Lymphocytes activation and immunoregulation by T cells

As previously stated, the consequence of immune response composes of a complex network of effector mechanisms including innate immune cells, phargocytes, antibody-secreting cells and T cell lineages. The sufficient activation of effector mechanisms is selectively regulated by the cooperative network between various cell types and mediators of the immune system. A crucial step in the initiation of an immune response is an appropriate activation of $CD4^+$ T cells. Although both antibody production and activation of cytotoxic T lymphocytes can be represented without the contribution of $CD4^+$ T cells, sufficient memory responses expressed by an increasing competency followed by secondary stimulation, are strongly restricted to primary activation of helper T cells (2, 7, 9).

2.2.3.1 The role of APCs on the activation of helper T cells

With respect to any T cells, helper T lymphocytes are only able to be activated after recognition of sufficient signal, i.e. an antigen fragment/MHC class II complex (2, 7, 9). In addition, the required signal referred as a peptide fragment/MHC complex can be powerfully generated by several immune cell types expressing MHC class II molecules however the specific capability to activate naïve helper T cells is strictly attributed to a rare subset of APCs known as dendritic cells (DCs). Recently, the theory of three signals has been developed and can inclusive explain the important role of DCs. Based on this theory, developing lymphocytes contain both naïve (immature) and mature (expressing developed functional helper and /or effector function) cells. Naïve T cells can develop to mature cells depending on

antigen recognition (i.e. peptide fragments/MHC complex) referred as signal 1 and a co-stimulatory signal carried by a set of membrane bound receptor expressed by DCs (including proteins of B7 family). Finally, the third signal is a unique set of secreted mediators known as cytokines produced by DCs. DCs-secreted cytokines affect the differentiation attribute of activated helper T cells towards a decided functional subset.

The property of DCs in activating naïve T cells seems to process in a stepwise manner including three separated steps referred as (1) antigen processing, (2) migration to lymphoid organ and eventually (3) activation of naïve T cells by preparation of three signals (2, 7, 9).

(1) Antigen capture mode. DCs are in peripheral tissue where they patrol and exploit potent endocytic activity. According to DCs ability to express various phargocytosis and endocytosis mediated receptors for antigens, pathogens and death cells, DCs utilize their receptors to internalize and degrade different protein antigens present in their environment. This simultaneous process of antigen presentation creates a series of peptide/MHC complexes that are present at the cell surface of DCs resided in peripheral tissues.

(2) Maturation and migration. According to an infectious event, DCs transit from an antigen-capturing mode to a T cell sensitizing mode referred as maturation. Mature DC are altered in their functions and become an antigen-loaded cell with the accumulation of high numbers of MHC class II molecules transporting from intracellular compartment to their plasma membrane. Mature DCs also lose their adherence with the surrounding tissues in associate with increase in capability to migrate to lymphoid organs where naïve lymphocytes reside.

(3) Co-stimulatory molecules expression, secretion of cytokines and activation of naïve lymphocytes. Upon their migration to nearby lymphoid organ, mature DCs express both a number of peptide/MHC class II complexes and co-stimulatory molecules deliver both two signals required for adequate activation of T cells, thereby inducing the differentiation of naïve T cells into a selective helper cell.

Based on their functional roles and residential location, DCs are therefore considered as a key player in the induction of immune response. DCs are resided in tissues such as skin and circulate in blood standing for the inherent entry

sites of pathogens. These cells have the prominent ability to shift from the infection sites to the lymphoid organs and become mature DCs that provide antigenic peptide and sufficient signals to T cells. Activation in association with sufficient signals promotes T cells to proliferate, differentiate and help in survival. Therefore, induction of DCs maturation is necessary prime to an effective immune response, and the character and quality of inducing signals are ultimate prominence in the initiation of immune responses.

2.2.3.2 Recognition of danger signals and maturation of DCs

DCs are one of the members of the innate immune response expressing receptors such as members of the TLR family. TLRs are able to recognize danger signals that are pathogen-derived molecules or signals delivering from endogenous compartment such as damaged or dying cells signals (2, 7, 9). Cytokine receptors like TNFs and IFNs are also expressed on DCs cell surface to allow DCs to recognize an occurring innate response in their environment. These series of receptors enable DCs to directly sense a set of organisms ranging from viruses to parasites or to recognize sequential signals of a local immune response. DCs sensed these signals through their receptors become mature therefore these signals functionally link between DCs and a local infection event. To link the development of an adaptive immune response to the primary recognition of innate receptors sensing from an infection event, DCs represent a confirmation signals referred as maturation and migration to lymphoid organs and expression of co-stimulatory molecules. Therefore, delivery of confirmation signals can be considered as a fail-safe strategy that helps in execute accidental event of self-components and dangerous invading from pathogens.

2.2.3.3 Generation of helper T cells diversity

As mentioned above, $CD4^+$ helper T cells activated through mature DCs signals differentiate into antigen-specific and selective helper cells. These cells are a key player in regulation of immune response by helping other effector cells to accomplish their tasks. Activity of other immune cells is regulated by helper T cells towards the secretion of a discriminating population of soluble mediators known as cytokines. Currently, the panel of cytokines secreted by activated T cells has been evaluated and exploits at least four different sub-lineages of helper T cells (2).

(1) Th1 cells are a distinct subset of helper T cells secreted mainly INF-γ and IL-2 (10, 11). Based on cytokines profile, Th1 cells play an important role in inducing expression of MHC molecules and expressing anti- viral effects. INF-γ and IL-2 are able to enhance the differentiation and activity of $CD8^+$ T cells and phargocytic cells. Therefore, Th1 cells seem to help in fighting against viruses and other intracellular pathogens.

(2) Th2 cells are associated with a major production of IL-4, IL-5, IL-10 and IL-13 cytokines (10, 11). According to their cytokines, these cells seem to particularly account for stimulating cells like eosinophils and mast cells that are responsible to immunity against large extracellular parasites. Moreover, due to supra-optimal stimulation of Th2 cells, high level release of IgE antibodies has been observed. Notably, IgE level is associated with the pathogenesis of allergic diseases and asthma.

(3) The follicular helper T cells (fTh) is another subset of helper T cells that is recently identified and often found in close relation with B cells in particular structures (follicules) of lymphoid organs. The fTh cells contribute to secret IL-21 which is a cytokines positively activate humoral immune responses *in vivo*. Based on production of IL-21, these fTh cells are able to enhance high levels of antibody production from abtigen specific B cells, and are therefore considered to be a key cell in regulating humoral immune responses after vaccination. Although fTh cells are originally considered to classify to Th2 sub-lineages, the recent study has shown that Th cells capable to activate B cells activity present a distinct set of genes. These cells also appear to lose ability to produce high level of the prototypic Th2 cytokines (i.e. IL-4 and IL-13). Thus, these fTh cells are currently classified to a distinct subset from a typical Th2 cells.

(4) Th17 cells, the forth Th subsets have recently been identified according to their ability to produce IL-17 and IL-22 cytokines. Both cytokines play a role in immune response to pathogens (i.e. several bacterium and fungal strains). Not only do Th17 cells appear to regulate the local immune response against gut and lung pathogens but Th17 cells are also the population caused autoimmune inflammation in several models.

During the early steps of antigen-specific stimulation, many soluble mediators, i.e. cytokines play a crucial role in selecting naïve $CD4^+$ helper T cells to differentiate into efficient effector cells (Th1, Th2, fTh or Th17) (2, 10). With respect to APCs role in Th cells differentiation, DCs are able to produce several cytokines previously referred as third signal helped in determining the choice of effector cells. In particular, DCs can directly polarize naïve $CD4^+$ T cells into Th1 cells through the production of several cytokines such as IL-12 that enhances INF-γ srcretion. In addition, IL-6 has been firmly established to promote differentiation of fTh and Th17 while the role of IL-4 and IL-10 cytokines able to skew Th cells differentiation into Th2 cells remain unclear. Notably, these cytokines seem to have ability to suppress each other's function however the nature of these responses is not completely confirmed. In particular, although the importance of these events remains to be firmly verified, Th1 and Th2 sub-lineages appear to both antagonize each other.

2.2.3.4 The humoral immune response

Different types of antigen or repetitive antigens (i.e. bacterial lipopolysaccharides or TLR ligands) are able to directly stimulate B cells to proliferate and differentiate into antibody secreting cells without contribution signals from T cells referred as T cell-independent manner. However, this stereotype "innate response" behavior identified by the secretion of low affinity antibody (mainly IgM) after encounters with the same repetitive antigen appears to fail to enhance a secondary, memory-like response. On the other hand, the ineffective of this type of response emphasizing the considerable role of T cells in enhancing protective humoral immune responses (2, 7, 9).

The typical secondary antibody response present after repetitive encounters to the same antigen is markedly noticed when B cells is activated by antigen in accordance with contributions from T cells referred as a T cell-dependent manner. Overall, the typical humoral response requires simultaneous activations of both B and T cells. B cells receptors (BCRs) can recognize a wide range of antigen types in contrary to T cells that are able to sense only the activation in response to protein antigens. The previous response exhibited following a primary injection of a protein antigen is slow and is identified by the low affinity IgM antibodies. If the identical protein antigen appears in a secondary exposure, the

secondary response emerges more rapidly and is mostly contained of higher affinity IgG antibodies. It is mentioned that antigen specific helper T cells provide the necessary required signals (both soluble and membrane borne) to B cells allowing these cells to achieve their ability to increase the production of high affinity IgG antibodies. In conclusion, a secondary response quantified by higher along with more sustained antibody titer, and qualified by class switch along with affinity maturation, tightly restricts to appropriate signals present by helper T cells.

2.2.3.5 Role of regulatory T cells (Tregs)

Although the assumption of existed cells that can suppress the immune response has been long considered, the established data of their nature and identification recently discovers. These T sub-lineages are characterized by expression of CD4 and constitutive CD25 markers along with the presence of Foxp3 transcription factor. The major function of these cells is to suppress an immune response or inflammation by impeding the activity of effector, helper and APCs cells. These T sub-lineages known as regulatory T cells or Tregs play an important role in immune tolerance (2, 7, 12). A genetic deficient autoimmune disease with the severe symptom can be simultaneously occurred with lacking of Tregs. As known, an autoimmune response can bring to tissue damage or deregulated hormonal responses. Thus, this autoimmune response can demonstrate the essence of Tregs existence and their important role in impeding the inappropriate immune reaction towards self-antigens. In contrast to instinct, the inhibitory effect of Tregs on the development of protective immunity against non-self antigens has recently been reported. It is recently postulated that Tregs help in resolving the excessive tissue damage that uncontrollably occurs in chronic inflammatory conditions although they directly react in the same way with non-self antigens. Therefore, Tregs bring a hope in using as an evolutionary tool to inhibit the excessive inflammatory responses. Interestingly, in mice model, constitutive and induced Tregs appear to be regulated by TGF-β and IL-10, a well-known immunosuppressive cytokines secreted by numerous immune cells (2, 7, 12).

2.2.4 Immune homeostasis by cytokines

The immune homeostasis is maintained by various mechanisms which are mainly governed by cytokines (9, 10, 13). The adaptive and innate immune cells are

received multiple cytokine signals throughout their life. The levels of multiple cytokine signals allow the immune cells to maintain survival, promote or inhibit their functions. As well as influence on homeostasis within the adaptive immune system, cytokines heavily affect the generation of specific-function effector cells. The production of different cytokines is a key influence on both maturation and specific functions of T cells as well as B cells. Cytokines acted as messenger molecules also help in communication of distinct elements of immune system (9, 10, 13). Communication between immune cell members triggers the amplification of immune reaction and is critical for orchestrating the appropriate reaction (i.e. whether prominently antibody-mediated or cell-mediated immune responses) depending on the character of invading pathogens and its entrance route into the body.

The structure of cytokines is diverse polypeptides. At present, many different cytokines have been studied and without doubt, some remain to be discovered. A major and the most important group of cytokines is the interleukin family. This family composes of cytokines that act as intercellular messengers between leukocytes. Members of interleukin family are structurally diverse proteins because the primary criterion to be included into this family is biological activity on leukocytes rather than conformation or amino acid sequence. To date, approximately 34 interleukin have been studied. Other cytokines family established on the principle of their capability to promote proliferation of hematopoietic precursors (colony stimulating factors) or cytotoxic activity against transformed cell types (tumor necrosis factors) or the interfering activity with viral replication (interferons). Notably, many cytokines have the diverse biological activities on different immune cell types (pleiotrophic effects). In addition, many factors like the stage of the cells, its position within the cell cycle and the presence of other cytokines, can all affect the response mediated by a certain cytokine. Here, we do not go in more detail of each cytokines but only give a detail concerning their sources and their biological influences of selected cytokines on their target cells, especially T cells.

2.2.4.1 Cytokines of the innate immune system

The cytokine members of the innate immune system include chemokines, IL-1, IL-6, IL-10, TNF-α and INF-α (1). Their biological activities and sources are presented in Table 2.1(1, 3, 10).

Table 2.1 Source and biological activity of innate immunity cytokines

Cytokine	Origin	Effector function
IL-1α, IL-1β	Monocyte, macrophage, DC, NK cell, B cell, endothelium	Helps in co-stimulating T cells activation by enhancing production of cytokines including IL-2 and its receptors, pro-inflammatory effects by including chemokines and adhesion molecules like I-CAM-1 and VCAM-1 on endothelium
IL-6	Th2, monocyte, macrophage, DC, bone marrow stroma	Myeloid stem cells differentiation, differentiation of B cells into plasma cells; induces T cell proliferation
IL-10	Th (Th2 in mouse), Tc, B, monocyte, macrophage	Inhibits secretion of INF-γ by mouse Th1 cells and IL-2 by human Th1 cells; down-regulates MHC class II and cytokines (e.g. IL-12) secretion by monocyte, macrophage, and DC thereby inhibiting Th1 cells differentiation; inhibit T cells proliferation; enhance B cells proliferation
TNF-α	Th, DC, mast cell, NK and B cells monocyte, macrophage	Tumor cytotoxicity; Enhances cytokine secretion; activates macrophage; antiviral
INF-α	Leukocytes	Inhibits viral replication; induce MHC class II

2.2.4.2 Cytokines of the adaptive immune system

Cytokines of the adaptive immune system mainly produce by T cell including IL-2, IL-4, IL-5, INF-γ, TGF-β, IL-13 and IL-17 (1, 3). Their properties and origin are summarized in Table 2.2 (1, 3, 13).

Table 2.2 Source and biological activity of adaptive immunity cytokines

Cytokine	Origin	Effector function
IL-2	Th1	Induces activated T and B cells to proliferate, enhance NK mediated cytotoxicity and killing of tumor and bacteria by monocytes and macrophages
IL-4	Th2, Tc2, NK, NKT, γδT, mast cells	Induces Th2 cells; induces proliferation of activated B,T and mast cells; down-regulates IL-12 production and thereby inhibits Th1 differentiation; induces IgG1 switching to IgE
IL-5	Th2 and mast cells	Induces activated B cells proliferation and eosinophils; induces switch to IgA
IL-13	Th2, mast cells	Inhibits activation and secretion of cytokine by macrophage; co-activates B cell proliferation; up-regulates MHC class II and CD23 on B cells
IL-17	T cells	Pro-inflammatory actions; induces production of TNF, IL-1β, IL-6, IL-8, G-CSF cytokines
IL-12	Monocyte, macrophage, DCs, B cells	1.) Critical for differentiation of Th1 cells; 2.) Induces proliferation and INF-γ secretion by Th1, $CD8^+$ and γδT and NK cells 3.) Enhance NK and $CD8^+$ mediated-cytotoxicity
INF-γ	Th1, Tc1, NK cell	1.) Inhibits viral replication 2.) Induce MHC class I and II 3.) Activates macrophage 4.) Antagonizes several IL-4 activities 5.) Inhibits proliferation of Th2
TGF-β	Th3, B, macrophage, mast cell	1.) Pro-inflammatory activity by chemotaxis of monocyte and macrophage, on the other hand also anti-inflammatory by inhibiting lymphocyte proliferation 2.) Induces switch to IgA

With respect to T cells deviation, several cytokines play an important role in polarization of naïve Th cells into their sub-lineages, Th1 and Th2. In this present study, the role of IL-2 and IL-10 in deviation of Th cells is further discussed in detail.

2.2.4.3 Human interleukin 2 (IL-2)

Human interleukin 2 (IL-2), also known as T cell growth factor (TCGF) is secreted from various immune cells including γδT cells, activated $CD4^+$ and $CD8^+$ cells, neurons, microglia, and haematopoietic stem cells. The role of IL-2 on T cell activation and expansion has been studied *in vitro* (3, 13, 14).

Moreover, the *in vivo* study has been shown that IL-2 is important for the development, survival and function of regulatory T cells which help in preventing against autoimmune diseases. However, for naïve $CD4^+$ T cells, the study indicated that IL-2 was not required during the primary immune response. The administration of IL-2 during priming stage of acute infection in mice seems to have a negative effect on number of effector and memory $CD4^+$ T cells. In contrast, during contraction phase IL-2 can clearly help in maintaining the survival of antigen specific $CD4^+$ T cells leading to increased number of $CD4^+$ memory cells for up to half a year.

Focus on $CD8^+$ T cells, IL-2 is critical for appropriate primary responses and differentiation into functional effector cells. Furthermore, IL-2 induces the development of activated $CD8^+$ T cells into memory cells and has been shown to regulate $CD8^+$ memory cells homeostasis. The *in vitro* study has also been shown that administration of IL-2 can augment proliferation and survival of $CD8^+$ cells at low cell densities and need for efficient INF-γ production and their cytotoxic function.

2.2.4.4 Human interleukin-10 (IL-10)

IL-10 is generally known as immunoregulatory cytokines primarily defined as cytokine synthesis inhibitory factor (CSIF) however IL-10 is a pleiotropic cytokine that can exploit either immunosuppressive or immunostimulatory activities on different cell types. IL-10 was primarily found from murine Th2 clones but in human, IL-10 can be produced from diverse cell types that are naïve Th cells, Th1 and Th2 $CD4^+$ T cells activated monocytes and peripheral blood T cells, including Th2-like $CD8^+$ cells and regulatory T cells (3, 15, 16). Nevertheless, a more novel study has been shown that IL-10 is early produced by APCs during infection.

IL-10 secreting by Th2 cells had been considered to suppress the production of Th1 cytokines responding to stimulation by antigen in the presence of monocyte/macrophage antigen presenting cells. IL-10 can inhibit antigen-stimulation cytokine synthesis, in the presence of antigen presenting cells (i.e. monocyte/macrophage) by PBMCs and NK cells. In addition, IL-10 can suppress a monocyte/macrophage-dependent manner, antigen specific proliferation of naïve Th, Th1 and Th2 cells (3, 15, 16).

IL-10 can influence on homeostasis of $CD8^+$ T cells. The presence of IL-10 during acute infection induces higher protective capacity of antigen-specific $CD8^+$ effector cells as well as memory cells. On the other hand, in chronic infections IL-10 production by APCs can cause T cell anergy.

It is now accepted that T cell homeostasis is affected by multiples cytokines, which provides an opportunities for selectively inducing the numbers of a defined subset or suppressing certain others.

2.3 Immunomodulatory activity focused on T cells

As previously described, the immune system is complex, dynamic and tightly regulated by organized mechanisms. The human immune system has evolved to preserve life by protecting body from invading pathogens or tumor cells. Foreign antigens are able to trigger the highly regulated networks of immune response however, in healthy immune status, proper responses are ensured. Technological advances made over the past few decades enable scientists to identify a wide range of immunomodulators. Within the body, a network of immunomodulators composes of cells, soluble mediators (e.g. antibodies, cytokines and chemokines) and membrane-associated molecules (e.g. receptors and co-stimulatory molecules) (8, 17). Moreover, many scientists believe that the imbalance of immunomodulators within the body has currently taken over as the one of the major causes for several disorders and an altered level of the activity of immunomodulators may be either beneficial or harmful to the body, depending on the overall degree of modulation and disordered conditions. Notably, immunomodulation is considered as normalizing process which restores weak immune status and to correct overactive immune system but it does not encourage the immune system. Moreover, there are also other factors that influence the immune status including age, sex, nutrition (18), endocrine hormones, antigen doses, antigen patterns (e.g. PAMP-expressing moieties) (19) and route of antigen administration (20). Based on the increasing knowledge of the immune based-disordered, the strategies of using immunotherapy in combination with standard approaches are evoked to improve the results of treatment (21-23). To treat and prevent such these immune disorders, the immunomodulators have gained much

interest from their ability to restore weak immune status and to moderate overactive immune system (19, 24).

2.3.1 Regulations of the balance of immune system

Recently, the philosophical concept of Yin-Yang has been concerned to describe in the sophisticated balance of immune reactions however it is stochastic process (18, 25). The immune system can orchestrate a whole immune homeostasis through their counterbalance functions that are pro- and anti-inflamatory functions in accordance with the Taoist principle. Moreover, the research of immunological-based diseases has shown that mulfunctions of internal control of immune reactions may be ascribed to their pathogenesis. In fact, while immune reactions require a rapidly and strongly response to pathogens invading through the body, limitations of its responses and durations simultaneously occur to restrict collateral damage to the host. For example, inflammation is general advantageous when occurs in appropriate location and limited duration but appears harmful to the body when chronic.

2.3.1.1 Immune based-pathological diseases

Nowadays, based on the increasing knowledge of the immunological functions, a wide spectrum of disease is currently considered as immune based-disorders. For examples, a number of studies suggest that immunosuppressive status and results in increased susceptibility to infections (26), tumor development, several autoimmune disorders and cancer development (27). On the other hand, overactive immune system (21, 28) leads to autoimmunity, chronic inflammatory diseases, hypersensitivity, systemic vasodilation, cancer, sepsis and anaphylactic shock. Current researches also indicate that immune system can affect brain aging and cognition.

