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Immunomodulatory Effects & Mode of Action of Ginsenoside Rb-1

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CERTIFICATE OF ORIGNALITY

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Abstract

Immune system protects the body against pathogens while spleen is crucial to the development of both innate and adaptive immune system. Upon activation, immune cells (e.g. B cells and T cells) communicate with each other through secretion of cytokines. Examples are interleukin-2, tumor necrosis factor-alpha, interferon-gamma, interleukin-1 alpha, interleukin-6 and interleukin-10. On another front, ginseng (*Panax quinquefolium*) is one of the most commonly used medicinal herbs. It is believed to have diverse pharmacological effects including immunomodulation. However, its mode of actions is unclear.

In this study, we aimed to investigate the immunomodulatory effects of ginsenoside Rb-1 and its mode of actions. With MTT assay, Rb-1 would neither induce growth nor inhibit proliferation of LPS-stimulated splenocytes. On the other hand, by ELISA assay, it was found that *(a)* Rb-1 alone did not induce IFN-γ and TNF-α production in splenocytes cultures; *(b)* various pre-incubation periods of Rb-1 prior to LPS stimulation did not alter the secretion of IFN-γ and TNF-α; *(c)* cytokines secretions were inhibited at highest dosage of Rb-1 such as 100 μg/ml significantly. Beside cytokines, LPS-induced nitrite production was also lowed after addition of Rb-1.

It should be stressed that both cytokines and iNOS expression share a universal transcription factor – nuclear factor-κβ (NF-κβ). Hence, with a liquid-protein suspension array system, we found that: *(a)* there was no difference in terms of total protein expression of I-κβ in either Rb-1 treated or Rb-1 untreated splenocytes; *(b)* however, phosphorylation of I-κβ was decreased in splenocytes culture treated with Rb-1; (*c)* ratio of (phosphorylated I-κβ : total I-κβ) in Rb-1-supplemented splenocytes was lowered than those without Rb-1. Furthermore, Western blotting experiment showed that translocation of NF-κβ into nucleus was decreased in Rb-1-treated splenocytes culture. These results explained why there was suppression of cytokines secretion and lower level of nitrite produced in Rb-1 treated splenocytes culture. Therefore, Rb-1 was concluded to have immunosuppressive effects on immune system.

Lastly, differential proteome expression of Rb-1-added splenocytes was investigated in order to exploit other possible action pathways involved by Rb-1. According to 2D-PAGE analysis, 32 protein spots were found to be differentially expressed in response to LPS and Rb-1. Of those 32 spots, 12 proteins could be identified by MALDI-TOF MS. Further comparison between LPS-stimulated splenocytes with or without Rb-1 incubation, 2 proteins (gelsolin and myovirus

resistance 1 protein) (out of 12 spots) were found to be differentially expressed upon the addition of Rb-1. However, their exact involvements in the actions of Rb-1 on splenocytes have to be further studied.

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ABBREVIATION

2D-PAGE	– Two dimensional-polyacrylamide gel electrophoresis
Ab	– Antibody
ACN	– Acetonitrile
Ag	– Antigen
Akt	– Protein kinase B Akt
AP-1	– Activated factor-1
APC	– Antigen presenting cell
ASD	– A-SMase activating domain
ATF-2	– Activated transcription factor-2
BCDF	– B cell differentiation factor
BCR	– B cell receptor
CCL	– CC-chemokine ligand
CD	– Cluster of differentiation
CLP	– Common lymphoid progenitor
Con-A	– Concanvalin-A
COS ligand	– Co-stimulatory ligand
CSIF	– Cytokine synthesis inhibitory factor
CTLL-2	– Cytotoxic T lymphocyte lymphoma-2
CXCL	– CXC-chemokine ligand
DD	– Death domain
DED	– Death-effector-domain
DHB	– 2,5-dihydeoxybenzoic acid
DTT	– Dithiothretol
ELISA	– Enzyme-linked immunosorbent assay
ERK	– Extracellular signal related kinase
FACS	– Fluorescent activated cell sorter
FITC	– Fluorescein isothiocyanate
GAF	– Gamma-activated factor
GAS	– IFN-γ activated sequence
HCCA	– a-cyano-4-hydroxycinnamic acid
HLA	– Human leukocyte antigen
HLDA	– Human leukocyte differentiation antigen
HRP	– Horseradish peroxidase
HSC	– Hematopoietic stem cell
I-κβ	– Inhibitor of NF-κβ
I-κβ-α	– Inhibitor of NF-κβ-alpha