2.3.1.2 Immune related mechanisms in allergy and asthma

Immune deviation has been considered to be associated with the symptoms and prevalence of allergic diseases and asthma. Many studies have been suggested that high serum levels of IgE of allergens, for examples, house-dust or pollen that are commonly exposed in environment, are thought to be a key determinat of allergic diseases like allergic asthma, allergic rhinitis and atopic dermatitis. The prevalence of allergic rhinitis alone is more than 155 million people worldwide. In

addition, asthama, one of the most common chronic disease, is affected people around 300 million worldwide. Asthma is also characterized as airway hyperresponsiveness (AHR) to bronchospasmogenic stimuli and approximately 50% and 80% asthma in adult and childhood respectively are allergic subtype (29).

Imbalance between Th1/Th2 mediated responses has been considered to play an important role in inducing allergic asthma and its exacerbation (30, 31). Th2 cells have been considered to mediate the initiation, progression and persistence of allergic diseases including asthma. Th2 cell mediated cytokines were associated in eosinophil chemotaxis, goblet cell hyperplasia with mucous hypersecretion, airway hyperresponsiveness (AHR) development and lung remodeling. Notably, allergrn-specific T cells clones isolated from the blood of allergic individuals exploit atypical Th2 cytokine patterns secreting IL-4, IL-5 and minimal levels of IL2 and IFN-g. Furthermore, presence of IL-3, IL-4, IL-5 and GM-CSF in bronchoavelolar cells in allergic asthma markedly support Th2 deviation. At present, Th2 cytokines IL-4, IL-5 and IL-13 are strongly associated with IgE production, airway eosinophilia, mucous hypersecretion and non-specific AHR. Therefore, since Th2-deviated immune response was observed in the allergic asthma pathogenesis, asthma is primarily considered as a Th2-mediated inflammatory disease (30, 31).

2.3.2 Immunomodulatory activity targeting on T cells

T cells are prominent cells of the adaptive immune response and play an important role in the regulation and persistence of the immunity (32). Complete activation of T cells relies on 2 signals. The signal 1 is generated from the interaction between T cell receptor (TCR) and antigenic peptide fragments/MHC complex whereas the interaction of co-stimulatory molecules provides signal 2. These two signals are required in immune responses to both endogenous and exogenous antigens. Numerous molecules are involved in these complex processes. Since there are many steps to regulate T cells responses, a number of promising molecules can be targeted to alter different T cells functions. Moreover, targeting on different T cell sub-lineages has been gained more interest to complete remission of several disorders. Immunomodulators that can alter or selectively act on T cells functions therefore are

promising novel approaches for the treatment and prevention of many disorders. Many novel strategies of T cell targeting modulations are presented below.

2.3.2.1 Modulation T cells

T cells activation is initiated by a presentation of antigen peptide/MHC complex expressed by APCs to a T cell receptor (TCR)/$CD3^+$ complex. Full T cell activation is ensured by a sufficient accessory signals produced by co-stimulatory molecules on APCs and this signal prevents the initiation of T cell tolerance, also known as T cell anergy which usually happens when T cells are activated without an appropriate accessory signals. CD80/CD86 expressed on dendritic cell that react with CD28 on T cells is the most potent accessory signals. After activation of TCR/CD3 complex on cell surface, a consequence phosphorylation reaction cascade is induced. First of all, non-receptor tyrosine kinases (e.g. the Src, Lck and Fyn) phosphhorylate a docking site for downstream signaling molecules located in the CD3 complex, namely immunoreceptor tyrosine-based activation motifs (ITAM). The phosphorylated downstream signaling molecules are activated and induce multiple signaling cascades including, for examples, the Ca^{2+} mobilization/calmudolin pathway, the mitogen activated kinases (MAPK), extracellular-regulated kinase (ERK) pathway. Finally, these activation cascades result in activation of the transcription factors, nuclear factor for activated T cells (NF-AT) and activator protein (AP-1) that bind to many promoters of T cell cytokine genes and increase their transcription. Full activation of NF-AT and AP-1 needs co-stimulatory signal by CD28. As mentioned above, therapies both activation of T cells and downstream cascade pathways may be a promising tool in treatment of immune-based diseases (32, 33).

2.3.2.2 Modulation of T cell differentiation into sub-lineages

Different T cell sub-lineages can be generated from different cytokine patterns. As previous mentioned, various patterns of cytokines produced by activated T cells are affected by the character of the cytokines it is exposed to primary encounter with antigens presented by a mature DC. Focus on promising molecules of signal transduction pathway, the expression of specific transcription factors influences the nature of a restricted Th1 or Th2 cytokine production. NF-AT and AP-1 seem to

involve in the expression of both Th1 and Th2 cytokines. Specific transcription factors convinced by the Th1 and Th2 cytokines, IL-12 and IL-4 are signal transducer and activator of transcription 4 (STAT4) and STAT6, respectively. STAT6 is crucial for Th2 cells development in response to IL-4 and negatively impacts on Th1 cells. Other selective transcription factors for differentiated Th2 cells are NF-IL-6 (C/EBP-β), c-Maf and GATA-3. As well as STAT6, GATA-3 expression is induced in polarized Th2 cells and down-regulation of Th1 cells. On the other hand a specific transcription factor of Th1 cells is T-bet. T-bet may be a key regulator in the development of Th1 cells. Modulation of immune deviation of Th1 and Th2 subsets may bring a hopeful method to treat Th1 or Th2 mediated immune responses (18, 32, 33).

2.3.2.3 Modulation of T cell co-stimulatory molecules

As mentioned, complete $CD4^+$ T cells activation requires two direct independent signals for optimal activation. Two signals are provided by TCR engagement with peptide/MHC class II complex and co-stimulatory signal. The most important co-stimulatory signal to activate naïve T cells may be the CD28 pathway (32, 34). CD28 is constitutively expressed on the cell surface of T cells. This molecule interacts with their 2 ligands, CD80 and CD86, present by APCs. Normally, CD86 is expressed at low level however it is rapidly up-regulated towards APC activation whereas inducible CD80 is later expressed after activation. CD28 signal is crucial for enhancing T cell activation through augmentation and sustaining T cells responses induced by TCR signals. Due to CD28 blockage of naïve T cells activation, T cells are anergic to secondary activation even when reactivated in the presence of CD28 ligation. Moreover, the data investigated in mice has been shown that co-stimulation with CD28 block the induction of Foxp3 expression and suppressive function. Therefore, blockage of CD28 co-stimulation renders T cells to tolerance induction and suppressed cytokine production. Another receptor for CD80 and CD86 ligands is known as cytotoxic T lymphocyte antigen 4 (CTLA4; CD152) that induces inhibitory signals. CTLA4 in contrary to CD28 is up-regulated after T cells activation and is a powerful negative regulator of T cells activation. In conclusion, CD28 and CTLA4 share their same ligands (CD80 and CD86 expressed on APCs) but they compete to counterbalance effects on T cells activation. Although there are various T cells co-

stimulatory molecules identified, the CD28 co-stimulatory molecule is currently used in clinic for the treatment of rheumatoid arthritis (32, 34).

2.3.2.4 Modulation of regulatory T cells

As previously described, Tregs are important regulatory cells that play a role in suppressing Th1 and Th2 mediated adaptive immune responses. Tregs are categorized in natural Tregs ($Foxp3^{+}CD25^{+}CD4^{+}$) and inducible (or adaptive) Treg cells (iTregs). These cells are thought to prevent immune response against self-antigen and adaptive immune response, respectively. iTregs are further subdivided into type-1 regulatory T cells (Tr1 cells), $Fox3^{+}$ inducible Tregs and T-helper type 3 cells (Th3 cells) that mediate suppression through the production of IL-10, IL-2 with TGF-β and TGF-β, respectively. In contrast to iTregs, natural Tregs mediate their suppressive effects via T cell:Tcell/APC contact (12, 15, 21, 33).

2.3.2.5 Modulation of T cell traffickling

Chemokines regulate immune responses by the attraction of inflammatory cells to target organ. Before the migration into inflammatory locations, lymphocytes must adhere on the endothelium. Chemokines are small chemotactic cytokines that are able to induce adhesion and transmigration of leucocytes through the endothelium. Therefore, chemokines are a prerequisite for leukocytes migration that they precede rolling process. A secretion of specialized integrins is involved in the adhesion of T cells to the endothelium. These molecules include very late antigen 4 (VLA-4) and leukocyte function-associate antigen 1 (LFA-1). They are ligands for the adhesion molecules, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), respectively. Both VLA-4 and LFA-1 are induced to present upon antigenic activation of T cells. VTA-4 and VCAM-1 highly affinity adhesive interactions express on differentiated Th2 cells. Focus on Th1 cells, another adhesion molecule P-selectin expressed on endothelium is involved in the transmigration of Th 1 cells by interacting with P-selectin glycoprotein ligand (PSGL-1) presented on Th1 cells. In addition to ingretin, different T cell subsets variously express chemokine receptor patterns. Those receptors are, for examples, CCR2, CCR3, CCR4, CCR5, CCR7 and CXCR3 that are induced by antigenic stimulation and only present on memory T cells. Interestingly, CCR5 has been reported to be a marker of Tregs and helps in recruitment of Tregs whereas Th1 cells and Th2 cells

plentifully express CCR2, CCR5, CCR7, CXCR3 and CCR2, respectively. Thus, therapies both directly interfere with T cell adhesion molecules or chemotactic mediators may be a beneficial strategy for immune based-disorders treatment (32, 34, 35).

2.3.2.6 Indirect modulation of T cells targeting on DCs

DCs are thought to be crucial for the induction (or priming) of naïve CD4+ T cells by providing signal 1 (TCR interaction with peptide/MHC class II complex), signal 2 (co-stimulation) and signal 3 (cytokine environment) resulting in activation of T cells (32, 36). They deliver signal 3 that is, polarizing cytokine, IL-12 family members (i.e. IL-12, IL-23 and IL-27 for Th1 cells generation and IL-10 as well as TGF-B for Tr1 and Th3 generation, respectively. Thus, DCs can police the generation of T cells subsets, Th1, Th2 and iTregs, Tr1 and Th3 cells. Generally, immature or semi-immature DCs can promote Tregs by displaying lower antigen uptake capacity, lower expression of co-stimulatory molecules and lower T cells stimulatory ability. Many pathogen-associated molecular patterns (PAMP) have also been to induce the secretion of either one or both of these cytokines. Therefore, inhibition or activation of DC will be of interest to therapeutically use for the treatment of immune-based diseases.

2.4 Immunomodulators

2.4.1 Immunomodulators definition and classification

Immunomodulators are biological, natural or synthetic compounds that originated from endogeneous or exogenous sources that can act on complex network of mechanisms of the immune system to stimulate, suppress or modulate any aspects of the immune system including both adaptive and innate arms of the immune system (37).

In clinical pharmacology, immunomodulators can be calssified depending on their functions into the following category (38, 39) (1) immunostimulants, (2) immunoadjuvants and (3) immunosuppressants.

(1) Immunostimulants are inherently non-specific as they are considered to serve as enhancers of the basic level of immune response (38). They can affect both innate and adaptive immune responses. In healthy individuals, immunostimulants are considered as normalizing agent that restores or prevents weak immune status however in patients underlined with immune-based disorders, they are contemplated to be immunotherapeutic agents.

(2) Immunoadjuvants are used to enhance the efficacy of vaccines. Immunoadjuvants hold the promise of being the actual modulators of the immune response because they act as specific immunostimulants by exploiting as selectors between cellular (Th1) and humural helper (Th2) cells, immunoprotective, immunodestructive, and reagenic IgE versus IgG type immune response.

(3) Immunosuppressants are defined as a structurally and functionally heterogeneous group of substances that can decrease immunocompetence.

2.4.2 Immunomodulators useing in clinical therapy

It is mentioned that several immunomodulators can contain both stimulation and suppression properties depending on which components of the immune networks they react.

2.4.2.1 Immunostimulants

Immunostimulants, also known as immunostimulators can enhance different components and mechanisms of the immune system (37). They are chemically diverse and have various mechanisms of action. A few immunostimulants have been developed to treat infection, immunodeficiency and cancer. They can classify using their modes of action as present in Table 2.3 (37, 40, 41).

Table 2.3 Immunostimulants and their mechanisms of action

Immunostimulants	Mode of action
BCG (Bacillus Calmette-Guerin)	Induction of a granulomatous reaction at the site of administration
Levamisole	Affect myeloid cells maturation
Thalidomide	Decrease circulating TNF-α in patients with erythema nodosum leprosum, but to increase it in patients who are HIV-seropositive
Isoprinosine	Augment production of cytokines such as IL-1, IL-2 and INF-γ, increase proliferation of lymphocytes in response to mitogenic or antigenic stimuli
Immunocynin	Elusive mechanism
Recombinant cytokines	
1. Interferons	1. Induction of certain enzymes, inhibition of cell proliferation, and enhancement of immune activities, including increased phagocytosis by macrophages and augmentation of specific cytotoxicity by T lymphocytes
2. Interleukins	2. Cellular immunity is profoundly activated with lymphocytosis, eosinophilia, thrombocytopenia, and release of multiple cytokines
3. Colony stimulating factor	3. Increases the number and differentiation of myeloid progenitors

Nowadays, clinical used immunostimulants primarily focused on the modification of the endogeneous enhancing components of the immune system. The major target where current immunostimulants act on is T cells. These biopharmaceutical products include recombinant interferon alpha (INF-α) as well as interleukins 2 and 12 which are used as anti-virus and anti-leukemia, respectively. In addition, the restoration of total certain immune cells using recombinant granulocyte colony-stimulating factors (G-CSF) and granulocyte macrophage colony-stimulating factors (GM-CFS) in patients receiving chemotherapy are included here (41). However the use of exogeneous compounds to enhance the adaptive immune responses is not actually constructed despite its historical traditional background. The induction of immune responses (known in the past as induction of fever and inflammation) has been recognized to benefit against many diseases (e.g. malaria and syphilis) for hundreds of year. William Coley is the first person who has reported to use toxin as immunotherapy in a treatment of cancer with a surprising result. He used

a killed bacterial mixture to induce a potent local inflammation at soft tissue sarcomas directly injected. Moreover, shock therapies or administration of oils (e.g. cotton oil) or milk protein by injection was used to restore a weak immune status (e.g. during chronic inflammation). However, such methods were not accepted because of a lack of certain mechanisms and severe side effects. At present, Bacillus Calmette-Guerin (BCG) is used in the therapy of superficial bladder cancer. This method seems to act via similar mechanisms of the historical method mentioned. The primary induction of pro-inflammatory cytokines (i.e. IL-1, IL-6, IL8 and TNF-α) secretion initiated a local reaction following by a consequence of infiltration of innate immune cells, first neutrophils then monocytes/macrophages. Although, the whole cell killed bacteria are still used in the therapy of superficial bladder cancer, the technological advances has led to a discovery of responsible immune receptors (Toll-like receptors, TLRs) accounted for the bacteria and to a preparation of chemically defined bacterial components which exert the same effect. Interestingly, in Asia, a preparation of Streptococcus pyogenes (picibanil, OK-432) has been used in the treatment of head and neck cancer. Lipopolysaccharide (LPS) well known as endotoxin is well established as mitogen for immune cells. The core molecule that is account for an ability to stimulate the immune response is lipid A (the phospholipid component of LPS). These non-specific immunostimulatory molecules including polysaccharides, polynucleotides or lipoproteins are considered to contain a structure common to various types of pathogens, the pathogen-associate molecular patterns or PAMPs. These molecules are rapidly recognized by the innate cell receptors (TLRs) of immune system at an early step of infection. These receptors have been extensively studied to drive a discovery of novel immunostimulants.

2.4.2.2 Immunosuppressants

In clinical applications, immunosuppressants have been widely developed (37). Nowadays, there are many reviews of existing immunosuppressive drugs including with non-steroidal anti-inflammatory agents (NSAIDs) (42). Immunosuppressants often administer in combination regimens to treat different types of organ transplantation rejection, autoimmune diseases, acute and chronic inflammatory conditions. They are categorized by their mechanisms of action shown in Table 2.4 (37).

Table 2.4 Immunosuppressants and their mechanisms of action

Immunosuppressants	Mode of action
Glucocorticoids	Inhibition of lymphocyte gene expression
1. Cyclosporine, Tacrolimus 2. Sirolimus, Everlimus	Inhibition of lymphocyte signaling 1. Calcineurin inhibitor 2. Mammalian target of rapamycin (mTOR) Inhibitors
1. Azathioprine, Mycophenolate mofetil 2. Cyclophosphamide	Reduction of lymphocyte proliferation (cytotoxic agents) 1. Antimetabolites 2. Alkylating agents
1. Etanercept, Infliximab, Adalimumab 2. Anakinra 3. Daclizumab, Basiliximab	Inhibition of cytokines (anti-cytokines antibodies) 1. TNF-α inhibitors 2. IL-1 inhibitors 3. IL-2 inhibitors
1. Antithymocyte globulin (ATG) 2. Muromumab (Anti-CD3, OKT-3)	Antibodies against specific immune cell molecules 1. Polyclonal antibodies 2. Monoclonal antibodies
Efalizumab (LFA-1 inhibitor)	Inhibition of immune cell adhesion
Rho (D) immune globulin	Miscellaneous

Immunnosuppressants like glucocorticoid, cyclosporine and tacrolimus has been used in organ transplantation and autoimmune disordered including chronic inflammatory conditions such as arthritis. A variety of mode of action is presented Notably, immunosuppressants are currently developed to be structural based on immune molecules including antibodies, for examples, recombinant forms of immune cell surface molecules, recombinant fusion proteins (e.g. abatacept targeting B7/CD28 in T cells), cytokines, signaling modulators (e.g. the sphingosine analogue FTY720) and kinase inhibitors. In clinical medicine, immunosuppressants are used to treat or alleviate imbalance of inflammation represented in the manner like pain, fever, or tissue damage related with acute or chronic inflammation. The review suggests that our inflammatory agents are relative diverse, plentiful and continuously growing. With respect to anti-inflammatory activity associated with typical inhibition of biosynthesis of prostaglandins and thromboxanes, in addition to glucocorticoides, there are non-steroidal anti-

inflammatory drugs (NSAIDs) , tumor necrosis factor alpha (TNF-α) monoclonal antibodies (e.g. infliximab, adalimumab, golimumab) and the engineered soluble TNF-receptor (etarnecept). These agents possessed anti-inflammatory activity have been shown to influence on many immune cellular components and express advantageous effects in immune based disorders such as rheumatoid arthritis, atherosclerosis, osteoporosis, metabolic syndrome, psoriasis, cardiovascular diseases and cancer (42, 43).

2.5 Plant-derived immunomodulators

2.5.1 Plant-derived medicine

Plant-derived medicines have been documented over five millennia utilizing in treatment and prevention of diseases. In addition, ancient civilizations such as Egyptian, Chinese, Japanese, Greek and Indian have been developed their traditional medicine recipes to maintenance health status. The most popular plant-derived medicines such as Ginseng, Echinacea and garlic remain to be used at present for indications related to those used historically (44). While modern medicine such as vaccination, the use of antibiotics and the developments in medical technology contributed to healthy and long life period, advent of modern medicine caused a rapid decline in herbal medicine use. However, herbal medicine consumption is increasing because of various reasons. In western countries, the increasing acceptance of herbal medicine causes from an inability of modern medicine to cure certain diseases (i.e. AIDs and cancer) and voluntariness to self-medicate as well as, for healthy individuals, maintenance healthy state. The botanical acceptance is assured by the global market at close to 60 billion US dollar or around 20% of drug market. Many modern pharmaceuticals originated from plant have been approved by the US FDA. Almost 30% of current available medicines such as aspirin, morphine, digoxin, salbutamol are example of botanical origin prescription. Nevertheless, the potential benefits of herbal medicine should be rigorously investigated to ensure or refute any perceived or actual biological properties. Moreover, scientific evaluations help in

supporting and establishing the use of herbal medicine by the professional healthcare providers (25, 36, 45).

In the present study, our interest has focused on the immunomodulatory activity of plant-derived medicines. The next review will discuss and give the examples of plant-derived medicines that have scientific data to modulate immune components particularly T lymphocytes.

2.5.2 Plants as immunomodulator

Many experimental data suggested that immune responses, for examples following vaccination or infection, can be affected by plants-derived compounds, namely plant-derived immunomodulators (39, 45, 46). The capability to alter immune functions both stimulation and suppression offers many benefits for maintaining health and fighting with diseases. Investigation and identification of such plant-derived compounds will be important in discovery of novel immunomodulators that augment existing immunotherapy or vaccination or suppress overactive chronic immune responses.

As previous described, the effective immune responses generally involve several critical steps including antigen presentation, activation of T and/or B cells and production of immune mediators such as antibodies and cytokines. Plant-derive immunomodulators could mediate immunomodulatory activity through those targeted key cellular or molecular components. However, rigorous scientific studies of plant-derived immunomodulators in their abilities, mechanisms of action and bioactive compounds to modulate immunity should be validated. In this review, the ability of plants as immunomodulator to alter various immune parameters has been discussed.

2.5.2.1 Plant-derived immunosuppressants

The findings of immunosuppressive drugs are increasing because of the rising incidence of chronic inflammatory diseases like allergy, arthritis (47) and cancer (25). In this study, the *in vitro* and *in vivo* scientific evidences that may be described the functional activity of plant-derived compounds on immune system, especially influenced on T, B and dendritic cells are illustrated in Table 2.5 (25, 48, 49) and the bioactive compounds from plant provided the immunosuppressive activity and their bioactivities are summarized in Table 2.6 (36, 39, 46, 50).

Furthermore, since the chemotherapeutic agents that can interfere with cell division mechanisms like anti-mitotic agents inhibit T cells proliferation, they should be considered as immunosuppressant as well as colchicine and plumbagin. Colchicine which can decrease infiltration of leukocytes and phagocytosis in joints, and plumbagin which is a topoisomerase inhibitor, thus have shown to exert anti-arthritis (anti-inflammatory) effects.

Table 2.5 Herbal medicine containing immunosuppressive properties

Herbal medicines	Immunosuppressive properties
Tripterygium wilfordii (Chinese Thunder God Vine)	- Inhibits transcription of the cytokine genes IL-2 and INF-γ, prostaglandin E2 secretion from monocytes - Inhibits T-cell and B-cell proliferation, - Inhibits immunoglobulin production by B-cells
Artemisia annua	- Suppresses Con A and LPS-stimulated mice splenocyte proliferation - Inhibits OVA-specific IgG, IgG1 and IgG2b antibody
Hemidesmus indicus	- Inhibits IgG production - Inhibits adenosine deaminase (ADA) activity of PBMCs related to lymphocyte proliferation and differentiation - Inhibits lipid peroxidation and scavenges hydroxide radicals
Tylophora indica	- Inhibits delayed hypersensitivity reaction - Inhibits splenocyte proliferation and suppresses IL-2 production in Con A stimulated splenocytes at higher concentrations
Aphanamixis polystachya	- Induces apoptosis of normal lymphoid cells. The potential - Inhibits mitogen-stimulated lymphocyte proliferation stimulated with T-specific mitogens (PHA, Con A, PWM and anti-CD3)
Achillea talagonica	- Inhibits antibody production - Inhibits both generation and effecter function of alloantigen specific cytotoxic T lymphocytes
Andrographis paniculata	- Relieves rheumatoid arthritis symptoms - Inhibits NF-kB up-regulation - Inhibits NO and prostaglandin E2 productions

Table 2.5 Herbal medicine containing immunosuppressive properties (continued)

Herbal medicines	Immunosuppressive properties
	-
Rehmannia glutinosa var. hueichingensis (Chinese foxglove)	- Decreases a hemolytic plaque forming cells (HPFC) formation - Suppresses mast cell-mediated, immediate-type allergic reactions and reduces plasma histamine levels in mice - Inhibits skin allergic reactions and TNF-α production in rat peritoneal mast cells induced by anti-DNP IgE
Bupleurum falcatum	- Induces cell cycle arrest and apoptosis leading to inhibition of T cell proliferation - Inhibits T cell activation through the modulation of NF-kB transcription factor
Hibiscus sabdariffa	- Induces production of IL-10 but reduces TNF-α level
Alternanthera tenella Colla	- Inhibits B-lymphocyte fuction through reduction of T-dependent antibody production
Argyrolobium roseum	- Downregulates Th1/Th2 cytokines release
Alstonia boonei	- Anti-complementary action
Clerodendron trichotomum	- Inhibits arachidonic acid and PGE_2 production - Inhibits NF-kB dependent pathway in macrophages
Periploca sepium	- Suppresses IL-17 production and inhibits differentiation of Th17 cells - Inhibits NKT-derived inflammatory cytokine productions
Salvia mirzayanii	- Inhibits lymphocyte proliferation - Induces apoptosis in activated lymphocytes - Inhibits humoral and DTH response to SRBC in mice - Inhibits IL-2 production
Dracocephalum kotschyi	- Inhibits lymphocyte proliferation - Induce apoptosis in lymphocytes
Campylotropis hirtella	- Inhibits mitogen-induced splenocyte proliferation
Artemisia vestita	- Inhibits IFN-γ signaling - Down-regulates the activation, adhesion and metalloproteinase production of T lymphocytes
Phlebodium aureum (Kalawala)	- Suppresses the production of Th1 cytokines (IL-2, IFN-γ and TNF-α in human PHA-stimulated PBMCs but increases Th2 cytokines (IL-10) level

Table 2.6 Examples of botanical active constituents targeting molecular mechanisms of inflammation

Immunosuppressive molecular mechanisms	Botanical active constituents
Inhibition of biochemical mediators of inflammation	
1. Targeting PLA_2, COX-2, LOX, PGE_2 and/or LTB_4	Quercetin, curcumin, ursolic acid, sinomenine, triptolide, triptonide, celastrol, resveratrol, gingerol, zingerone, oleanolic acid, 7-oxosandaracopimaric acid, boswellic acid, epigallocatechin-3-gallate
2. Targeting MMPs and/or TIMPs	Oleanolic acid, epigallocatechin-3-gallate, cibotinoside, cyathenosin A, magnolol, ursolic acid, paeoniflorin, sinomenine, guggulsterone, guggulsterol
3. Targeting NO, iNOS and/or SOD	Indole-3-carbinol, bis(helenalinyl)glutarate, celastrol, sinomenine
Inhibition of molecular mediators of inflammation	
1. Targeting cell signaling molecules (ERK, p38 MAP kinase and/or JNK)	7-Oxosandaracopimaric acid , phenylheptatriyne, linolic acid, linolenic acid, curcumin, ikarisoside A, parthenolide, zingerone
2. Targeting nuclear factors (NF-kB and/or AP-1)	7-Oxosandaracopimaric acid , Guggulsterone, cembranoids, Dictamnine, obacunone, fraxinellone, Ikarisoside A, Magnolol, Triptolide, triptonide, celastrol

Abbreviations: AP-1, activator protein-1; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; iNOS; inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LOX, lipooxygenase; LTB, leukotriene B; MAPK, mitogen-activated protein kinases; MMP, matrix metalloproteinase; NF-kB, nuclear factor-kappa B; NO, nitric oxide; PGE, prostaglandin E; PLA, phospholipase A; TIMP, tissue inhibitors of metalloproteinase; SOD, superoxide dismutase.

Notably, a number of plant-origin compounds have been shown to possess potent anti-inflammatory activities both *in vitro* and *in vivo* through separated molecular pathway (25, 48, 51). These molecular mechanisms include inhibition of mitogen-activated protein kinases (MAPKs), inhibition of certain

transcription factors such as NF-kB and NF-AT, activation of peroxisome proliferation-activated receptors (PPARS), TRPV channels, and activation of G protein coupled receptors leading to alleviation of inflammation (e.g. cannabinoid receptors, serotonergic and adrenergic receptors) as well as inhibition of cyclooxygenases (COX)

and lipooxygenaese (LOX). Besides well-defined molecular interactions with protein targets, a number of plant-derived medicines seem to exert antioxidant properties. Many plant-derive secondary compounds contained polyphenolic functional group (e.g. flavonoids, proanthrocyanidins etc.) act as antioxidant and lead to ultimately anti-inflammatory effect because of their oxygen radical scavenging activity that prevents an activation of pro-inflammatory gene expression (39, 52, 53). The free radical scavenging substances derived from plants have gained wide interest and broadly discussed in the context of anti-inflammatory properties. For instance, aspirin (acetylsalicylic acid) is the most well-known prototype of NSAIDs originated from plant and is one of the most consumed NSAIDs in medicine. Another increasing large group of anti-inflammatory plant-derive compounds has shown to inhibit activation of NF-kB by the various mechanisms and different levels of inhibition. As known, NF-kB inhibition leads to block a major signaling pathway of pro-inflammatory and pro-carcinogenic gene expression. In addition a wide range of medicinal plant preparations has been used in clinical to treat chronic and acute inflammatory disorders (e.g. rheumatoid arthritis) and its target is study, at least *in vitro*, to act on NF-kB pathway .

In conclusion, focus on ethnopharmacological view, traditional herbal formulas for therapy of inflammatory disorders and associated symptoms like pain and fever have been used in all human civilizations. Therefore, their utilities, functional activities and mechanisms of action including molecular targets have been widely studied and discussed (42, 48, 51).

2.5.2.2 Plant-derived immunostimulants

The immunostimulatory principle is not empirical and not strongly established. This concept is based on the theory of a balance in immune system through looking for the homeostasis in health and has been fostered by the concept of Chinese philosophy (Taoist) and Buddhism. Moreover, the ancient concepts such as the Chinese zheng qi, Indian rasayana (39, 46) and Japanese

kampo(54) have been strived for healthy immune defense. To fortify or strengthen the immune defense, numerous of herbal medicines and traditional recipes has been used as a general tonic and fortifying agent to improve the defense mechanism of the body, prevent the body stress and claimed for exerting immune enhancing properties (46, 51), for examples, shitake mushroom (*Lentinula edodes*), *Andrographis paniculata*, *Panax ginseng* (Korean ginseng), *Panax quinquefolium* (American ginseng), *Eleutherococcus senticosus* (Siberian ginseng). These concepts and medicinal formulas could be positively influence on the using of phytotherapy to treat unhealthy immune defense. However, there are a few scientific evidences (i.e. in a double-blind randomized controlled study) have been provided to ensure that recipes actually work in a clinical medicine (25, 45).

Currently, the healthy immune system is generally postulated to fight infections and destroy tumor cells and should be balance. The popular concept of a healthy and stronger immune reaction strengthened by medicinal plants has been found in the literature on adaptogens (55). An adaptogen is believed to help the body to work close to its maximum capacity or the most well-being. However, this concept is more popular among food scientists and food companies who claim supplementary properties like pro-and prebiotics but these positive effects have not been clinically investigated on the immune system. It is noteworthy that malnutrition can cause dysfunction of immune system and more susceptible to infection. Although, the poor diet that, for examples, lacks of protein, zinc and vitamin D deficiencies negatively affects the immune status, it is questioned that we can use general or special compounds, especially from medicinal plants, to fortify certain immune components. In addition, the immune system has evoked to prevent the body against invading pathogens but it seems to not react with the small bioavailable plant constituents which make up a major portion of our food (25, 45).

Since the evidences of plant-derived immunostimulants have almost been studied on *in vitro* cell culture or *ex vivo* preparations or mainly studied on systemic direct injectable administration that may exclude the activation of innate immune system. Although, an aqueous or hydro-ethanolic plant extract tested with immune cells *in vitro* exploited the activation and a consequence of pro-inflammatory response (Table 2.7) (36, 44-46, 56), it is questioned that such experiment has no

adequate endotoxin control. Contamination of bacterial endotoxin may be responsible to those positive effects since endotoxin is known as a potent pro-inflammatory agent.

Table 2.7 Herbal medicine containing immunosuppressive properties

Herbal medicines	Immunostimulatory properties
Azadirachta indica	- Stimulates both specific and nonspecific immune responses.
Withania somnifera	- Counteracts undesirable effects of myelosuppressive drugs
Astragalus mongholicus	- Enhances antigen presentation capacity of DC by up-regulating murine DC expression of CD11c and MHC Class II molecules
Ganoderma lucidum	- Induces maturation of human DC related with increased expression of HLA-DR, CD40, CD80 and CD86
Amomi semen	- Activates CD86on murine bone marrow-derived DC
Lycium barbarum	- Up-regulates expression of CD40, CD80, CD86 and MHC Class II molecules on DC
Tridax procumbens	- Increases SRBC-specific antibody and almost double anti-tetanus toxoid (TT) IgG level
Polygala senega	- Induces a four-fold production of serum anti-OVA IgG compared to control mice
Polygala tenuifolia	- Elevates the production of anti-DT, anti-PT and anti-TT IgG compared to control mice
Glycyrrhiza uralensis	- Stimulates OVA-specific IgG, IgG1 and IgG2b productions equivalent to the human vaccine adjuvant
Mangifera indica	- augments the serum anti-SRBC titre almost 20-fold above control mice
Pinellia ternata	- Induces the anti-influenza IgG titre above control mice
Panax ginseng	- Elevates mouse lung IgA 15 days after challenge with influenza virus
Echinacea angustifolia	- Significantly elevates the IgG titre to keyhole limpet hemocyanin (KLH) compared to control rats
Bupleurum falcatum	- Enhances the proliferative response of ConA - stimulated mouse splenocytes - Activates IL-2 production
Dong quai	- Stimulates mouse splenocyte proliferation induced by Con A
Echinacea purpurea	- Upregulates the level of the NF-kB in LPS and PMA-stimulated human Jurkat T-lymphocytes

Table 2.7 Herbal medicine containing immunosuppressive properties (cont.)

Herbal medicines	Immunostimulatory properties
Lentinus edodes	- Increases activity of $CD8^+$ T-lymphocytes (CTL) associated with susceptibility to IL-2
Aeginetia indica	- Increases the T cell proliferative
Silybum marianum (milk thistle)	- Induces mouse splenocyte proliferation stimulated by Con A - Induces polarization to Th1-mediated effect

Interestingly, the *in vivo* studies have been carried out with certain types of plant-derived immunostimulants. Those plant-derived compounds are plant lectins (57) , plant polysaccharides (58, 59) and saponins (60) however it is mentioned that those compounds were usually intraperitoneally administered not by oral route. It is mentioned that polysaccharides from medicinal mushroom may compose of a component of pathogenic fungi considered as PAMP-like molecule, thus act as a non-specific immunostimulant. The plant compounds are able to induce life threatening-immune processes *in vivo*. For instance, the well-known reaction caused by plant-induced allergies is contact dermatitis. In addition, plant-derived irritants (e.g. phorbol esters, lectins and carrageenan) are used to study the mechanistic immunostimulatory pathways in immunological research. However, several serious questions such as what part of the immune system is stimulated and by what mechanisms have not been clearly investigated. Polysaccharides from a number of medicinal plants seem to positively stimulate immune cells. It may be the PAMP-like structure classified as arabinogalactan proteins that has been well established to activate cellular pro-inflammatory cascades (24). Nevertheless, their certain large molecules (a molecular weight of 20-1000 kDa) make them generally to be not bioavailable and ineffective orally. Moreover, although the cytokine secretion is an *in vivo* phenomenon, numerous plant-derived compounds are able to induce cytokine secretion from immune cells *in vitro*.

Several studies have been shown that some small organic bioactive compounds have been reported to induce immune functions both *in vitro* and *in vivo* (Table 2.8) (24, 45, 46, 48, 56) however the mechanisms of action remain unclear because of multiple cellular process interferences. The plant origin natural

products are, for examples, phytol, aristolochic acid as well as levamisole. Notably, levamisole was originally synthetized as anti-helminthic but it is used as immunomodulator or adjuvant in both human and veterinary. Phytol, a plant terpene, has been reported to possess adjuvant property and induce reactive oxygen production suggesting that it contains both pro-and anti-inflammatory properties. Interestingly, several cytotoxic compounds such as vincristine, paclitaxel and isopteropodin which are plant natural products have been shown to possess immunostimulatory activities over their anti-cancer properties suggesting a potential dual action. Moreover, it is considered that an initial immunostimulant may ultimately act as immunosuppressant when consume them long-term because of their potential cytotoxic properties. For instance, artistolochic acid, a small organic plant compound not only can significantly enhance phargocytosis but also damage tissues leading to indirect activation of innate immune system.

Table 2.8 Examples of botanical active constituents targeting immunostimulatory activity

Plant constituents	Biological properties
Alkaloids	
Aristolochic acid	Enhances phagocytosis
Cepheranthine	Stimulates antibody production
Camptothecin	Induces interferon
Saponins	
Ginsenoside	Induces lymphocyte proliferation
Oleanolic acid	Increases phagocytosis
Rb1 ginsenoside	Enhances the rabbit HI antibody titre to an inactivated rabbit hemorrhagic disease (RHD) vaccine
Quil A	Enhances the OVA-specific IgG, IgG1 and IgG2b levels in mice
Polysaccharides	
Heteroxylan	Stimulates phagocytosis, enhances production of antibodies against SRBC
Epimedin C	Induces lymphocyte proliferation
Astragalan	Increases HLA-DR, CD80, CD86 expression on DC
Lectins	
Viscumin	Activates lymphocytes
Concanavalin A (57)	Activates lymphocytes

Table 2.8 Examples of botanical active constituents targeting immunostimulatory activity (cont.)

Plant constituents	Biological properties
Terpenoids	
Echinadiol	Stimulates phagocytosis
Cardioside	Activates macrophages
Euperfolin	Stimulates phagocytosis
Quinines	
Limonoids	Enhances phagocytosis
Lawsone	Stimulates neutrophils
Gossypol	Induces interferon

At present, there are only a few actual exogeneous immunostimulants used in clinic in the exception with levamisole and all of those are not originated from plants. Nevertheless, the ISCOM, a novel vaccine adjuvant developed from saponin derived from *Quillaja saponaria* bark, was successful in many clinical trials. The ISCOM provides an important evidence to use a plant-derived compound as potential immunostimulant (36, 60).

In conclusion, although there are several studies on plant compound possessed immunostimulatory properties, the mechanisms of action and active ingredients need to be extensively investigated. It is noteworthy that many plant-derived compounds mediated *in vitro* or intraperitoneal injected immunostimulatory activities however those studies were performed based on *in vitro* studies and have not been discussed on bioavailability. Nevertheless, at least in theory, plant-derived immunostimulants are beneficial as exogeneous compounds used in enhance immune functions and homeostasis and need to develop further.

2.5.3 Flavonoids as immunomodulator

Flovonoids, also well-known as bioflavonoids are a set of polyphenolic compounds that are secondary metahbolites produced by the plant kingdom. Approximately 4000 flavonoids have been identified to date and are becoming increased numbers. They are intrinsically found in fruits, vegetables, common beverages like vine, coffee, cocoa and tea and our diet. However the flavonoids content contained in the same is highly inconstant. Structural variations on the core

structure result in different classes of compounds (51, 52, 61). The structural variations also lead to a wide range of biological activities observed in nature and in this regard, help in further dividing flavonoids into seven sub-groups. Nevertheless, the flavonoids mostly studied are in five major subclasses including flavones, flavonols, isoflavones, flavan-3-ols ans flavanones (51). In addition, each subclass of flavonoids can be further modified with additional processes such as methylation, dimerization, hydroxylation, and bisulfate formation. Notably, favonoids present in nature do not occur as aglycones in the exception with catechin and glycosylation becomes therefore the most important modification of flavonoids.

Flavonoids have been shown to exert several biological/pharmacological activities including antioxidant, anti-tumor, antiangiogenic, antithrombotic, anti-inflammatory, antiallergic and antiviral effects (62). Flavonoids are typical phenolic compounds and these properties may explain their potent metal chelators and free radical scavengers and powerful chain-breaking antioxidants. Therefore, their structural properties can exert a wide range of biochemical and biological/pharmacological effects that influence a variety cellular system of mammalian. It is mentioned that most studies on the potential benefits of these flavonoids have been carried on *in vitro* studies. In addition, a number of studies have been described that numerous flavonoids do not exert biological activities *in vivo* because of a lack of bioavailability and requirement of whether microbial or human metabolism.

The immunomodulatory activities of flavonoids have been investigated using numerous assays tested on a diverse series of distinct flavonoids classes (51, 52, 61). To date, many reviews associated to the immunomodulatory effects including anti-inflammatory properties of these compounds have been reported and recently their immunomodulatory capacities have gained more interest. It is noteworthy that several flavonoids contain the same biological effect on mammalian system however they may act on different mechanisms. The potential mechanisms related with the immunomodulatory property of flavonoids and examples are summarized in Table 2.9 (24, 39, 51, 61). Moreover, the *in vivo* preclinical and several clinical evidences correlated with their immunomodulatory benefits and bioactive flavonoids applied oral are listed in Table 2.10 (61).

Table 2.9 Examples of flavonoids and their immunomodulatory mechanisms of action

Mechanisms of action	Flavonoids
Enzyme regulation	
- Several flavonoids can modulate enzymes associated in critical metabolism pathways of mammalian cells which are regulation of cell survival, proliferation and cell signaling.	
1. Inhibition of protein tyrosine kinases (PTKs)	
a. Inhibitiom of myosin light chain kinase	Quercetin
b. Inhibition of protein kinase C, mitogen-activated protein kinase (MAPK)	Genistein, quercetin, fisetin, luteolin
2. Inhibition of enzymes involved in detoxification metabolism	
a. Glutathione S transferase, epoxide hydrolase, xanthine oxidase, and the cytochrome p450 system	Kaemferol
Antioxidant properties	
- During an inflammation considered as an immune reaction, a stress oxidative condition is caused by the activation of phargocytes, especially macrophages	
1. Inhibition of enzymes related with inflammation (e.g. phospholipase A2, ATPases, lipoxygenases, cyclooxygenases and phospholipase C	Quercetin, baicalein, hypolaetin, luteolin
2. Direct antioxidant properties and chelate metal ion	Typical flavonoids
3. Inhibition of enzymatic and non-enzymatic lipid peroxidation	Quercetin
Interference with cell signaling	
- Interaction with a certain free radicals and several key enzymes can regulate signaling cascades involving cytokines and regulatory transcription factors	
1. Interference with free radicals including ROS and iNOS	Quercetin, kaemferol
2. Interference with subsequent signal-cascade transcription factors (e.g. NF-kB, PPAR-γ)	Amentoflavone
Modulation of cytokines and immune cells	
1. Inhibition of T cell proliferation	Quercetin, genistein
2. Inhibition of cytolytic T lymphocytes activity	Luteolin
3. Inhibition of mitogen-activated antibody secretion by B cells	Quercetin
4. Enhancement of NK cytotoxicity against tumor cells	Flavone acetic acid, quercetin
5. Decrease release of histamine from mast cells	Myricetin, kaemferol, quercetin
6. Inhibition of secretion of pro-inflammatory cytokines or activation of anti-inflammatory cytokines	Hesperidin

Table 2.10 The *in vivo* and clinical immunomodulatory benefits of several flavonoids

Conditions	*In vivo*	Clinical
Chronic inflammation		
1. Rheumatoid arthritis	Hesperidin, luteolin, quercetin, rutin	Quercetin
2. Inflammatory bowel disease	Hesperidin, quercetin, rutin, diosmin, morin, silymarin	-
3. Atherosclerosis or cardiovascular diseases	-	Catechins, procyanidins, genistein, resveratrol
Allergy and hypersensitivity		
1. Asthmatic airway inflammation	Nobiletin*, genistein*, apigenin*, naringenin*, quercetin*, isoquercitrin, luteolin, narirutin	Genistein,
Prevention of infection		
1. Antibacterial activity	Luteolin (prevents chlamydiasis)	Quercetin anthrocyanidins**, proanthrocyanidins***
2. Septic shock	Naringin, luteolin, silymarin, wogonin, glabridin	Rutin
3. Anti-hepatitis C	-	Kushenin, silibinin

* Intraperitoneal administration

** Reduction of upper respiratory tract infection

*** Prevention of urinary tract infection

2.6 Chemical and pharmacological properties of *Thunbergia laurifolia*

Thunbergia laurifolia Lindl (Acanthaceae) is commonly known as blue trumpet vine or laurel clock vine. It is grown in tropics as ornamental vine and being a medicinal plant in some countries. Its leaves are dark green, opposite, heart-shaped, with a pointed tip and slightly serrated leaf margin.