IAA	– Iodoacetamide
ICAM-1	– Intercellular adhesion molecule-1
IEF	– Isoelectric focusing
IFN	– Interferon
IFN-γ	– Interferon-gamma
IFNGR	– Interferon-gamma receptor
IFN-R1	– IFN-receptor α chain
IFN-R2	– IFN-receptor β chain
Ig	– Immunoglobulin
L-NMMA	- N(G)-monomethyl-L-arginine
IL-1α	– Interleukin-1alpha
IL-1R	– Interleukin-1 receptor
IL-1R-AcP	– Interleukin-1-receptor accessory protein
IL-2	– Interleukin-2
IL-2R	– Interleukin-2 receptor
IL-4	– Interleukin-4
IL-4R	– Interleukin-4 receptor
IL-5	– Interleukin-5
IL-6	– Interleukin-6
IL-6R	– Interleukin-6 receptor
IL-10	– Interleukin-10
IL-10R	– Interleukin-10 receptor
IL-13	– Interleukin-13
IVAM	– intracellular vascular adhesion molecule
iNOS	– Inducible nitric oxide synthase
IPG	– Immobilized pH gradient
IPG buffer	– Immobilized pH gradient buffer
ITAM	– Immunoreceptor tyrosine-based activation motif
JAK	– Junas activated kinase
JNK	– c-Jun N-terminal kinase
KIR	– Killer-cell inhibitory receptor
LAK cell	– Lymphokine activated killer cell
LPS	– Lipopolysaccharide
LT	– Lymphotoxin
mAb	– Monoclonal antibody
MALTI-TOF	– Matrix assist laser desorption and ionization-time of flight

MALTI-TOF MS	– Matrix assist laser desorption and ionization-time of flight mass spectrometry
MAPK	– Mitogen activated protein kinase
β-ME	– β-Mercaptoethanol
MHC	– Major histocompatibility complex
mIg	– Membrane-bound immunoglobulin
MS	– Mass spectrometry
MTT	–3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MZ	– Marginal zone
NC membrane	– Nitrocellulose membrane
NF-κβ	– Nuclear factor-κβ
NFAT	– Nuclear factor activated T cells
NH_4HCO_3	– Ammonium carbonate
NK cells	– Natural killer cells
NO	– Nitric oxide
NP-40	– Nonident P-40
NSD	– N-SMase activating domain
p38 MAPK	– p38 mitogen activated protein kinase
p90RSK	– p90 ribosomal subunit kinase
pAb	– Polyclonal antibody
PAGE	– Polyacrylamide gel electrophoresis
PALS	– Periarteriolar lymphoid sheath
PAMP	– Pathogen-associated pattern molecule
PBS	– Phosphate buffer saline
PE	– Phycoerythrin
PH domain	– Pleckstrin homology domain
PHA	– Phytohemagglutinin
PI3K	– Phosphatidylinositol-3'-OH kinase
PLC	– Phospholipase Cγ
PMF	– Peptide mass fingerprint
PMN	– Polymorphonuclear neutrophils
PS	– Polystyrene
PSD	– Post source decay
PTM	– Post-translational modification
RIP	– Receptor interacting protein
ROS	– Reactive oxygen species
RP	– Red pulp