In Thailand, *T. laurifolia* is well known as "Rang Chuet". Thai traditional medicine used their leaves as an antidote for poisons and drugs including anti-inflammatory, anti-diabetic, and antipyretic properties. Local herbal companies produce herbal tea and capsules from leave of Rang Chuet and are marketing for detoxifying effects.

2.6.1 Phytochemistry

There are a few studies of phytochemistry profile of *T. laurifolia* however phytochemistry profile from several parts has been elucidated (63, 64). Kanchanapoom et al. have studied the phytochemistry of its leaves (65). They have isolated two novel iridoid glucosides along with seven phenolic constituents including grandifloric acid, benzyl β-glucopyranoside, benzyl β-(2'-O-β-glucopyranosyl)-glucopyranoside, 6-C-glucopyranosyl apigenin, 6,8-di-C-glucopyranosyl apigenin, (E)-2-he[enyl-β-glucopyranoside, and hexanol-β-glucopyranoside. The two novel iridoid glucosides are 8-epi-grandifloric acid and 3'-O-β-glucopyranosyl-stilbericoside. Leaves, as well as flowers, of *T. laurifolia* have been found to compose of other phenolic compounds including delphinidin-3,5-di-O-β-D-glucopyranoside, apigenin, apigenin-7-O-β-D-glucopyranoside, chlorogenic acid and rosmarinic acid (66).

The water extract of leaves of *T. laurifolia* has been found the presence of phenolic constituents including flavonoids like apigenin and apigenin glucosides along with phenolic acids of caffeic, gallic, and protocatechuic. It is noteworthy that a study on the variations of phenolic compounds within *T. laurifolia* plant suggested that leaves and flowers had comparable phenolic content and free radical scavenging ability (63, 64).

2.6.2 Ethno-pharmacology

It is mentioned that, in the present review, we emphasize on the pharmacological effects of *T. laurifolia* as an aqueous extract preparation that can be comparative with our TLL extract.

2.6.2.1 Antioxidant activity

Dried leaf powder of *T. laurifolia* has been extracted using water ethanol and acetone and evaluated for antioxidant activity. Total phenolic content (TPC) profile showed that the most effective extract was the water extract (2430 mg GAE/100 g), whereas ethanol and acetone extract had values of 565 mg GAE/100 g and 142 mg GAE/100 g, respectively. Based on free radical scavenging ability, water extraction was also found the highest free radical scavenging. The EC_{50} value of water extraction was lowest as compared to ethanol and acetone extraction (0.13 mg, 0.26 and 0.61 mg GAE/ml, respectively. Additionally, the water extract exploited the highest (FRP) (0.93 mmol/g), compared to extracts of ethanol (0.18 mmol/g) and acetone (0.04 mmol/g) (64).

However, the variations of antioxidant properties of *T. laurifolia* have been reported. Many studies showed that antioxidant activities were affected by the optimum extraction time, extraction solvents, manufacturing methods, drying methods, the different ages, collection times and locations.

When compare to other 13 commercial herbal teas, antioxidant properties of *T. laurifolia* tea based on TPC, AEAC and FRP were categorized in the low antioxidant group. It had antioxidant properties as comparable as herbal teas of *Alpinia zerumbet*, *Garcinia atroviridis*, and *Cymbopogon citratus*. *T. laurifolia* tea prepared by proper methods such as microwave-dried tea, had significantly higher antioxidant properties than the commercial *T. laurifolia* herbal teas. Nevertheless, the antioxidant values of a well prepared *T. laurifolia* tea were not superior to common beverages like tea and coffee (63).

2.6.2.2 Antimicrobial activity

T. laurifolia leaf extracted with ethanol did not exert any antibacterial or antifungal activities against UV light-activated *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida*

albicans, and *Aspergillus fumigatus* with the exception of UV light-activated *B. subtilis* (64).

2.6.2.3 Antiproliferative activity

Antiproliferative activity of *T. laurifolia* ethanolic extracts against human breast cancer cells (SKBR3) was analysed using MTT assay. Dried leaf powder extract of *T. laurifolia* was also screened for this activity against two normal cells (BHK and L929) and two cancer cells (HepG2 and Caco-2) using MTT method. Similarly, these studies showed that *T. laurifolia* did not exhibit any cytotoxic activities against those cells (64).

2.6.2.4 Hepatoprotective activity

T. laurifolia extract from its leaf showed the hepatoprotective activity in mice from hepatic injury induced by ethanol (63, 67). In addition, aqueous extract of *T. laurifolia* protected rats from ethanol induced liver injury and in primary cultures of rat hepatocytes. The cell viability of primary cultures of rat hepatocytes treated with ethanol increased by 2-3 folds when the hepatocytes were incubated with *T. laurifolia* aqueous extract and decreased ALT and AST release levels. Severe rat liver injury induced by ethanol also recovered, as reflected by normalization in hepatic triglyceride, ALT, and AST levels, after 14 days of treatment of *T. laurifolia* aqueous extract.

2.6.2.5 Detoxifying effects

A study of the effects of *T. laurifolia* aqueous extract from its leaf to alleviate lead poisoning in the brain of mice has been conducted (68). The results suggested that the aqueous extract can protect the mice brain deterioration and memory loss from lead uptake inducing neuronal cell death. These results were dedicated to the capacity to maintain antioxidant enzymes and restore the levels of caspase-3 activity in the brain.

The study of *T. laurifolia* effects on endogenous dopamine release from rat striatal in compared with amphetamine effects has been reported (69). Rat striatal slices were investigated using *in vivo* functional nuclear magnetic resonance imaging to compare the effects of hot water extracts of dried *T. laurifolia* leaves on K^+ stimulated dopamine release. The result showed that release of dopamine

from rat striatal slices stimulated with *T. laurifolia* water extract showed the similar positive results to amphetamine.

A study of effects of *T. laurifolia* in the toxicity and addiction treatment was determined. This study had used *in vivo* functional nuclear magnetic resonance imaging to follow up the alteration on rat brain region activity. The result was reported that the methanolic extract of leaf of *T. laurifolia* can stimulate brain activity by increasing signal intensity in various regions of the brain. These effects were similar to amphetamine and cocaine effects. However, the effects of this plant to treat drug addiction should be confirmed whether *T. laurifolia* itself can cause addiction or not.

2.6.2.6 Antidementia effects

A study of effects of aqueous *T. laurifolia* leaf extract (TLL) on impaired cognitive function and emotional behavior in a mouse model has been clarified. The study used olfactory bulbectomized (OBX) mice that loosed their cognition and memory without object recognition impairment. The results showed that TLL decreased OBX-induced cognitive deficits in the objective recognition test (ORT) as well as tacrine. Furthermore, the results suggested that TLL extract could ameliorate the cognitive deficits by reversing of OBX-induced down-regulation of the central cholinergic function. In addition, TLL extract at 500 mg/kg as well as imipramine ameliorated depression-like behavior in OBX-induced mice model (70).

2.7 Properties of control substance

Plant lectins are glycoproteins that have ability to agglutinate (clump) red blood cells *in vitro*. Many lectins are found in storage tissues (almost present in seeds) that they constitutively accumulate in these tissues during a certain developmental period. Famous examples that are preferably used in immunological researchs are members of the legume lectin family such as the lectins expressed in the seeds of jackbean (*Canavalia ensiformis*, ConA), pea (*Pisum sativum*, PSA) or bean (*Phaseolus vulgaris*, PHA). Lectins also play important roles in the immune system because of their recognizing carbohydrate structure that similarly exert on pathogens (71).

Concanavalin A

The ability of Concanavalin A or Con A which usually extracted from the jack-bean to activate T lymphocytes was initially recognized in the studies with murine lymphocytes performed by Dutton in 1972. To date, it is well-established that Con A can potentially induce T cell proliferation, thus act as T cell mitogen and can activate functions of their subsets. The study performed with mouse lymphocytes had been shown that at least four functions of T cells were influenced by Con A. Activation of T cells by Con A can result in industriously proliferation, stimulation of helper T cells leading to efficient B cells production of antibodies, on the other hand, activation of suppressor T cells lead to suppression of T cell responses. Therefore, since Con A can potentially act via T cell-mediated mechanisms, it can be applicably used as model system to study the immunomodulatory mechanisms of potential substances (57, 71).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and solutions

3.1.1.1 Isolation and culture of PBMCs

Name	Catalog No.	Company
Lymphoprep™	1114547	Axis-Shield
Penicillin G/Streptomycin	A2210	Biochrom AG
RPMI 1640 medium	T121-01	Biochrom AG
Sodium bicarbonate	478537	Carlo Erba
FBS superior	S0615	Biochrom AG
Trypan Blue	T6146	Sigma

3.1.1.2 Determination of the cytotoxic effect of TLL

Name	Catalog No.	Company
MTT	19265	USB corporation
Dimethyl sulfoxide	67-68-5	Merck

3.1.1.3 Determination of cytotoxicity of NK cells

Name	Catalog No.	Company
MEM-Earle's	T031-10	Biochrom AG

3.1.1.4 Flow cytometric analysis of lymphocyte proliferation using CFSE dye

Name	Catalog No.	Company
CFSE	-	-
Concanavalin A	234567	Calbiochem

3.1.1.5 PBMCs phenotyping by flow cytometry

Name	Catalog No.	Company
A tetrachrome anti-CD panel for lymphocyte distribution	340503	Becton, Dickinson
A tetrachrome panel for T lymphocyte subpopulations	340503	Becton, Dickinson

3.1.1.6 Quantitative determination of cytokine production

Name	Catalog No.	Company
Human IL-2 ELISA kit	D2050	R&D Systems
Human IL-10 ELISA kit	D1000B	R&D Systems

3.1.2 Instruments

Name	Model	Company
Biohazard laminar air flow	CYTAIR	Flufrance
Flow cytometry	FACSCalibur	Becton, Dickinson
Flow cytometry	FACSCanto II	Becton, Dickinson
Microplate reader (UV scan)	InfinitedM200	Tecan
Microplate reader	Anthos 2010	Anthos

3.2 Methods

Many *in vitro* techniques have been developed and validated (11, 72). A PBMCs assay has been considered to be very advantageous to assess the immunogenicity of a small compound. PBMCs assay has been developed, validated and implemented to seek for interaction between reactive immune cells and a compound. Since PBMCs contain a major cellular component corresponding to immunogenicity, the use of PBMCs in various assays can comprehensively demonstrate overall immunogenicity of compounds (38). In PBMCs assay, a

promising compound is added to different lymphocyte populations therefore a promising compound may interact directly or indirectly with PBMCs and changes in a variety of immune responses can be quantified. In addition, PBMCs assay can be used to assess the mechanisms of such compounds intended to target immune system (72, 73). Nevertheless, it should be stated that no validated test systems are available yet and the fact that *in vitro* test system may not demonstrate the cellular complex interaction occurred within *in vivo* setting (74).

Although, a universal protocol for immunomodulatory screening is not established, as a general strategy, *in vitro* testing for an immunomodulatory activity should be done in a sequential step (75). A potential first step in this approach should include analyzing a prominent immunotoxic potential. Lymphotoxicity should be tested because a profound immunotoxic substance could eliminate or limit a rapid cell division, or interfere with cell activation affecting cell transduction pathway of lymphocytes which are the effectors and regulators of acquired immune system. After immunotoxic potential are excluded, basic immune cell functions should be analyzed by performing specific functional testing to characterize the mechanistic pathway of a promising compound. A wide range of *in vitro* methods are available to evaluate immune cell functions including lymphocyte proliferation assay, cytokine production, NK cell activity, and the T-dependent antibody response (TDAR) (38, 74, 75).

3.2.1 Ethical consideration

All experiments conducted on human blood materials were approved and exempted by Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review Board, Project number MU-DT/PY-IRB 2014/040.1710 (Appendix B).

3.2.2 Preparation and identification of *Thunbergia laurifolia* water extract (TLL)

Crude water extract powder from leaves of *Thunbergia laurifolia* has been prepared by Assistant Professor Dr. Piyanuch Rojsanga, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University (70).

Thunbergia laurifolia Linn. or "Rang Chuet" leaves were collected from Wangnamyen district, Sa Kaeo Province, Thailand, in May 2011. The plant samples were identified by Mr. Pinit Chinsoi, a pharmacist at Wangnamyen Hospital.

The leaves were cleaned and then dried using a hot air oven at 60°C for 6 hr. Dried leaves were further powdered with an electronic mill (20 mesh sieve). The 200 g of powdered leaves were boiled with 2 liters of distilled water for 8 hr and repeated for three times. The concentrate was filtered through Whatman Filter paper No.1. The combined concentrate was dried by lyophilization to give a dried leaf decoction extract (TLL) at a yield of 20.80% w/w (70).

Preliminary chemical analysis was performed by thin layer chromatography (TLC). The TLC profile revealed that TLL extract was contained phenolic compounds corresponding to caffeic acid. The further analysis also showed that the powder of TLL extract contained several flavonoids screening by aluminium chloride method. LC-MS analysis was performed using Shimadzu LC-IT-TOF mass spectrometer equipped with an ESI interface. The result revealed peaks corresponded to schaftoside and vitexin (70).

3.2.3 Preparation of stock solutions

TLL extract stock solution

The TLL extract powder was diluted with RPMI-1640 medium (GIBCO, USA) into a final concentration at 10 mg/ml. The solution was allowed to dissolve in water bath at 37°C for 30 minutes. Thereafter, the diluted preparation was subsequently filtered through a 0.22 μm filters. The filtered solution had been dispensed into individual aliquots at 500 μl and stored at −20°C until it was used.

Concanavalin A stock solotion

Concanavalin A powder (Calbiochem®) was dissolved in PBS into final concentration at 1 mg/ml and was dispensed into individual aliquots at 100 μl. The solution had then been stored at −20°C until it was used.

CFSE stock solution

5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) was kindly provided by Prof. Dr. Kovit Pattanapanyasat, Office of Research and

Development, Faculty of Medicine Siriraj Hospital, Mahidol University. CFSE was dissolved in DMSO as 5 mM stock solutions and stored at -20°C until it was used.

3.2.4 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were prepared using buffy coats of healthy blood donors received blood bank of Ramathibodi Hospital. The buffy coat samples (20 ml) were diluted with an equal volume of PBS and gently mix. The diluted suspension (30 ml) was slowly layered on the surface of 10 ml of the Lymphoprep™. After centrifugation at 800×g, 18°C for 30 min with no brake, a white cloud-like PBMCs band could be observed on an interface and was collected by carefully pipetting out. PBMCs were washed three times with excess PBS by centrifugation at 250×g at room temperature for 10 min and then resuspended in the RPMI-1640 medium containing 10% FCS (76, 77). A cell number was counted using a haemocytometer by trypan blue dye exclusion technique. The PBMCs number was adjusted to 2×10^6 cells/ml (78) and then cultured in the RPMI-1640 medium (Appendix A) containing 10% FCS at 37°C in a humidified incubator.

3.2.5 Determination of the cytotoxic effect of TLL on PBMCs

TLL extract may damage lymphocytes which are the primary effectors and regulators of acquired immunity. Using non-toxic concentrations of tested TLL extract is a crucial component of immune functional evaluations (cell viability > 80%) and a range of non-toxic concentrations of tested TLL extract should be primary identified (11, 75). A wide range of methods are available for investigate the immunotoxity of compounds, for examples colorimetic and flow cytometric assay.

3.2.5.1 Incubation and culture of PBMCs

TLL stock solution was diluted with RPMI-1640 medium containing 10% FCS to a final concentration at 500 µg/ml. A 200 µl aliquot was transferred to be the starting solution in the wells of first column of the plate. Subsequently, the 100 µl aliquot was removed from the starting solution to perform serial 2-fold dilutions. Finally, the various TLL extract concentrations ranging from 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 µg/ml were obtained. RPMI-1640 medium containing 10% FCS was used as negative control (79).

PBMCs at 2×10^6 cells/ml prepared as described above were transferred at 100 µl to a well of 96-well flat-bottom cell culture plate. The cells were incubated with TLL extract at final concentrations of 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 µg/ml. Each concentration of TLL extract was tested in triplicate. The total volume of each well was 200 µl. The plate was then incubated at 37°C in a humid atmosphere with 5% CO_2. After 24 and 48 hr, the cells were subjected to MTT assay. The four individual experiments were performed and analyzed.

3.2.5.2 MTT cell viability assay

The MTT colorimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies and was first developed by Mosmann. This assay fundamentally depends on the property of the yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is cleaved to form an insoluble purple formazan product by mitochondrial enzyme succinate-dehydrogenase (80). The number of formazan product is directly proportional to the number of living cells with functional mitochondria exposed to MTT at a time (79, 81).

Before the PBMCs were subjected to MTT assay, the 150 µl of supernatant of wells treated with various concentrations of TLL extract had been discarded. Each well had been washed three times with 200 µl PBS to remove TLL extract residual by centrifugation at 300×g 18°C for 10 min. After washing, 50 µl of freshly prepared MTT (2 mg/ml) dissolved in PBS was added in triplicate and another well, as background well was added with 50 µl of PBS. The incubation continued for another 4 hr then the plate was centrifuged (300×g at 18°C for 10 min). The supernatant and untransformed MTT was carefully removed by centrifugation (300×g at 18°C for 10 min) and pipetting. The resulting precipitate was dissolved by adding 200 µl of DMSO. After shaking for 15 min, the absorbance was measured in a microplate reader (Anthos 2010, Austria) at a wavelength of 590 nm. The result was expressed as the percentage of MTT absorbance with respect to vehicle-treated control cells as calculated by following equation:

$$\text{Cell viability (\%)} = \frac{(\text{ODsample} - \text{OD background})}{(\text{OD control} - \text{OD background})} \times 100$$

OD_{sample} = Optical density value of test sample wells containing TLL extract, PBMCs and MTT

$OD_{control}$ = Optical density value of the negative control wells containing culture medium, PBMCs and MTT

$OD_{background}$ = Optical density value of background wells containing test samples or culture medium, and PBMCs without MTT

3.2.6 Determination of cytotoxicity of NK cells

In addition to activation through mechanisms of cell death, cytotoxicity is defined as the cell-killing property of a substance or a mediator cell. The classical assay to evaluate NK cell function is the chromium 51-labeled sensitive tumor cells lysis such as K562 erythroleukemia cells (11, 75). NK cells in PBMCs were used as the effector cell and sensitive tumor cells were used as a target cell line in cell-mediated cytotoxicity assays. Previous established sensitive tumor cells are incubated with lymphocytes containing NK cells at different effector to target (E:T) ratios within a 96-well culture plate. However, in this present study, NK cell-mediated cytotoxicity was measured using MTT-based assay as previously described. NK cells are perfectly sensitive to modulation by toxic compounds however the predictive potential is still questioned because NK cell activity is not often associated to pathological conditions of the immune system (11, 72).

3.2.6.1 Preparation of effector cells

NK cells in PBMCs prepared as described above were used as the effector cell. PBMCs at 2×10^6 cells/ml were seeded into a 96-well flat-bottom cell culture plate as a starting number of effector cells. Then PBMCs were transferred from the starting well to perform the total four serial 2-fold dilutions of effector cells (76,

82, 83). The cells were then incubated with increasing concentration of TLL extract in triplicate.

TLL stock solution (10 mg/ml) was diluted with RPMI-1640 medium containing 10% FCS to a final concentration 200 µg/ml and was further prepared as serial 2-fold dilutions. The serial-2 fold dilutions of TLL extract were added to incubate with effector cells. Finally, effector cells were incubated with various final concentrations of TLL at 0, 6.25, 12.5, 25, 50 and 100 µg/ml. The total volume of each well was 200 µl. After incubation at 37°C for 24 hr. in a humid atmosphere with 5% CO_2, the effector cells were subjected to NK cells–mediated cytotoxicity assay.

3.2.6.2 Preparation of target cells

SK-N-SH cell lines

SK-N-SH is a neuroblastoma cell line which is widely and extensively used as a target cell line in cell-mediated cytotoxicity assays. It was first developed by J. L. Biedler et al. (1973), and was then established in cell culture from human metastatic neuroblastoma tissue (84). SK-NSH cells have been characterized by high dopamine β-hydroxylase activity, a very low activity level of glutamic acid decarboxylase, an ability of the conversion of glutamate to GABA by its enzyme, a negligible activity of choline acetyltransferase activity. These findings indicate that SK-N-SH cells exhibit a neuronal phenotype, and have multiple neurochemical markers.

SK-N-SH cells were cultured and maintained in an Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS). The cells were cultured in monolayer in 25-flask at 37°C under 5% CO_2 and 95% humidified air. Medium was changed every 3 days, and the cells were passaged if they reached approximately 80% cell confluence. The cells were expanded the culture to the sufficient number of cells before use (84).

3.2.6.3 NK cells-mediated cytotoxicity assay

Before the effector cells were subjected to NK cells–mediated cytotoxicity assay, the 150 µl of supernatant of wells treated with various concentrations of TLL extract had been discarded. Each well had been washed three

times with 200 µl PBS to remove TLL extract residual by centrifugation at 300×g 18°C for 10 min.