SAPK	– Stress activated protein kinase
SD rat	– Sprague-Dawley rat
SDS	– Sodium dodecyl sulfate
SH domain	– *Src* homology domain
sIg	– Surface immunoglobulin
STAT	– Signal transducer and activator of transcription
T_C cells	– Cytotoxic T cells
T_H cells	– Helper T cells
TBS	– Tris-buffer saline
TBST	– Tris-buffer saline-Tween
TCGF	– T cells growth factor
TCM	– Traditional Chinese Medicine
TCR	– T cell receptor
TD	– Thymus-dependent
TGF-β	– Tumor growth factor-β
TI	– Thymus-independent
TLR	– Toll-like receptor
TNF-α	– Tumor necrosis factor-alpha
TNFR	– TNF-α receptor
TRAF	– TNF-receptor associated factor
Tyk	– Tyrosine kinase
WP	– White pulp
WS motif	– WSXWS motif

Chapter 1) Literature Review

1.1) Immune system

Immune system confers an interacting mechanism inside our bodies which defends against surrounding harmful agents (Baeuerle *et al.*, 1994; Alam, 1998). Microorganisms and pathogens that are foreign to our body can invade to cause diseases. Our immune system has two important properties which are the abilities to (1) respond to all foreign antigens, and (2) capable to mount an enhanced response (or called memory) against previously encountered antigens (Geha, 1980).

Immune system is classified into two different (but inter-dependent) modalities: innate immune response and adaptive (acquired) immune response (Alam, 1998; Hoebe *et al.*, 2004). Innate immune system provides an in-born defense and first barrier against common microorganisms. It consists of physical, chemical and biological barriers against bacterial invasion. Physical and chemical barriers are essential for immediate control of most bacterial invasions (Nussenzweig *et al.*, 1997). Biological barriers consist mostly of competition among pathogen and normal microflora of the body (especially skin) for essential nutrients. However, these systems are not specific and cannot eliminate all pathogens. Biological defense in the adaptive immunity is more specific and plays a crucial role in the arsenals of body defense (which takes a few days to develop) (Hedrick, 2004).

Once adaptive immunity is initiated, numerous immune cells such as B lymphocytes, T lymphocytes and macrophages are activated against the invaded pathogens via different mechanisms (O'Garra *et al.*, 2004). First of all, immune

cells start to proliferate by the process of cell differentiation. Differentiated effector immune cells are triggered to produce various anti-bacterial substances called cytokines. Cytokines not only attack the bacteria or pathogens, but also regulate the immune response by its signaling properties (Fitzgerald, 2001). In the meantime, activated cells differentiate into memory cells that acquire an advanced level of protection from subsequent re-infection of the same pathogens.

In this review section, a brief background of innate and adaptive immune system will be introduced. Subsequently, various signaling transduction pathways in lymphocytes will also be discussed.

1.2) Innate immunity

Innate immunity is conferred by all elements of the body, from chemicals to systems, which are already present since birth (Schulenburg *et al.*, 2004). Such pool of defense components is considered as relatively non-specific components. For instances, physical barrier such as the skin system or chemical property like acidic pH in stomach, both have a defense role in innate immunity. With the aid of immune cells, innate immunity is able to tackle most of the biological substances that the body encountered. Hence, immune cells play an important role in innate immunity in which specialized immune cells can directly destroy the invading microorganisms by either ingestion or inflicting extracellular damages (Nussenzweig *et al.*, 1997).

1.2.1) Intracellular digestion

During infection, bacteria and pathogens may escape from both physical and chemical barriers. However, they will encounter immune cells at or adjacent to the infection site. Generally, macrophages engulf the bacteria and then digest them. Intracellular ingestion can be achieved by two different mechanisms: endocytosis and phagocytosis (Winfield, 1971; Varesio *et al.*, 1980).