Pre-incubated PBMCs as effector cells were co-cultured with SK-N-SH cell as target cells in triplicate (76, 82, 83, 85). SK-N-SH cells were trypsinized from culture flask and counted using a haemocytometer by trypan blue dye exclusion technique. The cells were adjusted to 8×10^5 cells/ml and resuspended in RPMI 1640 medium containing 10% FCS then 50 µl of cells was seeded in 96-well flat-bottom cell culture plate to achieve different effector: target (E:T) cell ratios of 50:1, 25:1, 12.5:1 and 6.25:1. In each ratio of effector cell incubated with a concentration of TLL extract, the triplicate wells were seeded with SH-N-SH to obtain OD_{sample} whereas another triplicate wells was placed with 50 µl RPMI 1640 medium containing 10% FCS to obtain $OD_{effector}$. The effector and target cells were incubated for another 4 hr at 37°C in a humid atmosphere with 5% CO_2 in the incubator. SK-N-SH cells were separately seeded in four wells of 96-well flat-bottom cell culture plate at 4×10^4 cells/well in RPMI 1640 complete medium to achieve OD_{target}. Thereafter, the plate was subjected to MTT assay. The NK cell-mediated cytotoxicity was analyzed from three independent experiments. NK cells-mediated cytotoxicity against SK-N-SH cells was calculated as the percentage of total dead SK-N-SH cells as calculated by following equation:

$$\text{NK activity}(\%) = \frac{(\text{ODtarget} - (\text{ODsample} - \text{ODeffector}))}{\text{ODtarget}} \times 100$$

OD_{sample} = Optical density value of test sample wells containing the test samples, PBMCs, target cells and MTT

$OD_{effector}$ = Optical density value of the effector cell control wells containing the test samples, PBMCs and MTT

OD_{target} = Optical density value of target cell wells that containing the test culture medium, target cells and MTT

3.2.7 Flow cytometric analysis of lymphocyte proliferation using CFSE dye

The capability of lymphocytes to proliferate properly is a principal requirement for a healthy immune response. An appropriate proliferation requires the suitable stimulus including antigen presentation, cell contact dependent signalings from antigen presenting cells and cytokine microenvironment. Proliferation assay does not provide a specifically sensitive indicator for immunotoxicity but it is used to predict the direct ability of compounds to suppress or stimulate the metabolic events required for proliferation (11, 86).

The mitogen-stimulated proliferative response widely performed in immunotoxicology, immunomodulatory study and in clinical immunology, is an *in vitro* test associated to activation and proliferation of *in vivo* specific-antigen sensitized lymphocytes (38, 72). *In vitro* lymphocyte proliferation assay is an easy promising assay. The lymphocyte mitogens widely used to stimulate T- or B- cells are plant lectins (e.g. PHA, Con A, PWM, etc.) as well as anti-CD3, CD-28 antibodies, PPD and LPS. *In vitro* mitogen non-specific stimulation of lymphocytes leads to innumerable biochemical events, including calcium influx, protein kinase C activation, and phospholipids synthesis, culminating in DNA synthesis and cell division. Therefore, a compound interfering with these signal transduction pathways may have a capability to modulate lymphocyte proliferation. Moreover, the immunosuppressive and immunostimulatory properties may result from the inhibition of rapid cell dividing by necrosis or apoptosis, and from augment of metabolic activity, respectively.

The original method of investigating *in vitro* lymphocyte proliferation is a tritiated thymidine incorporation (87). However, currently, lymphocyte proliferation assay can be performed using a variety of techniques including colorimetric, flow cytometric and luminescent assays.

The discovery of the cell labeling non-radioactive fluorescence dye is critical for the progress of immunological studies. Carboxyfluorescein diacetate succinimidyl ester (CFSE) dye has been widely used in scientific immunological researches. The cell division analysis using CFSE dye relies on the simple basis that when a dye-labeled cell divides, the CFSE fluorescence intensity is half-decreased in the two daughter cells (88).

CFSE labeling dye possesses two important chemical natures making it ideal for flow cytometry assay (87). The first chemical attribute of the dye is the presence of two acetate groups, which permits the dye rapidly across the plasma cell membrane. Then acetate groups are removed by intracellular esterase. The removal lessens the membrane permeability of the dye, thus allowing the dye to concentrate within cells. The second attribute is the amino reactive succinimidyl side chain of the dye, which allows CFSE to covalently couple to a number of intracellular proteins. This results in a high degree of stable fluorescence. Therefore, CFSE are allowed to concentrate within cells up to 8 peaks of fluorescence (or 7 cell cycle divisions) to be assessed by flow cytometry and provides the information on the kinetics of proliferation (87).

3.2.7.1 CFSE dye labeling

PBMCs at 4×10^6 cells/ml were resuspended with CFSE (at final concentration of 10 μM/ml) in RPMI 1640 medium. After immediate vortex, The PBMCs were incubated for 10 min at 37°C in the dark. Then a cold RPMI 1640 containing 10% FCS was added to stop the staining reaction. The CFSE-labeled cells were further incubated on ice for 10 min then washed three times with cold RPMI 1640 containing 10% FCS (78, 87, 89).

3.2.7.2 Flow cytometric analysis

CFSE-labeled PBMCs, 4×10^6 cells/ml prepared as describe above were seeded at 1 ml into 24-well flat-bottom plate. PBMCs were incubated with final concentrations of TLL prepared as previous described, at 0, 6.25, 12.5, 25, 50 and 100 μg/ml in either unstimulated or pre-stimulated with a mitogen, Concanavalin A (Con A, 5 μg/ml). The total volume in each well is 2 ml. The plate was incubated at 37°C in a humid atmosphere with 5% CO_2 for 48 hr.

Pre-stimulated and unstimulated PBMCs depleted in adherent monocytes were collected and then washed twice with PBS. The cells were kept at 4-8°C in PBS in the dark until the next day of analysis. Fluorescence intensity was measured by flow cytometry using a BD FACSCanto II flow cytometer. A minimum of 20,000 cells was analyzed for each sample. Lymphocyte proliferation was analyzed with BD FACSDiva software. The lymphocyte proliferation was analyzed from three independent experiments (87, 88, 90).

3.2.8 PBMCs phenotyping by flow cytometry

Since T cells play an important role in the development of immune response, the evaluations based on these prominent T cells have become tools for investigating the immunogenicity of a new compound. Furthermore, besides APCs, among the known sub-lineages of T cells, Th cells are thought to be the most correlated T cells for immunogenicity of a substance. Therefore, extensive analysis of relevant T cell sub-lineages may give a better understanding of mechanistic basis, such as specificity, activation and expansion of the promising target cells.

Prediction of the immunogenicity caused by various compounds by investigation of lymphocyte and T cell sub-lineage epitopes requires a tool allowing for the evaluation of proteins expressed on the cell surface. Immunophenotyping and flow cytometry has recently become a valuable method for the research of immunomodulatory and immunotoxic effects.

Immunophenotyping of lymphocytes in peripheral blood, using fluorescence-labeled monoclonal antibodies directed to specific cell surface epitopes (markers) and flow cytometric analysis has become an important tool in immunomodulatory study (86). Optimal immunophenotyping using multicolor fluorescently labeled-monoclonal antibodies followed by flow cytometry allows better definition of immune cell subsets. Alterations in cell phenotypes have proved useful in some setting to identify the immunomodulatory potentials of different compounds. Nevertheless, phenotypic analysis alone is often not sufficient to indicate the immunomodulatory activity at low dosage of compounds.

Flow cytometry has been markedly developed over the last decade. Flow cytometry is literally the assessment of various natures/properties of single cells (particles) as they flow in single file past a beam of laser light. The scatter light from the cells or fluorescent emission is collected, filtered and then converted to digital values that are categorized and analyzed by a computer program. Flow cytometry can help in assessing cells at the single-cell level, one cell at a time made on thousands of individual cells within a very short period of time. Therefore, discrete population of cells can be identified and characterized without physical purification (86).

Evaluation of the corresponding lymphocyte epitopes in *in vitro* model may provide valuable data that point towards the specific effector cells affected by a

promising compound (11). The standard immunophenotyping panel for immunomodualtory evaluation typically includes total lymphocytes, mature $CD4^+$ and $CD8^+$ T cells, mature B cells, mature NK cells, and mature T cells. The past work has shown association between changes in PBMCs and immunotoxicity detection in rodents, especially immunosuppression.

3.2.8.1 PBMCs preparation for immunophenotyping

TLL stock solution (10 mg/ml) was diluted with RPMI-1640 medium containing 10% FCS to a final concentration 200 µg/ml. Subsequently, an aliquot at 1 ml was removed from the starting solution to further perform a serial 2-fold dilution to obtain TLL concentrations of 12.5, 25, 50, 100 and 200 µg/ml. A 1 ml aliquot of RPMI-1640 medium containing 10% FCS was placed into a well as negative control (62, 76, 78, 83).

PBMCs at 2×10^6 cells/ml prepared as described above were seeded at 1 ml to a well of 24-well flat-bottom plate in either unstimulated or stimulated conditions. For stimulated condition, PBMCs, 4×10^6 cells/ml prepared as describe above were activated with Con A at final concentration 10 µg/ml before seeding. The aliquot of each treatment was seeded into a well that had been placed with different concentrations of TLL extract. Finally, the cells in either unstimulated or stimulated conditions were incubated with final concentrations of 0, 6.25, 12.5, 25, 50 and 100 µg/ml of TLL extract and the final concentration of Con A in stimulated group was 5 µg/ml. The total volume of suspension in each well was 2 ml. The plate was incubated at $37^{\circ}C$ in a humid atmosphere with 5% CO_2 for 48 hr.

3.2.8.2 Immunofluorescence staining

Pre-stimulated and unstimulated PBMCs depleted in adherent monocytes were collected into disposable 12x75-mm BD Falcon™ capped polystyrene test tubes and then washed twice with 2 ml PBS. The 100 µl aliquot was then incubated in the dark for 15 min at room temperature with the following mAbs (BD Multitest IMK Kit, Becton Dickinson, Franklin Lakes, NJ, USA) 1) a tetrachrome anti-CD panel for lymphocyte lineages distribution (anti-CD3-FITC, anti-CD16+56-PE, anti-CD45-PerCP and anti-CD19-APC); and 2) tetrachrome panel for T lymphocyte sub-lineages using anti-CD3-FITC, anti-CD8-PE, anti-CD45-PerCP and anti-CD4-APC. After staining, the cells were washed twice with 1 ml PBS by

centrifugation (300×g, room temperature, 10 min). The cells will be kept at 4-8°C in the dark until the next day of analysis (62, 76, 83).

3.2.8.3 Flow cytometric analysis of lymphocyte lineages and T cell sub-lineages distribution

Immunofluorescence was measured by flow cytometry using a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). A minimum of 5,000 cells was analyzed for each sample. Lymphocyte distribution and T lymphocyte sub-lineages were further analyzed with CELLQUEST software. The lymphocyte lineages and T cell sub-lineages were analyzed from three independent experiments. The lymphocyte distribution results were checked to control accuracy based on the principle that the total T cells ($CD3^+$), B cells ($CD19^+$) and NK cells ($CD16^+$+$CD56^+$) should be equal to the total lymphocytes ($CD45^+$) (between 100±5%). Cross-checks within T lymphocyte subsets can be performed based on the principle that the total T cells ($CD3^+$) should be equal to $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (between 100±5%) (62, 76, 78, 83).

3.2.9 Quantitative determination of cytokine production by ELISA

Cytokines are protein produced by immune cells that have compensatory activities and innate redundancy of action. Therefore, the influence of cytokine on a certain immune cell should be viewed not as discrete entities with single actions but as integrated biological network. Immunomodulation produced by cytokines is the results of modification of these networks and typically occurs from over- or under-production due to the effects of compound on immune target cells (75).

The modulation of any immune response is dependent upon the production and secretion of cytokines. Cytokines are secreted as one of the primary steps in the immune response and the altered quantitative levels can be used as a prediction of immunomodulation. Furthermore, the relevant adverse events of immunostimulatory drugs are almost associated with cytokine release (38). Moreover, due to species-specific differences, cytokine release from human cells is considered to be a more reliably non-clinical tool than *in vivo* test in monkey.

Numerous techniques are available for quantitative measuring cytokines and their receptors including ELISA, flow cytometry, and molecular biology

techniques such as PCR (11, 72). Due to human peripheral blood cells can produce various cytokines originating from several blood immune cell populations, the highly pleiotropic and redundant nature of cytokines, it is advisable to measure the wide panel of cytokines possible in any *in vitro* system evaluating this end point (11, 75). Depending on the selective stimuli, culture of lymphocytes with a compound in the presence of stimulus allows quantitative measurement of influences on production of different cytokines releasing from several lymphocyte populations (78). The model has been shown to reflect several perspectives of immunomodulation such as immunostimulation, priming and inhibitory effects. Interestingly, it is also more believed that Th cells ($CD4^+$) may polarize into either Th1 cells or Th2 cells by the types of cytokines they produce. Th1 cells can produce IL-2, IL-12 and IFN-γ to enhance macrophages and cytotoxic T lymphocytes to kill virus-infected cells or tumor cells whereas Th2 cells can secret IL-4, IL-5, IL-10, IL-13 to induce B cells to initiate antiboby production (11). Therefore, quantitative assessment of over- or under-produced level of cytokines can point towards a skewing into a discrete phenotype of T cells.

3.2.9.1 PBMCs preparation for cytokine detection

As previous described, PBMCs at 2×10^6 cell/mL in RPMI-1640 medium containing 10% FCS were treated at 48 and 72 hr, for IL-2 and IL-10 detection, respectively with Con A at final concentration of 5 μg/mL in the absence or presence of different final concentrations of TLL extract prepared as previous described at 0, 6.25, 12.5, 25, 50 and 100 μg/ml in 24-well flat-bottom plate. Unstimulated PBMCs as negative control were seeded at 1 ml and added with 1 ml RPMI-1640 medium containing 10% FCS. The total volume of suspension in each well was 2 ml (62, 76, 78).

3.2.9.2 Detection of cytokine production by ELISA kit

Cell-free supernatant from each well was collected by centrifugation (300×g at 18°C for 10 min). The supernatant had been kept at −20°C until it was used to quantitatively determine the cytokine level. The amount of IL-2 and IL-10 in cultured supernatants was analyzed using commercially available cytokine-specific ELISA kits (Quantikine®). The analysis was performed according to the manufacture's protocol. The optical density was measured using a microplate

reader (Tecan™). The sensitivities of IL-2 and IL-10 were 2,000 and 500 pg/ml, respectively. The cytokine level of IL-2 and IL-10 were analyzed from three independent experiments (62, 76, 91).

3.2.10 Statistical analysis

The data were analyzed using SPSS statistics version 20 and expressed as mean ± SD of raw data and as the ratio (expressed in percentage) between the TLL treated value and the baseline value (set at 100%). Either one-way ANOVA with Turkey test or Dunnett's test for the normal distribution data or Kruskal-Wallis H test following with Mann-Whitney U test for the nonparametric data was used to analyze statistical significance of the difference between control and treated samples. Statistical significance level was set at $p<0.05$.

CHAPTER IV
RESULTS

4.1 Preparation and activation of peripheral blood mononuclear cells (PBMCs)

PBMCs were prepared by using a buffy coat of healthy blood donor obtained from the blood bank of Ramathibodi hospital. We used Lymphoprep™, a density gradient solution, to isolate PBMCs from the blood samples. Because of the different density between Lymphoprep™ and other blood components, we can take an advantage of the density differences to separate them (11). After centrifugation, a white cloud-like PBMCs band was observed on the interface between Lymphoprep™ and plasma. Red blood cells and granulocytes were found at the bottom of Lymphoprep™ layer in contrast to plasma and platelets that found on the top of Lymphoprep™ (Figure 4.1A). The PBMCs were resuspended in the RPMI 1640 supplemented with 10% FCS (Figure 4.1B).

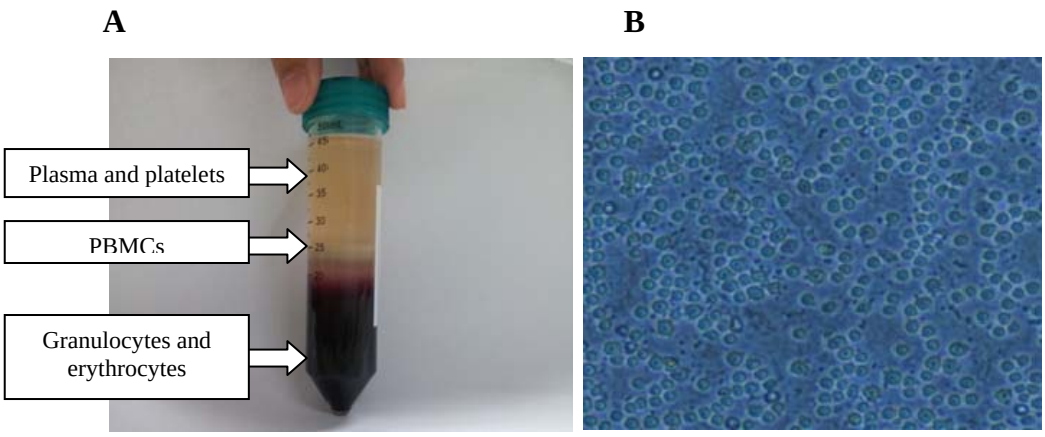

Figure 4.1 (A) An isolated white cloud-like band of PBMCs (B) The microscopic picture of cultured PBMCs at 2×10^6 cells/ml.

4.2 Effects of TLL extract on PBMCs viability

In order to determine any immunotoxicity of TLL extract on normal immune cells, PBMCs viability was evaluated using a MTT colorimetric based assay. PBMCs were incubated with various concentrations of TLL extract (3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 µg/ml) for 24 and 48 hr. The results showed that the PBMCs were not significantly sensitive to TLL toxicity whether 24 and 48 hr compared to untreated resting control cells as shown in Figure 4.2A and 4.2B, respectively. The optical density averages ± SD of untreated cells at 24 and 48 hr were 0.354 ± 0.039 and 0.382 ± 0.041, respectively. These optical density averages were calculated to obtain 100% baseline values.

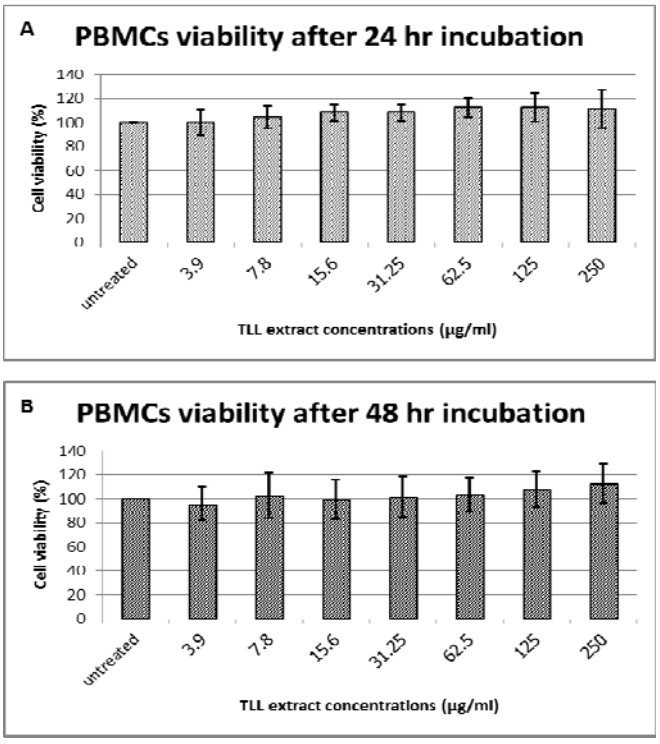

Figure 4.2 Effects of TLL extract on PBMCs viability. PBMCs were incubated for (A) 24 hr and (B) 48 hr with different concentrations of TLL extract (0-250 µg/ml). Cell viability was assessed using MTT colorimetric based assay. The values were mean ± SD (n=4). The untreated groups were calculated to obtain 100% cell viability as baseline values.

In this study, more than 95% of cell survival was obtained from any TLL incubated groups in comparison to untreated cells. We also observed a more increase in cell viability at higher concentrations of TLL compared to untreated cells with both 24 and 48 hr incubation. The compounds can be determined as immunotoxic compounds if those compounds reduce cell viability more than 20% or greater (74). However, a cell number in all experimental groups was assumed to remain more than 80% of primary cell cultures. Since TLL extract at all concentrations tested did not exhibit any toxicity on normal PBMCs up to 48 hr, we could further investigate the immunomodulatory effects of TLL (74).

4.3 Effects of TLL extract on NK cells-mediated cytotoxicity activity

NK cells play a crucial role in innate immune response. Immunomodulatory activity of compounds can be indicated by an increase or decrease in target cell lytic activity of NK cells. Moreover, NK cells mediated cytotoxicity is perfectly sensitive to modulation by immunosuppressive agents (11). For measurement of potential effects on cell-mediated innate immune response, we optimized the NK cells-mediated cytotoxicity against SK-N-SH sensitive tumor target cell quantified by MTT assay. PBMCs incubated with various concentrations of TLL extract (6.25, 12.5, 25, 50 and 100 μg/ml) for 24 hr were used as the effector cells. We also performed this assay at differential effector to target cell (E:T) ratios starting at 50:1 ratio to 6.25:1 ratio (92).

To be effective, the number of NK cells must achieve E:T ratio estimated to exert a visible antitumor activity. The averages of cytotoxicity effects among untreated cells at differential E:T ratio (6.25:1, 12.5:1, 25:1 and 50:1) were calculated as percentage baseline values (%). These cytotoxicity baseline values were not directly proportional to the increasing 6.25:1, 12.5:1 and 25:1 ratio (46.39 ± 46.42, 48.75 ± 44.41 and 44.78 ± 18.01%, respectively) however there was increasing of cytotoxicity baseline value at 50:1 ratio (60.81% ± 43.05%). There were no significant differences between averages of cytotoxicity values among these E:T ratios. In addition, whether

the E:T ratios, our results showed that there were no significant changes in NK mediated-cytotoxicity values between various TLL concentrations (Figure 4.3.1).