Endocytosis describes the process that macromolecules present in extracellular fluids are ingested by cells. It occurs at different locations at where endocytes or macrophages are available, for example, at epithelium of respiratory tract or peritoneal cavity. Once fusion of enzyme and these macromolecules occur, macromolecules will be digested or broken down. Subsequently, such digested fragment is presented on cell surface for a recognition process called antigen presentation (Neefjes *et al.*, 1993). Different from endocytosis, phagocytosis is an active process of ingestion and destruction of invaded pathogen. Similarly, phagocytes engulf bacteria directly by forming phagosomes. Phagosome fuses with lysosome and destroy the bacteria eventually. Besides, phagocytes are capable of converting molecular oxygen into reactive oxygen species (ROSs) which are strong oxidizing agents to bacteria (Vliet A *et al*., 2000).

1.2.2) Extracellular damage

In cases where pathogens can escape the initial body defense such as the attack by phagocytes, they can infect normal cells subsequently. Extracellular killing of these invaded-pathogens is achieved with cell-mediated cytotoxic activities (Darmon *et al.*, 1998). Cytotoxic activities are mediated by natural killer (NK)

cells and cytotoxic T cells (T_C). In 1995, D'Andrea *et al* first cloned the killer-cell inhibitory receptor (KIR) in NK cells that was responsible for self-antigen recognition. (D'Andrea *et al.*, 1995). Therefore, NK cells were considered for extracellular damage of infected cells. Subsequently, activated NK cells secret cytotoxic agents which induce membrane pores, DNA fragmentation etc.

1.3) Adaptive immunity

As mentioned before, induction of innate immune response is probably sufficient to protect against common invading microorganisms. Further, with the activation of the adaptive immunity, our body is highly effective against more sophisticated pathogens (Pancer *et al.*, 2006). In adaptive immunity, subsequence of immune events is triggered by interaction among a wide variety of immune cells. Various elements that participate in adaptive immune response exhibit strict specificity. Different from innate immunity, adaptive immune response is a more specialized and complicated process which responds relatively late (Flajnik *et al.*, 2004).

It is well known that immune system is build of network of immune cells in that each immune cell type has their distinct and specific functions (Sehra *et al.*, 2006). B lymphocyte (cell) and T lymphocyte (cell) are two major cell types and contribute to facilitation of acquired immunity. Moreover, acquired immune response is usually divided into two mechanisms: humoral and cellular adaptive immunity (Helm, 2004). B cells are the core mediators in humoral immunity while T cells take the responsibility in cellular immunity. In most cases, activated B cells play an important role in antibodies production (LeMaoult *et al.*, 1997).

On the other hand, T cells not only regulate cell-mediated immune response but also command the development of adaptive immunity (O'Garra *et al.*, 2004). Furthermore, T cells secret numerous cytokines that will be discussed in later section.

1.3.1) B lymphocyte (B cell)

B cells differentiate from a common lymphoid progenitor and gets mature in bone marrow. B cell development is an extreme complex development (Baeuerle *et al.*, 1994). Each stage of development can be identified by distinct phenotypes or expression of unique surface markers called cluster of differentiation (CD). Subsets of differentially expressed surface markers are used to distinguish various B cells (Hoven *et al.*, 1989). Further, mature B cells migrate from bone marrow and retain in spleen. In spleen, B cells are usually mature but naive until they encounter their complementary antigens. Therefore, splenic B cells are for antigens recognition and elimination of blood-borne pathogens. On the other hand, activated B cells differentiate into effector plasma cells which produce massive specific antibodies (Brown, 1992).

1.3.1.1) Humoral immunity

As bacteria or pathogens invade the body, these invaders will proliferate in the extracellular spaces and spread through the extracellular fluid or humor immediately. Furthermore, intracellular pathogens are able to migrate from cell to cell by using body fluid as a medium. Hence, to combat this, antibody from B cells or humoral effector is used to fight against extracellular microorganisms and prevent their spreading (LeMaoult *et al.*, 1997).

1.3.1.2) B cell receptor (BCR)

Structure of immunoglobulin (Ig)

In humoral immunity, B cells are genetically programmed to encode surface receptors for particular antigens binding. These surface receptors are called immunoglobulin (Ig) (Wildt *et al*., 2002). Immunoglobulin (Ig) is a Y-shape hetero-tetramer molecule consisting of two identical heavy chains (H chain) and two identical light chains (L chain) in a symmetrical orientation (Figure 1.1) (Virella *et al.*, 1993; Bengten *et al.*, 2000). Several disulfide bondings confer the linking between (i) heavy chains and heavy chains at hinge site and (ii) heavy chain and light chain. In addition, each heavy chain and light chain carries both constant and variable region. Hence, immunoglobulin composed of two distinct region – constant region (C_H + C_L region) and variable region (V_H + V_L region). Variable region is responsible for antigen binding while constant region designates the isotype of Ig.

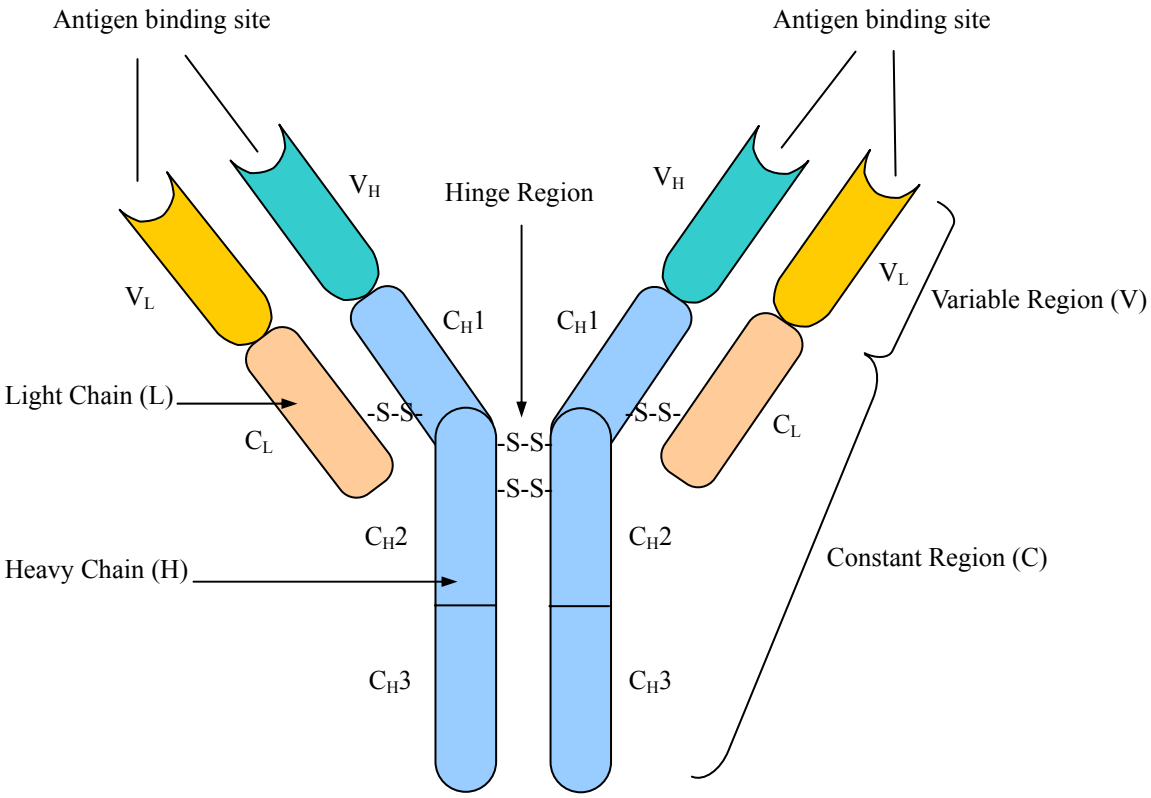


Figure 1.1 – Structure of immunoglobulin (Ig) [S-S: disulphide bond]

Structure of BCR

B cell receptor (BCR) is a membrane-bound immunoglobulin (mIg) which is ubiquitously expressed on the B cells surface. Extracellular Ig domain is always glycosylated and responsible for antigen binding by two variable regions (MacLennan, 1998). However, in case of mIg, constant region sequence elongates through membrane as cytoplasmic tail and contributes to effector functions. Several polypeptides associate with mIg to form the complete BCR signaling complex. For instance, two trans-membrane molecules immunoglobulin-α (Ig-α) and immunoglobulin-β (Ig-β) forms a disulfide-linked heterodimer (Figure 1.2). Extracellular region of Ig-α/ Ig-β dimer associates with the extracellular heavy chain of mIg and contribute no effects on antigen binding.