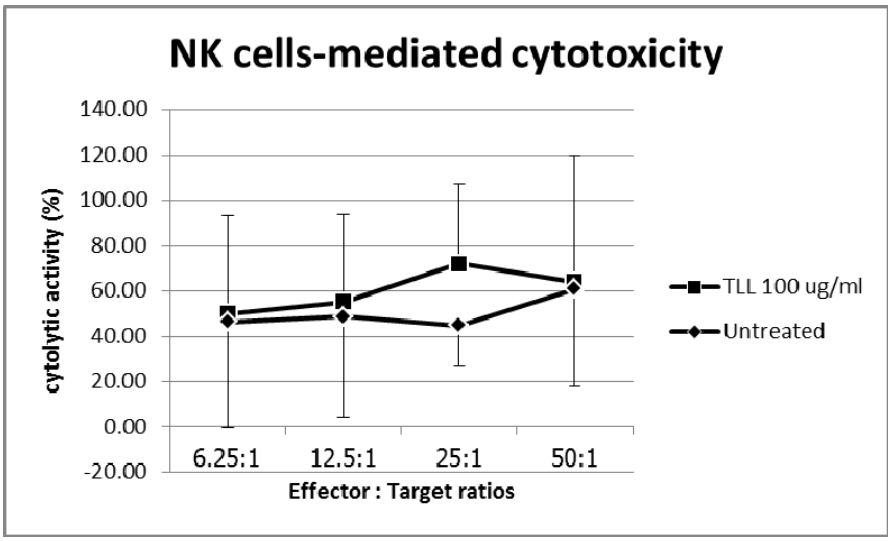

Figure 4.3.1 *In vitro* effects of TLL extract at 100 µg/ml on natural killer (NK) cells cytotoxic activity against SK-N-SH cells after 24 hr treatment in comparison with untreated NK cells at expected 6.25:1, 12.5:1, 25:1 and 50:1 E:T ratio. NK cells cytotoxic activity was measured using MTT assay. The values were expressed as cytolytic activity percentage means ± SD (n=3).

It was also mentioned that the standard variations of baseline value whether E:T ratios seemed to be in a wide variable degree (Table 4.1)

Table 4.1 NK cells cytolytic activity against SK-N-SH cell at different E:T ratios

E:T ratio	TLL extract concentrations (µg/ml)					
	0	**6.25**	**12.5**	**25**	**50**	**100**
6.25:1* ratio	46.39±46.43	44.11±12.90	46.52±15.30	41.71±51.16	49.89±47.38	49.88±43.65
12.5:1* ratio	48.75±44.41	44.77±16.45	45.30±15.00	68.61±30.15	58.01±36.36	55.19±38.81
25:1* ratio	44.78±18.01	49.28±14.09	49.41±11.74	51.24±44.86	69.19±27.42	72.22±36.95
50:1* ratio	60.81±43.05	45.87±6.57	51.98±24.79	55.58±40.17	45.46±50.61	64.14±93.35

* Results were expressed as lytic activity percentage mean ± SD.

We also observed that the higher concentrations of TLL extract whatever E:T ratios, especially at 100 µg/ml seemed to strengthen the cytotoxicity activity of NK cells in the exception with 12.5:1 ratio (Figure 4.3.2). Although, we could not observe any significant modulations of NK cells mediated-cytotoxicity after incubation of various concentrations of TLL, but we observed the tendency to increase the NK cells lytic activity at higher TLL concentration in most E:T ratios.

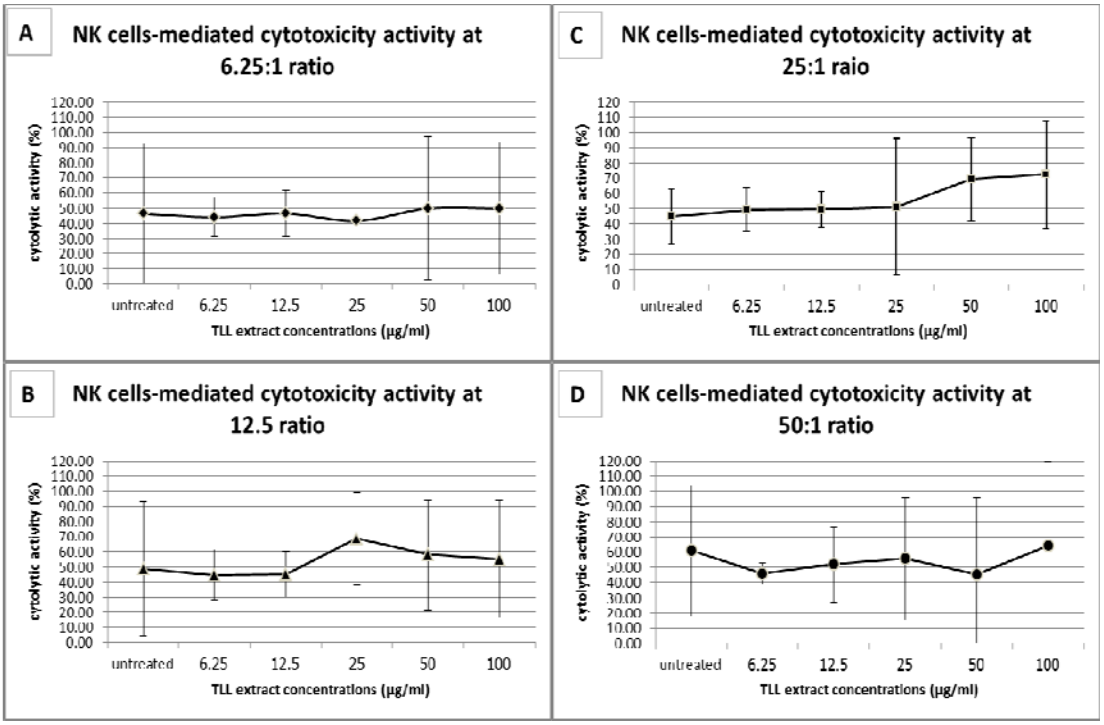

Figure 4.3.2 *In vitro* effects of various TLL extract concentrations on natural killer (NK) cells cytotoxic activity against SK-N-SH cells after 24 hr treatment at expected (A) 6.25:1, (B) 12.5:1, (C) 25:1 and (D) 50:1 E:T ratio. NK cells cytotoxic activity was measured using MTT assay. The values were expressed as cytolytic activity percentage means ± SD (n=3).

4.4 Effects of TLL extract on CFSE-labeled lymphocyte proliferation

Lymphocyte proliferation is a significant event of responses of adaptive immunity. Activated lymphocytes, especially T cells play an important role in pathogenesis of several diseases like allergy, asthma (31) and autoimmune diseases. Thus, in this study, we examined the effects of TLL extract on lymphocyte proliferation focused on T cells proliferation. We used the generally known T cells mitogen, Concanavalin A (Con A), at 5 µg/ml to stimulate T cells for 48 hr and determined the effects of various TLL extract concentrations (6.25, 12.5, 25, 50 and 100 µg/ml) on T cells proliferation using CFSE dye following by flow cytometry (93, 94).

In the absence of Con A, data analysis of unstimulated cells exposed with higher concentrations of TLL extract showed a dose dependent reduction of lymphocyte proliferation however there were no significant differences. The proliferative capacity of untreated resting cells presented as percentage mean ± SD (%) was 15.93 ± 13.70% and was normalized to 100%. The proliferative capacity of unstimulated cells treated with concentrations of TLL extract at 6.25, 12.5 and 25 was slightly increased but no significant differences. In contrast, The proliferative capacity of unstimulated cells treated with the higher concentrations of TLL extract at 50 and 100 µg/ml were markedly reduced by 13.96 and 76.65%, respectively in comparison to TLL untreated control cells however there were no significant alterations (Figure 4.4.1A). These results were consistent with our lymphocyte lineages distribution data that remained unchanged after incubation with TLL extract. We suggested that the unstimulated PBMCs proliferative capacity was not activated or suppressed by TLL extract

In the presence of Con A, various concentrations of TLL extract showed a dose dependent reduction on the proliferative capacity of lymphocytes and these proliferative capacities seemed to be more sensitive suppression in comparison with unstimulated PBMCs groups. Our study showed that lymphocytes induced with Con A for 48 hr in the absence of TLL extract seemed to proliferate properly. The proliferative capacity of TLL untreated control cells, expressed as percentage mean ± SD was 23.03 ± 17.98%. The proliferative capacity was averaged to 100% as baseline value. We also observed that lymphocytes had undergone a round of cell cycle

division as shown in Figure 4.4.2. TLL extract at concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml was gradually reduced the proliferative capacity of Con A by 4.98, 9.10, 11.00, 61.82 and 92.37%, respectively compared with stimulated control cells (Figure 4.4.1B). Interestingly, our data indicated that TLL extract at 50 and 100 µg/ml could significantly inhibit the proliferative capacity of Con A stimulated cells ($p<0.01$). As described above, we concluded that the proliferative capacity of lymphocytes was interfered with potential suppressive effects of TLL extract as a dose dependent tendency and T cells might be the major cells affected by suppressive effect of TLL extract.

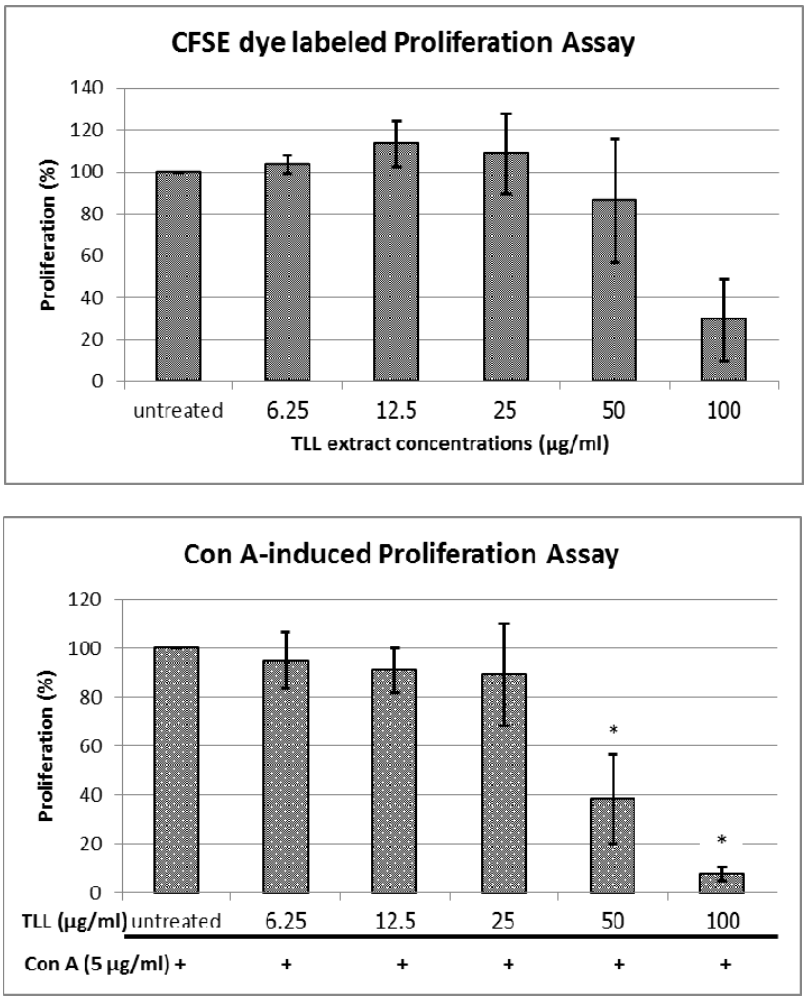

Figure 4.4.1 Suppressive effects of various concentrations of TLL extract on lymphocyte proliferation. CFSE-labeled lymphocytes were incubated with (A) increasing concentrations of TLL extract without Con A; (B) increasing concentrations of TLL extract in the presence of Con A (5 µg/ml). Lymphocyte proliferation was investigated using CFSE dye following by flow cytometry. The values were normalized to TLL untreated control in the presence/absence of Con A (=100 %). The data was presented as percentage of cell proliferation ± SD (n=3). Statistical significance, *p<0.01 when compared to TLL untreated control cells.

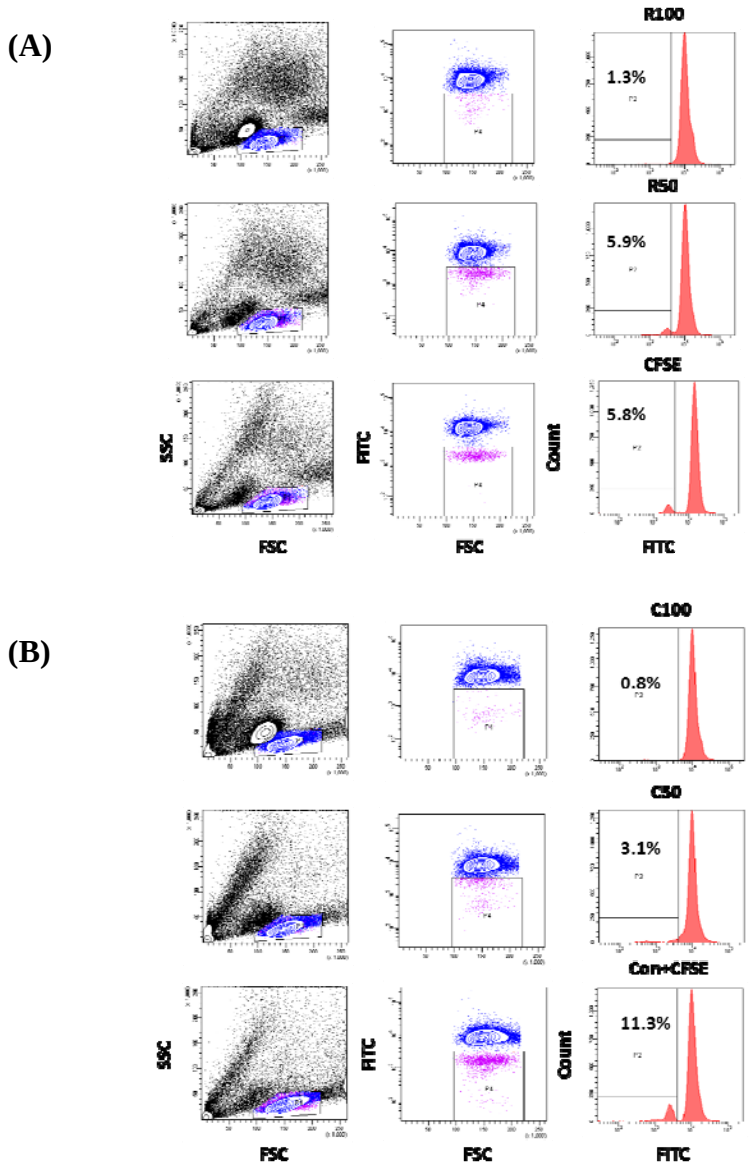

Figure 4.4.2 Effects of TLL extract on cell division of CFSE-labeled lymphocytes assessed by flow cytometry and pictured as representative dot plots (middle panel) and histograms (right-sided panel) in culture with (A) increasing concentrations of TLL extract without Con A (R100 and R50 were CFSE-labeled lymphocytes incubated with TLL at 100 and 50 µg/ml, respectively); (B) increasing concentrations of TLL extract with Con A (5 µg/ml) (C100 and C50 were CFSE-labeled lymphocytes incubated with TLL at 100 and 50 µg/ml, respectively). FSC is forward scatter, SCC is side scatter and FITC is fluorescein isothiocyanate.

4.5 Effects of TLL extract on lymphocyte lineages and T cell sub-lineages distribution

Plant-derived immunomodulatory agents are able to alter lymphocyte phenotypes in many ways including alterations in cell proliferation, activation, suppression or differentiation. Thus, lymphocyte lineages distribution data can be useful in investigating a specific target cell of plant-derived immunomodulators (11, 75). In this study, we emphasized to characterize the effect of TLL extract on distribution of T lymphocytes, the prominent cells of adaptive immune response. We evaluated the effects of TLL extract at 6.25, 12.5, 25, 50 and 100 µg/ml whether in the presence or absence of Con A (5 µg/ml) on the distribution of lymphocyte lineages (T, B and NK cells) and T lymphocyte sub-lineages ($CD4^+$ and $CD8^+$). Lymphocyte lineages data can be obtained from immunophenotyping followed by flow cytometric analysis. Distribution of lymphocyte lineages data was evaluated after 48 hr incubation on the basis of cell surface expression of $CD45^+$, $CD3^+$, $CD16^+$+56^+, $CD19^+$ whereas T lymphocyte sub-lineages data was investigated on the basis of expression of $CD4^+$ and $CD8^+$ (8).

In the absence of Con A, lymphocytes treated with TLL extract whatever concentrations had no remarkable effects on the lymphocyte lineages distribution in comparison with untreated resting cells as presented in Table 4.2.

Table 4.2 Distribution of lymphocyte lineages and T lymphocyte sub-lineages in the absence of Con A

Cell Types	TLL extract concentrations (µg/ml)					
	0	**6.25**	**12.5**	**25**	**50**	**100**
T cells ($CD3^+$)[a,*]	72.73±6.24	73.76±7.22	75.78±6.89	73.78±6.65	75.12±8.89	75.06±8.74
B cells ($CD19^+$)[a,*]	4.30±1.36	4.03±1.16	2.89±0.52	3.23±0.92	2.68±0.47	2.65±0.32
NK cells ($CD16^+$+56^+)[a,*]	22.97±7.21	22.22±7.97	21.33±6.52	23.00±6.02	22.20±8.66	22.30±8.80
Helper T cells ($CD3^+CD4^+$)[b,*]	8.21±2.12	7.47±2.02	8.75±2.19	8.93±1.45	9.24±1.26	11.88±4.05
Cytotoxic T cells ($CD3^+CD8^+$)[b,*]	43.96±16.26	45.58±15.14	46.98±17.70	45.92±14.91	45.20±16.20	44.93±17.13

[*] Results were expressed as percentage mean ± SD.

[a] T cells, B cells and NK cells as a percentage of total lymphocyte population.

[b] Helper T cell and cytotoxic T cells as a percentage of total T cells.

In accordance with T cells distribution, the proportion of T cells sub-lineages ($CD8^+$ and $CD4^+$ T cells) after treatment with TLL extract did not showed any significant changes (Figure 4.5.1A and 4.5.1B, respectively). However, the data showed that TLL extract at 100 µg/ml slightly exhibited stimulation on $CD4^+$ expression compared to untreated resting cells. These results were consistent with our proliferation assay results which TLL extract itself did not demonstrate any significant alterations of lymphocyte proliferation after treatment with TLL extract alone.

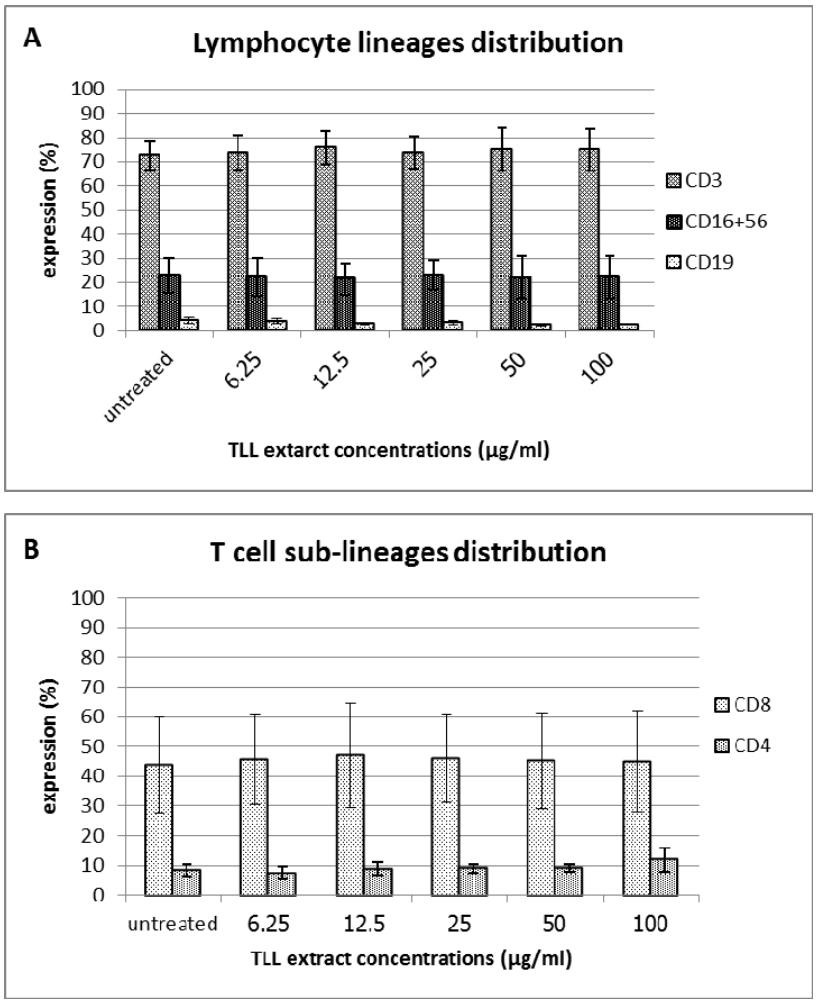

Figure 4.5.1 Percentage of expression of (A) T, B, and NK cells population and (B) T cells sub-lineages after incubation with increasing concentrations of TLL extract in the absence of Con A for 48 hr. Distribution data was obtained from immunophenotyping following by flow cytometric analysis. Results are expressed as mean ± SD of three independent experiments.

In the presence of Con A, our data showed that TLL extract at all test concentrations (0-100 µg/ml) did not modify the distribution of lymphocyte lineages. Moreover, our experiments exploited that T cell subsets were not polarized to any $CD4^+$ and $CD8^+$ T cells by TLL extract. In the present study, $CD3^+$ T cells distribution was decreased as compared with unstimulated resting cells contrary to NK cells (Table 4.3). The decrease of distribution percentage of T cells was ascribed to Con A proliferative effects and might be caused by our gating criteria (data not shown). During cell division a size of lymphocyte is increased and can be detected using forward scatter with flow cytometry. However, lymphoblasts that undergo incomplete cell division and expression of specific CD on their cell surface because of short time of incubation might be excluded from our gating (78, 87, 94, 95). Notably, Con A stimulatory effects has been shown most effective at day 5 (78, 87, 88). Thus, several $CD3^+$ cells that were major cells activated to proliferative effect of Con A might simultaneous divide and expand out of our gating. Those cells might be excluded whereas percentage of non-divided NK cells was increased. Furthermore, it was mentioned that when a general T cells expression after stimulation with Con A was decreased in comparison with unstimulated resting cells whatever TLL concentrations, $CD8^+$ expressing T cells percentages were slightly decreased (96) in contrast to $CD4^+$ T cells that showed a little increase in all TLL concentrations.

Table 4.3 Distribution of lymphocyte subpopulations and T lymphocyte subsets in the presence of Con A

Cell Types	TLL extract concentrations (µg/ml)					
	0	**6.25**	**12.5**	**25**	**50**	**100**
T cells ($CD3^+$)[a,*]	67.85±9.85	67.66±10.17	68.38±7.70	67.60±7.71	67.27±7.72	65.91±13.16
B cells ($CD19^+$)[a,*]	2.78±0.53	3.61±1.31	4.20±0.92	3.35±0.65	3.26±1.42	2.13±0.63
NK cells ($CD16^+$+56^+)[a,*]	29.37±9.89	28.73±9.38	27.41±8.34	29.06±7.80	29.48±7.39	31.96±13.62
Helper T cells ($CD3^+CD4^+$)[b,*]	9.18±3.14	8.14±2.22	9.58±3.76	9.29±3.34	9.20±3.16	11.87±4.59
Cytotoxic T cells ($CD3^+CD8^+$)[b,*]	39.44±7.79	40.47±8.64	40.60±9.18	40.83±6.99	42.07±7.22	43.67±12.67

[*] Results were expressed as percentage mean ± SD.

[a] T cells, B cells and NK cells as a percentage of total lymphocyte population.

[b] Helper T cell and cytotoxic T cells as a percentage of total T cells.

TLL extract at various concentrations did not show any modulations on T, B or NK cells distribution. Nevertheless, TLL extract showed a slight suppressive tendency in B cells (Figure 4.5.2A). The TLL extract concentration at 100 µg/ml demonstrated the maximal suppression in B cells in comparison with Con A stimulated control cells. As well as T lymphocyte distribution, T cells sub-lineages did not alter to polarize into any sub-lineages (Figure 4.5.2B). TLL extract showed a slight dose-related increasing in $CD8^+$ T cells expression. The maximal stimulatory effect was observed at a concentration of 100 µg/ml compared to Con A stimulated control cells. Furthermore, $CD4^+$ T cells expression also exhibited maximal stimulatory effects at 100 µg/ml of TLL extract compared with Con A stimulated control cells however we could not observe a dose dependent manner. Nevertheless, the proportion of $CD8^+$ and $CD4^+$ remained unchange.

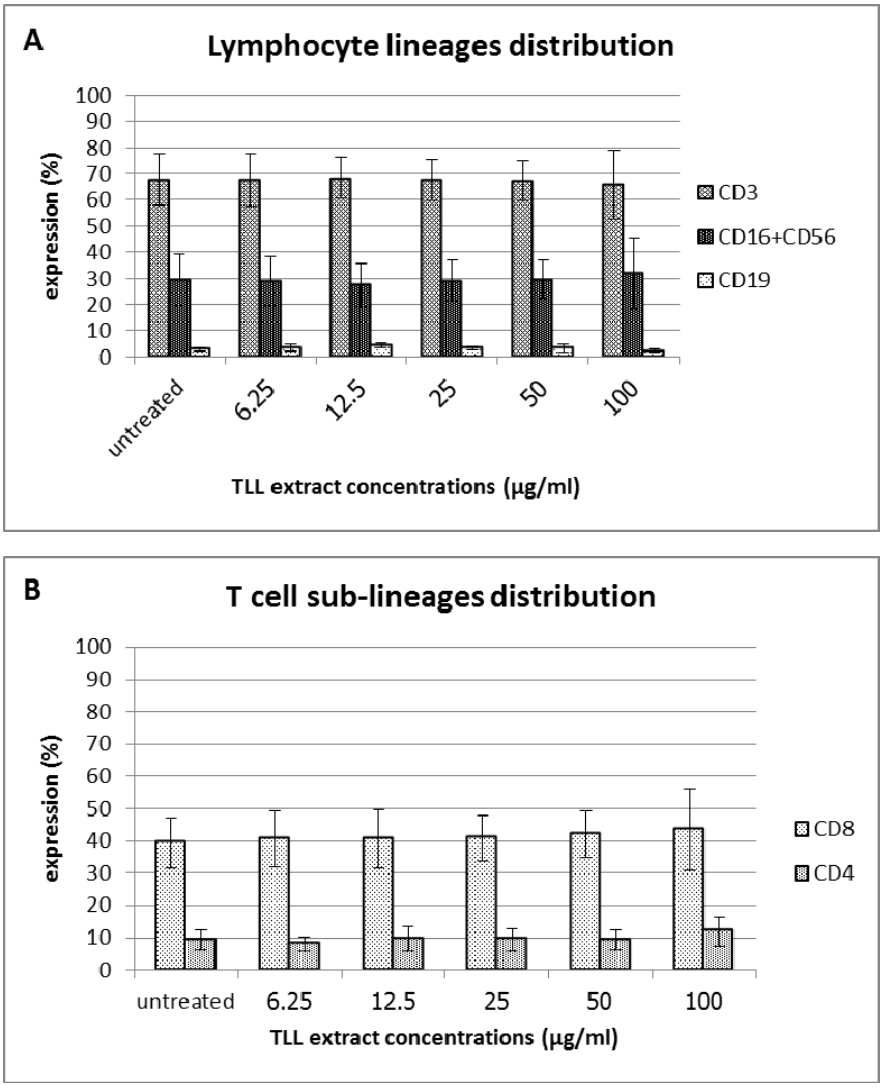

Figure 4.5.2 Percentage of expression of (A) T, B, and NK cells populations and (B) T cell sub-lineages after incubation with increasing concentrations of TLL extract in the presence of Con A (5 µg/ml) for 48 hr. Distribution data was obtained from immunophenotyping following by flow cytometric analysis. Results were expressed as mean ± SD of three independent experiments.

4.6 Effects of TLL extract on modulation of cytokines production

Cytokine secretion is an initial response of immune cells to antigenic stimulation (97). Besides cell to cell interactions, cytokine production is a crucial mode for triggering progressive activation or sequential inhibition of lymphocytes responses. The elucidation of cytokine pattern is used as a prediction of mechanisms of plant-derived immunomodulators (98). In addition, a predominance pattern of helper T cells (Th cells) cytokine secretion reflects the skewing of Th cells into their sub-lineages, Th1 and Th2 cells. In this study, we examined how TLL extract particularly modulated cytokine secretions of T cells. We focused on the production of IL-2 which is Th1 pro-inflammatory cytokine as well as IL-10 which is regulatory T cells (Tregs) and Th2 inhibitory cytokine. Stimulation with Con A for 48 to 72 hr leads to the release of IL-2 (82, 91) and IL-10 from T cells, respectively. Thus, we determined the effects of TLL extract at 6.25, 12.5, 25, 50 and 100 µg/ml on Th1 and Th2 cytokine secretion after stimulation with Con A (5 µg/ml) for 48 to 72 hr using ELISA detection.

Our study showed that TLL extract, especially at 100 µg/ml promoted Con A-induced IL-2 production by human lymphocytes in a proportional manner to the concentrations of TLL extract. It was noteworthy that Con A influenced properly the release of IL-2 from lymphocytes (19.93 ± 17.68 pg/ml) in comparison with unstimulated resting cells (9.78 ± 11.12 pg/ml). The analysis of IL-2 production showed sequential increasing levels as a dose dependent manner. The production of IL-2 was increase by the treatment of 6.25, 12.5, 25, 50 and 100 µg/ml of TLL extract (21.45 ± 18.04, 24.73 ± 23.21, 177.33 ± 283.56, 244.39 ± 394.63 and 271.93 ± 312.92 pg/ml, respectively) compared with untreated resting control cells (Figure 4.6.1). At the concentration of 100 µg/ml, IL-2 release was marked different from stimulated control by 32 folds however there was no significant difference in comparison with stimulated control. This result indicated that TLL extract might polarize Th cells into Th1 sub-lineages.

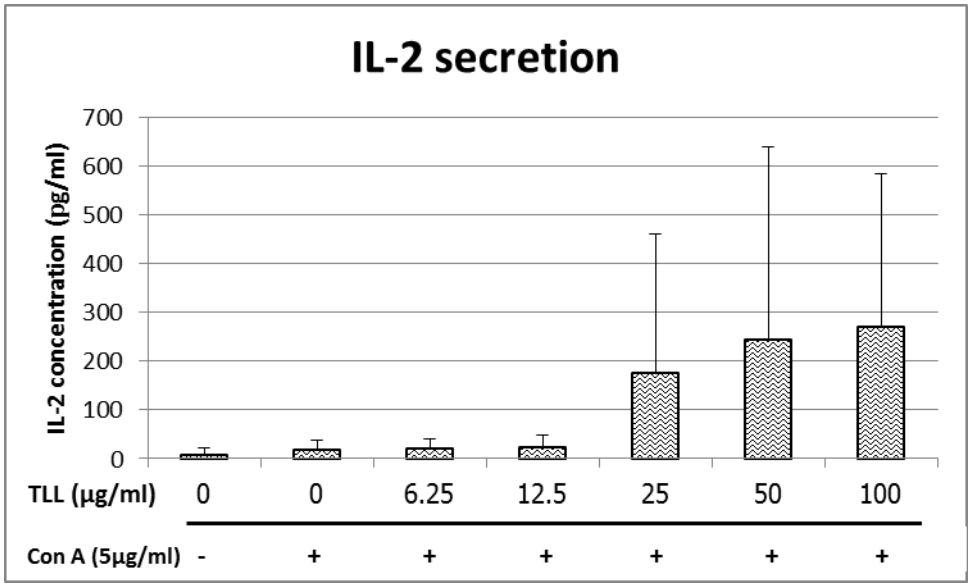

Figure 4.6.1 Influence of TLL extract on IL-2 secretion of Con A-activated lymphocytes. Resting PBMCs were cultured alone to indicate the IL-2 baseline level. PBMCs were incubated in the presence of Con A (5 µg/ml) with various TLL extract concentrations for 48 hr. The level of IL-2 was analyzed in the supernatant of cultured PBMCs using ELISA detection. Data from three independent experiments were presented as secreted amount of IL-2 mean ± SD.

The data analysis showed that TLL extract at 100 µg/ml following stimulation with Con A, significantly inhibited the release of IL-10 from lymphocytes. Incubation of Con A at 5µg/ml with lymphocytes markedly affected IL-10 production in an increasing manner (602.66 ± 15.08 pg/ml) compared to unstimulated resting cells (302.08 ± 197.59 pg/ml). TLL extract at 6.25, 12.5, 25, 50 and 100 µg/ml slightly decreased IL-10 production to 598.86 ± 10.88, 606.24 ± 8.45, 573.91 ± 65.46, 566.63 ± 20.53 and 382.39 ± 42.23 pg/ml, respectively (Figure 4.6.2). The IL-10 production analyzed in response to 100 µg/ml of TLL extract was significantly decreased compared to stimulated control cells (p<0.05). IL-10 release was markedly decreased from stimulated control cells by 1.61 times and IL-10 release was still higher than unstimulated resting cells by 1.2 times. This result was contrary to the previous IL-2 production profile and again supported that TLL extract might directly polarize Th cells into Th1 lineages.

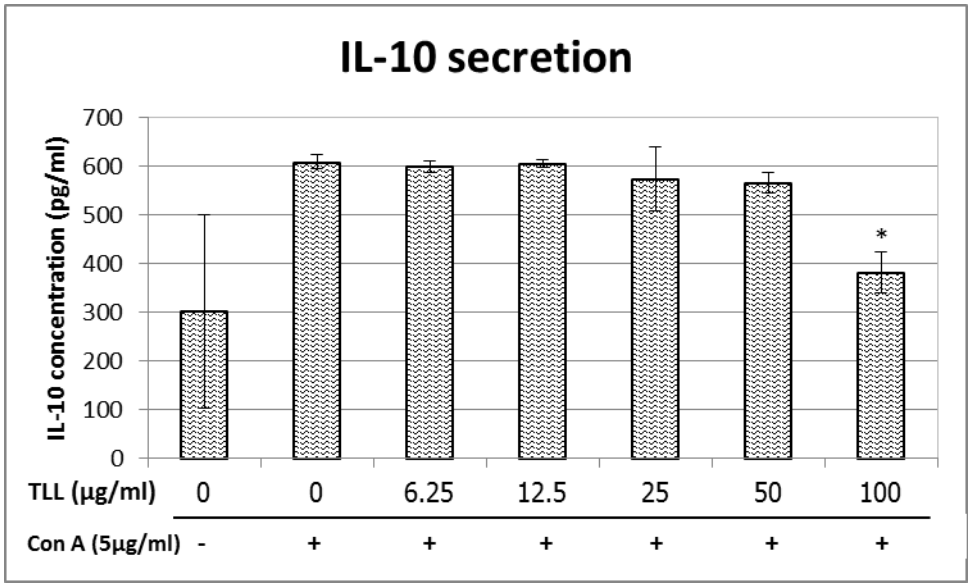

Figure 4.6.2 Influence of TLL extract on IL-10 secretion of Con A-activated lymphocytes. Resting PBMCs were cultured alone to indicate the IL-10 baseline level. PBMCs were incubated in the presence of Con A (5µg/ml) with various TLL extract concentrations for 72 hr. The level of IL-10 was analyzed in the supernatant of cultured PBMCs using ELISA detection. Data from three independent experiments were presented as secreted amount of IL-10 mean ± SD. Limit of quantitation for the IL-10 ELISA was 500 pg/ml. The asterisk represents significant differences of TLL extract treated cells in comparison with Con A treated control cells.

CHAPTER V
DISCUSSION

In this present study, we demonstrated for the first time that TLL extract contained high rich flavonoids contents possessed an *in vitro* immunomodulatory activity on human immune system by suppressing on lymphocyte proliferation and stimulating $CD4^+$ helper T cells cytokine secretion.

Researches of modulation of immune functions correlated with autoimmunity, allergy, asthma, infection and cancer are an area of active interest. Several studies have been confirmed that medicinal plants possessed immunomodulatory activity as anti-inflammatory, anticancer, anti-oxidative properties are beneficial in such disease conditions. Investigation of plant-derived molecules that enhance or inhibit any immune components brings a hope to discover a drug used in immunotherapy of those conditions (24).

With a focus on allergic diseases, immunity imbalance of the adaptive immune system targeting T lymphocytes is mainly ascribed to play important role in asthma and allergy pathogenesis in addition to the genetic and environment factors. In these conditions, Th1/Th2 imbalance has been attributed as a prominent mechanism to induce allergic asthma since Th2 derived cytokines are considered as a pro-activated factor in asthma exacerbation. Besides conventional first-line drug which may have several side effects and poor efficacy for controlling such pathogenesis in long term, immunotherapy has evoked to improve the treatment of these allergic conditions. Since this pathogenesis is characterized by a pro-activated Th2 lymphocytes, immunomodulator that acts as immunosuppressant reversing the Th1/Th2 imbalance is an alternative treatment of these conditions.

5.1 Effects of TLL extract on PBMCs viability

Besides TLL non-toxic effects on cancer cells, there is no data about its toxicity on normal or immune cells (64). To ensure the further immunomodulatory effects of TLL extract on normal human lymphocytes, we evaluated the cytotoxicity of TLL extract using MTT colorimetric viability assay. MTT colorimetric assay is the rapid *in vitro* method to determine viable cell number (81). The MTT assay is based on the ability of mitochondrial enzyme succinate dehydrogenase to cleave the yellow color tetrazolium bromide into purple-color formazan. That enzyme is present only in living cells with functional mitochondria. Thus, it was assumed that the formation of formazan during a given exposure period is directly proportional to the viable cell counts. MTT assay is a reliable established method for measuring the number of viable cell (81).

TLL extract ranging from 3.9-250 µg/ml did not demonstrate cytotoxicity on human lymphocytes. More than 95% of cell viability was obtained from TLL treated cells in comparison with untreated control cells up to 48 hr of incubation. In most studies, the compounds can be determined to immunotoxicity if they can decrease the cell viability more than 20% or greater compared with resting immune cells (74, 99). Those compounds possessed immunotoxic properties are difficult to clearly differentiate from specific immunosuppressive (89). A decrease of cell number strongly restricts on immune cells to express several sensitive effects like activation, proliferation, cytokine secretion, inflammatory processes, etc. because those effects require complex interdependences and sufficient number of cell types (73, 78). The cell numbers after treatment of TLL extract at 3.9-250 µg/ml remained more than 95% of cell cultures. Therefore, TLL extract clearly revealed non-immunotoxicity up to 250 µg/ml. This result was consistent with the previous studies that showed non-toxicity of TLL extract on transformed or normal cell lines (100). Moreover, the chronic toxicity of aqueous leaf extract of *T. laurifolia* has been studied on Wistar rat. The result suggested that aqueous leaf extract of *T. laurifolia* at concentrations ranging from 20-2000 mg/kg/day did not implicate in general health of rat. Since TLL extract did not exhibit any cytotoxicity on human lymphocytes, the further immunomodulatory activity analysis of TLL extract could be correctly predicted (64).

5.2 Effects of TLL extract on NK cells-mediated cytotoxicity activity

NK cells constitute a major cell of the innate immunity. They are large granular lymphocytes that are able to be activated in order to destroy target cell without the need for prior exposure (83, 92). Although alterations in NK cells-mediated cytotoxicity are not related to pathological condition of allergy and asthma, this cytotoxic activity is predictive potential to modulation of innate immune response (101). NK cells-mediated cytotoxicity usually evaluates *in vitro* using sensitive target tumor cells such as K562 and YAC-1 cells. SK-N-SH is a neuroblastoma cell line that is sensitive to NK cytotoxic activity. NK cells have been evaluated to have potential to lyse neuroblastoma *in vitro* and helped in enhancing immune response against neuroblastoma (92, 102).

To ensure the sufficient numbers of effector cells to be test, the appropriate effector to target cell (E:T) ratio should be selected to perform the NK cells-mediated cytotoxicity assay. The appropriate E:T ratio depends on the potency and sensitivity of effector cells to lyse target cells (103). Since we did not know the degree of sensitivity of NK cells-mediated cytotoxicity against SK-N-SH measured by MTT assay and to ensure the appropriate number of cell, this assay was performed at different E:T ratios. Our study showed that the percentage averages of cytotoxicity effects among untreated resting cells at 6.25:1, 12.5:1 and 25:1 ratio were not direct proportional to the increasing ratio but there was insignificant slight increasing of cytotoxicity baseline value at 50:1 ratio. These variations might be caused from a variable number of NK cells in each individual. NK cells account for 7-41% of the total PBMCs number (11) and this wide range of cell number may affect the NK cell counts in each experiment (11, 92). In addition, time of incubation results in a variable degree of cytotoxicity activity of NK cells. Even under optimal conditions, differences in NK cells-mediated cytotoxicity can be as large as 100% although cells of certain individuals can be preserved without losses. From these results, we could assume that SK-N-SH cells would be more sensitive to NK cells if we increased the number of effector cells. These results showed that an appropriate E:T ratio was critical parameter for evaluating the NK cells-mediated cytotoxicity. However, our results showed a sufficient degree of sensitivity to detect the cytotoxicity of NK cells against SK-N-SH

cells. The degree of sensitivity of NK cells mediated cytotoxicity against SK-N-SH confirmed the sufficient numbers of NK cells in our study.

Our results demonstrated that SK-N-SH cells were not sensitive to the cytotoxicity of NK cells treated with various concentrations of TLL extract whatever E:T ratios. However, SK-N-SH cells seemed to be more sensitive to NK cells at higher concentrations of TLL extract. It was worth mentioning that our IL-2 analysis profile showed the increasing levels of IL-2 as a dose dependent manner after treatment of higher concentrations of TLL extract. IL-2 secretion is crucial for activation of NK cells and enhancement of NK cells mediated cytotoxicity (104, 105). This indirect effect may be one of the mechanisms that contributed to higher NK cell cytotoxic activity although these increasing levels of IL-2 insignificantly promoted NK cells mediated cytotoxicity (101). NK cells stimulated with IL-2 at 50:1 ratio have also been demonstrated more effective cytotoxic activity against SK-N-SH than unstimulated NK cells (74% and 13%, respectively) (105). Nevertheless, IL-2 can be detected from 48 to 72 hr incubation with mitogens (11, 78). Because of a short incubation time in this study, therefore we could not observe a clear effect of IL-2 on NK cells mediated cytotoxicity. On the other hand, TLL extract may have direct ability to stimulate NK cells-mediated cytotoxicity without significant differences. NK cells can be directly activated to destroy target cells through two major signaling pathways by activation of granule exocytosis and stimulation of the death receptor pathway (92). Many previous studies showed that flavonoids demonstrated both stimulatory (83) and suppressive effects (105) on NK cells mediated cytotoxicity by direct activation on cell surface TLR. Therefore, flavonoid constituents in TLL extract may contribute to NK cells-mediated cytotoxicity against SK-N-SH in stimulatory manner.

5.3 Effects of TLL extract on lymphocyte proliferation

T lymphocytes play a central role in adaptive immune response to regulate homeostasis and conduct proper responses. The initial step of effective T cells-mediated responses is a rapid T cells proliferation (106). Pro-activated T lymphocytes have been implicated to the initiation and prolongation of some chronic immune

diseases such as allergy and asthma (107). Finding the potentials of plant-derived molecules that stimulate or suppress lymphocyte proliferation contributes to study immunomodulation and to classify the drug category. To determine immunosuppressive effects, mitogen stimulated assay is the most promising method for prediction of that property of compounds (73).

With respect to mitogenic activity of Con A, our CFSE-based proliferation assay confirmed that lymphocytes incubated with Con A alone for 48 hr underwent cell division properly (78). We also observed a round of cell cycle division after treatment with Con A. These results confirmed the suitable incubation time, sufficient concentration of Con A and the appropriate precursor cell number to observe the modified effects of TLL extract (78, 88).

Our study showed that lymphocyte proliferation was suppressed by TLL extract at higher concentrations in the dose dependent manner. We also demonstrated that TLL extract could inhibit proliferation either in the presence or absence of Con A. In the absence of Con A, the high concentration at 100 μg/ml showed a reduction of cell proliferation more than 20% (70.65%) without significant differences. In addition, TLL extract acted more suppressive in Con A-stimulated groups than unstimulated groups by suppressing lymphocyte proliferation more than 20% at the concentrations of 50 (61.82%) and 100 (92.37%) μg/ml, respectively. As known, Con A mitogen stimulated proliferation can act restricted on immune cell types. Con A is well recognized to stimulate T cells. Therefore, our findings might be postulated that TLL extract appeared to act as a T cell suppressor (79, 83, 89, 106). To support these results, in accordance with CFSE proliferation assay, our preliminary data performed using MTT assay (105) to determine the viable cell numbers obtained in the exposure with Con A 5 μg/ml along with the treatment with different TLL extracts, had shown that the proliferative effect of Con A was diminished in the incubation with various concentrations of TLL extract (6.25-100 μg/ml). The results also showed a reduction of cell viability by 20% as compared with Con A treated cells whereas, the bulk number of living cells was unchanged in comparison to unstimulated resting cells (87, 89). This finding also helped in assuming that the cell viability in the presence of Con A was more than 85% in all TLL extract incubations. As known, MTT assay is only directly proportional to the bulk viability of living cells at specific time point and not

to dividing cells (78, 81). Therefore, we might conclude from these findings that TLL extract could interfere with the proliferation mechanisms of dividing cells but not resting cells (74, 79, 87).

If any compounds can reduce the proliferative capacity than 20%, those compounds could be determined as immunosuppressant (78, 79, 108). Mitogen stimulated proliferation assay can be used to correctly predict immunosuppressive effects and help in limiting certain cell types affected by compounds. TLL extract strongly affected both resting and Con A stimulated lymphocytes proliferation. This result occurred without any cytotoxicity of immune cells. Therefore, it was assumed that TLL extract had a potential to be a T cells suppressant by inhibiting with T cells division process but it did not interfere with undivided resting T cells.

A theory of T cell anergy (T cell unresponsiveness) may describe our results (109). T cells activation is initiated by a presentation of antigen peptide/MHC complex expressed by APCs to a T cell receptor (TCR)/$CD3^+$ complex. Full T cell activation is ensured by a sufficient accessory signals produced by co-stimulatory molecules on APCs and this signal prevents the initiation of T cell tolerance, also known as T cell anergy which usually happens when T cells are activated without an appropriate accessory signals (109). T cell anergy is also induced after subsequent challenge with antigen at an appropriate immunogenic concentration (110). Moreover, *in vitro* data has been shown that T cell anergy is more susceptible to higher concentration of repetitive antigens compared to low concentrations. With a lack of sufficient number of APCs, we assumed that several compounds in TLL extract might be responsible for this suppressive effect by modulating TCR induced signal transduction during T cell activation (89).

With respect to anti-inflammatory activity of *Thunbergia laurifolia* extract, *T. laurifolia* has been reported that its ethanolic extract possessed anti-inflammatory activity (66, 111). The ethanolic extract significantly inhibited carrageenan-induced paw edema in mice and demonstrated significant activity against PGE_2 induced paw edema. Rosmarinic acid was attributed to be one of the responsible compounds for these effects (66). These results suggested that polyphenolic compounds in TLL extract may contribute to the anti-inflammatory effects. As mentioned previously, the rapid proliferation of lymphocytes is crucial event involved

in inflammation mechanisms (108). Therefore, in consistent with the anti-inflammatory activity, suppressive effect of lymphocyte proliferation of TLL extract might also regard to one of the mechanisms supporting these anti-inflammatory effects (79).

On the other hand, our study notably presented that IL-10 secretion was dramatically decreased after incubation with higher concentrations of TLL extract in the treatment of Con A. The reduction of IL-10 level in this study was in accordance with most of our results in the exception with lymphocytes proliferation data. Focused on T cells, IL-10 plays crucial role in immunoregulatory mechanisms with ability to suppress Th1 mediated responses and can cause T cell exhaustion. The direct effects of IL-10 on T cells proliferation have been reported however that results are equivocal. IL-10 has also been reported to affect T cells functions by interfering with antigen presentation by APCs thereby indirectly regulating T cell activation. IL-10 level itself can cause negative feedback loop controlling its production. Furthermore, IL-10 is secreted by many cell types including macrophages, Tregs, Th1, Th2, Th17 and antigen presenting B cells. Moreover, a panel of cytokines should be investigated to detect immunosuppressive effects of compounds since those classified immunosuppressants do not act restricted on a shared cytokine pattern (99, 112, 113). The cytokine secreting pattern depends on type of compounds, concentrations and effector mechanisms. For example, several classified immunosuppressants like cyclosporine A have been reported to mildly decrease IL-10 level after treatment on post-transplant rat (114). As mentioned, it is difficult to point to a direct correlation between the decreased IL-10 level and suppressive effects on T cell proliferation. Therefore, the effector mechanisms of TLL extract to decrease IL-10 levels in contrary to suppression of lymphocytes proliferation should be further investigated.

5.4 Effects of TLL extract on lymphocyte lineages and T cell sub-lineages distribution

Immunomodulatory agents are able to alter lymphocyte lineages expression. Immunophenotyping data obtained was performed by using fluorescently

labeled monoclonal antibodies directly interacting at specific cell surface markers of lymphocytes. Analysis of the data is useful in detecting any changes in lymphocyte phenotypes (76, 96, 115). Flow cytometry method provides tools for measurement of differential characteristics or properties of single cells. Moreover, flow cytometric analysis is a rapid, sensitive and reliable assessment of lymphocyte functions that requires small amount of lymphocytes (78, 86, 89).

Our proliferation data revealed that the mechanisms of TLL extract might correlate with immunosuppressive effects of T cells. Thus, a further study of impacts of TLL extract on lymphocyte lineages distribution had been performed to ensure the above conclusion and to look for its effects on balance between lymphocytes subpopulations. The ratio of $CD4^+$ and $CD8^+$ T cell sub-lineages which are the main type of T cells to regulate the balance of the immune system has become a potential parameter to exploit the balance of the immune response (96, 115). In addition, typical immunosuppressants generally suppress the $CD4^+$ population (78). Moreover, the imbalance between T cell sub-lineages is considered as a key mechanism in several disease pathogenesis like allergy and asthma (32, 116). Therefore, we had observed more on the possible effects of TLL extract on T cells sub-lineages proportion.

Evaluation on lymphocyte lineages distribution using immunophenotyping following by flow cytometry revealed that TLL extract alone whatever concentrations, or with Con A stimulation had no remarkable effect on the lymphocyte lineages distribution (T, B and NK cells) and T cell sub-lineages ($CD4^+$ and $CD8^+$ T cells) expression. With respect to T cells, in the incubation with Con A, lymphocytes treated with TLL extract ranging from 0-100 μg/ml did not show suppression or stimulation in T cells distribution compared to stimulated control cells. However, TLL extract slightly showed a dose dependent stimulatory effect on $CD8^+$ T cells expression with no significant difference whereas $CD4^+$ T cells did not exhibit any prominent effects in the exception with a concentration of 100 μg/ml that showed a slight increase. Thus, the ratio of $CD8^+$ and $CD4^+$ expression was slight decrease with no significant change. Due to TLL effects on IL-2 and IL-10 secretion profile, TLL effects on T cells might be associated with decrease in IL-2/IL-10 ratio. Since, IL-2 is known to mainly produce by $CD4^+$ T cells (62), especially Th1 cells and IL-10 is secreted by Tregs and Th2 cells following cell activation by mitogens or antigens, the increase of IL-2 level

and decrease of IL-10 level at dose of 100 μg/ml of TLL extract was in accordance with the higher $CD4^+$ T cell expression. Furthermore, increasing expression of $CD8^+$ T cells was observed as a dose dependent manner along with the increase of IL-2 and the gradually decrease of IL-10. Therefore, we assumed that the stimulatory effects of TLL extract at 100 μg/ml on $CD4^+$ and $CD8^+$ expression might be accounted for increasing of IL-2 and decreasing of IL-10 secretion.

On the other hand, our result showed that lymphocytes treated with TLL extract alone ranging from 0-100 μg/ml again did not show suppression or stimulation in T cells distribution and their $CD4^+$ and $CD8^+$ sub-lineages expression. The ratio of $CD4^+$ and $CD8^+$ expression was not sensitized to TLL extract effects. However, at 50 and 100 μg/ml, TLL extract slightly stimulated $CD4^+$ expression. In contrast, the dose dependent stimulation of $CD8^+$ T cells expression in Con A stimulated group was diminished and we could not observe any clear effects of TLL when lymphocytes were not activated by Con A. The results indicated that TLL extract itself slightly up-regulate $CD4^+$ expression. However, because of lacking of cytokines profiles, due to Con A was responsible for a significant secretion of both IL-2 and IL-10 cytokines, and inconsistent result with the above results, we could not assume that in the absence of Con A, TLL extract alone could trigger the expressions of $CD4^+$ T cells.

$CD4^+$ naïve T cells can be polarized to their sub-lineages, Th1 and Th2 cells through imbalance of cytokines secretion (115). IL-2 is produced by Th1 cells and is also responsible for stimulation of polarization of $CD4^+$ naïve T cells into Th1 cells by down-regulating the Th2 mediated cytokine secretion in contrary to IL-10 that is able to mediate $CD4^+$ naïve T cells into Th2 expression by inhibiting the Th1-mediated responses (96). In addition, Th1-mediated responses promote the cell-mediated immunity (60, 91). This assumption is in accordance with our increasing of $CD8^+$ T cells expression as compared with control cells. In conclusion, these results suggested the possible property of TLL extract at higher concentration to drive T cells expression into Th1 and $CD8^+$ sub-lineages as a result of strengthened Th1-mediated response.

5.5 Effects of TLL extract on modulation of cytokines production

T lymphocytes including $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and $CD4^+CD25^+$ regulatory T cells (Tregs) are prominent cells of cell-mediated immunity. These T cells play crucial role in immunoregulatory mechanisms in healthy status. The $CD4^+$, $CD8^+$ T cell and Tregs sub-lineages are the major population of T cells and their balances are crucial for immune homeostasis (96). Moreover, it is believed that T cells, especially $CD4^+$ helper T cells can influence the balance of immune responses through production of various cytokines (13). The $CD4^+$ helper T cells (Th), including Th1 and Th2 cells mediate cellular immune responses through Th1 cytokines (IL-2, IL-12 and INF-α,) and humoral immune responses through Th2 cytokines (IL-4, IL-5 IL-10 and IL-13) (7, 11, 30).Thus, the attribute of immune responses can be dramatically affected by these secreted cytokines. Depending on the relative concentrations and timing, these secreted cytokines can regulate the polarization of arms of immune responses. Currently, it is clearly suggested that Th1 and Th2 cells mutually exhibit inhibitory effect to each other through production of each different cytokines (29).

The imbalance of T cells and their cytokines can result in several pathological conditions. For examples, Th2-cytokine bias is correlated with allergic conditions and asthma (30, 31, 116) whereas Th1-cytokines are involved in pathogenesis of autoimmune diseases (62, 117). In such conditions, therefore modulation of cytokines levels through stimulation or suppression may help in supporting a therapy or improving a disease state (30).

Our results showed that TLL extract dramatically stimulated IL-2 secretion at higher concentrations in a dose dependent manner. On the other hand, IL-10 level was significantly decreased after TLL treatment at higher concentrations in a dose dependent fashion. The increasing level of IL-2 together with suppressive level of IL-10 indicated that TLL extract elevated the Th1/Th2 cytokine ratio suggesting that TLL extract strengthened the Th1 mediated responses (30, 82, 96). Although IL-10 can be produced from different cell types including macrophages, Tregs, Th1, Th2, Th17 and antigen presenting B cells, in our study we assumed that IL-10 might be mainly secreted by $CD4^+$ T cells which were Th2 cells and Tregs since the level of IL-10 was markedly increased after exposure to Con A (113), a T cell mitogen. With respect to

IL-2 properties, IL-2 has been reported to support tolerance and maintenance of Tregs survival (14) and Tregs number might be insignificantly induced by the Th1 immune deviation (30) thus their ability to produce IL-10 should not be affected by increasing of IL-2. Conversely, IL-10 secreted Th2 cells might be suppressed by the inhibitory effects of IL-2. Therefore, we could conclude that a reduction of IL-10 level in present study should be accounted for suppression of Th2 cells after exposure to the increased IL-2 levels (118).

Furthermore, other meaningful data may help us in execution between Th1 and Th2 mediated responses (119). First of all, our present study showed that T cells proliferation was suppressed when exposed to higher concentrations with TLL extract. A feasible mechanism in account for this observation is T cell anergy. T cell anergy is defined as antigen induced specific non-responsiveness to subsequent challenge with immunogenic concentration of antigen. T cell anergy followed by high dose allergen stimulation is ascribed for a preferential decrease of Th2 cells (29, 32, 120-122). Second, Th1 mediated responses can be indicated by stimulation of NK cells-mediated cytotoxicity (115). IL-2 secreted by Th1 cells is also needed by NK cells to promote their development and activity (82, 123). In our study, TLL extract might be presumably assumed that it was capable to stimulate NK cells-mediated cytotoxicity function by increasing IL-2 production however we observed the promiscuous effects because of lower level of IL-2 and short incubation time. Finally, there is a close relationship between an increase in expression of $CD4^+$ and $CD8^+$ T cells and Th1 mediated responses (96, 115, 124). IL-2 has been shown to enhance survival, stimulation, accumulation and differentiation of $CD8^+$ as well as $CD4^+$ T cells (62). In this study, in consistent with the IL-2 profile, $CD8^+$ and $CD4^+$ T cells expression was slightly increased after incubation with TLL extract and Con A. On the other hand, $CD8^+$ T cells proportion and NK cells lytic activity have been shown suppressive responses in the presence of IL-10 (16, 125) in contrary to our results. In addition, the increased IL-10 and TGF-β levels following allergen-specific immunotherapy (SIT) have been observed when polarization from Th2 to Th1 mediated responses simultaneously occurred (110, 125). As mentioned above, these distinct signals indicated that TLL extract could lead T cells to polarize into Th1 mediated responses.

It should be stated that TLL extract contains several constituents including flavonoids and iridoid glucosides (126-129) that are responsible for modulating immune functions. Previous data confirmed that the aqueous extract from leaf of *T. laurifolia* contained at least flavonoids and phenolic acids. The preliminary analysis has also been shown that the powder of TLL extract contained a number of flavonoids screening by aluminium chloride method and possessed good scavenging properties using DPPH assay. Flavonoids are secondary metabolite produced from various plants. They have been extensively investigated to possess a wide range of pharmacological function including antioxidant properties, anti-tumor activity and anti-inflammation (52). Several flavonoids have shown immunomodulatory potentials on various cellular components and cytokines of immune system (98). Flavonoids, for example apigenin presented in *T. laurifolia* aqueous extract have been reported to exert immunomodulatory properties and may contribute to its immunomodulatory activity (130). Interestingly, apigenin, a flavones contained in TLL extract has been shown to markedly inhibit OVA-induced airway inflammation in rodents (i.e. guinea and mice). This *in vivo* study has demonstrated that intraperitoneal administration of apigenin (5 and 10 mg/kg) reduced eosinophils infiltration and a consequence activation leading to inhibition of inflammatory mediators release including IL-4, the Th2 cytokines (131, 132).

In conclusion, the results under present study suggested that TLL extract possessed immunosuppressive activity by interfering with T cells proliferation without cytotoxicity and also suggested the potentiating Th1 mediated effects by increasing IL-2 level along with decreasing IL-10 level. These results pointed to their potential attribute as immunosuppressant used in Th2 mediated pathological conditions like asthma and allergy. In addition, the results obtained point to the effects of flavonoids containing in TLL extract on the lymphocyte proliferation and cytokine production. These results provided scientific and experimental information for the beneficial applications of TLL. Future studies should be focused on isolating along with identifying the active constituents and its impacts in *in vivo* conditions.

CHAPTER VI
CONCLUSION

The management of patients underlining with immune-based disorders, such as allergic diseases and asthma remains a task of clinical practice. Besides genetic and environmental factors, imbalance of the adaptive immune system, especially T cells, is recently considered to play an important role in their pathogenesis. The role of cytokines in the pathogenesis of allergic asthma has been intense studied in a past decade. Imbalance between Th1/Th2 mediated cytokines (i.e. excessive Th2 cytokine production) has been considered to play an important role in inducing allergic asthma and its exacerbation. Therefore, asthma is primarily considered as a Th2-mediated inflammatory disease.

Typical treatment using corticosteroids and β_2-agonist can totally control these allergic diseases. However, typical drugs to treat and maintain such conditions may have many side effects and sometimes not be sufficient to cure or control the diseases in long term. To decrease side effects but maintain typical drug efficacy, attempts have been made to reduce a dosage of a typical drug together with adding an additional compound that has a similar or augment properties in the therapy. Immunomodulator targeting on suppression of T-lymphocyte proliferation together with activation of Th1-mediated response, is an alternative and gains more interest for the treatment of allergic and asthma diseases.

Currently, plant-derived compounds offer many benefits for maintaining health and fighting with diseases. Plant-derive compounds exerted immunomodulatory activity through the immune cellular or molecular target of allergic disorder, have been thought to use as complementary or alternative medicine to treat allergic disorders. Notably, there are a number of traditional herbal medicines with potential efficacy to treat these allergic disorders however these are not proven in clinical efficacy.

T. laurifolia water extract (TLL) contains several promising constituents displayed immunomodulatory activities including apigenin, apigenin glucosides and iridoid glucosides. Apigenin has been shown to markedly inhibit OVA-induced airway inflammation in rodents (i.e. guinea and mice), and reduce eosinophils infiltration and a consequence activation leading to inhibition of inflammatory mediators release including IL-4, the Th2 cytokines. In addition, TLL has been demonstrated to display moderate antioxidant and anti-inflammatory properties that is considered to modulate immune response by prevention of oxidative stress and counteracting the activation of redox-regulated signaling pathways occurred during pro-inflammatory immune response. Based on the previous report, TLL attracted more attention for developing as new immunomodulator to treat such allergic diseases.

In the present study, we determined the immunomodulatory effects of TLL extract with various concentrations on normal immune cells, PBMCs. The results suggested that TLL extract did not exhibit any toxicity on normal PBMCs up to 48 hr. Moreover, TLL extract at various concentrations did not show any modulations on T, B or NK cells distribution and expression of T lymphocyte subsets. Furthermore, NK cells mediated cytotoxicity remained significant unchanged among all evaluated conditions. In contrast, our study showed that T cells proliferative capacities were interfered with potential suppressive effects of TLL extract in a dose dependent manner. TLL extract, especially at 100 μg/ml also promoted Con A-induced IL-2 production by human lymphocytes in a proportional manner to the concentrations of TLL extract. Moreover, TLL extract at 100 μg/ml following stimulation with Con A, significantly inhibited the release of IL-10 from lymphocytes. These results supported that TLL extract was immunosuppressive agent and might directly polarize Th cells into Th1 lineages. Collectively, the results under present study suggested that TLL extract may provide benefit as immunosuppressant used in Th2 mediated pathological conditions like asthma and allergy.

In conclusion, our study demonstrated the potential use of TLL extract as phytotherapy for allergic disorders and may be possible to apply in combination with current established therapy. Nevertheless, further studies to investigate its active constituents, potential risks, modes of action, preclinical and clinical efficacy, have to be performed.

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APPENDICES

APPENDIX A
MEDIA AND REAGENTS

RPMI 1640 medium

RPMI (Roswell Park Memorial Institute) 1640 medium	10.43	g
$NaHCO_3$	2	g
Streptomycin (100 µg/ml)/Penicillin G (100 units/ml)	10	ml
Sterile distilled water to make	1000	ml

Dissolve and mix the ingredients in sterile distilled water and make the total volume to 1000 ml using volumetric flask. Then adjust the pH of the solution using 1N HCL or 1N NaOH until the pH are within the range of 7.0-7.2. Sterilize the media by filtration with cellulose acetate filter (pore size 0.22 µm).

MEM for SK-N-SH culture

MEM (Minimum Essential Medium Eagle)	9.5	g
$NaHCO_3$	1.5	g
Sodium pyruvate	0.11	g
Streptomycin (100 µg/ml)/Penicillin G (100 units/ml)	10	ml
Sterile distilled water to make	1000	ml

Dissolve and mix the ingredients in sterile distilled water and make the total volume to 1000 ml using volumetric flask. Then adjust the pH of the solution using 1N HCL or 1N NaOH until the pH are within the range of 7.4. Sterilize the media by filtration with cellulose acetate filter (pore size 0.22 µm). Aliquot 100 ml of the media and supplement with 100X NEA (non-essential amino acid) 1 ml and add sterile FCS (Fetal Calf Serum) 10 ml before using the media.

Phospate buffer saline (PBS) pH 7.4

0.1 M PB (Phosphate buffer stock solution)

NaH_2PO_4	0.1 M	(pH 4.5)	27.998 g/l
NA_2HPO_4	0.1 M	(pH 9.4)	35.598 g/l

Add NaH_2PO_4 to Na_2HPO_4 dropwise to adjust the pH to 7.4

0.01 M PBS (Phosphate buffer saline)

PB 0.1 M	100	ml
NaCl	8.76	g
Distilled water to make	1000	ml

APPENDIX B
ETHICAL EXEMPTION DOCUMENT

Certificate of Exemption

COE. No. MU-DT/PY-IRB 2014/040.1710

Documentary Proof of Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review Board

Title of Project:	Immunomodulatory Evaluations of *Thunbergia Laurifolia* Crude Water Extract on Human Peripheral Blood Mononuclear Cells.
Project Number:	MU-DT/PY-IRB 2014/PY088
Principle Investigator:	Mr.Thanapol Acharakul
Name of Institution:	Faculty of Pharmacy
Date of Recommendation:	October 17, 2014

Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review Board is in full compliance with International Guidelines for Human Research Protection such as Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Signature of Chair:

(Associate Professor Dr.Choltacha Harnirattisai)
Chair

Office of Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review, Board
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PUBLICATION / PRESENTATION	Poster presentation: *Thunbergia laurifolia* water extract does not exhibit immunomodulatory effects and toxicity on human peripheral blood mononuclear cells International Graduate Research Conference 2014 Chiang Mai, Thailand 12 December 2014