On the opposite terminal, each cytoplasmic tail of Ig-α and Ig-β contains an immunoreceptor tyrosine-based activation motif (ITAM) which is responsible for signaling transduction after antigen binding and BCR activation (Matsuuchi *et al.*, 2001).

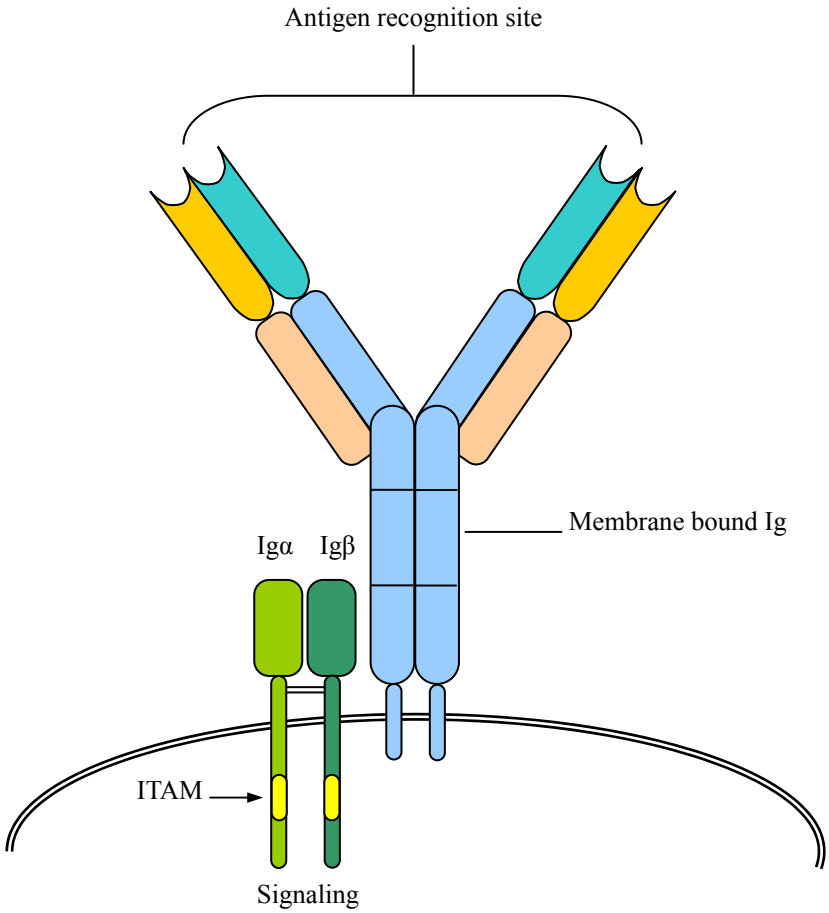


Figure 1.2 – Structure of B cell receptor (BCR) complex

1.3.1.3) B cell activation

Mature B cells enter the blood circulation and migrate to the spleen. Brown had reviewed the functions and significance of splenic B cells and suggested that spleen is the most functional compartment in development of the humoral adaptive immune response (Brown, 1992). For instances, in the spleen, naive B

cells can easily encounter its specific blood-borne antigen via its BCR. Activated BCR produces signals that initiate humoral immune activities. Subsequently, activated B cells undergo proliferation and differentiate into effector cells and memory cells. Effector plasma cells are active in producing antibodies whose antigen binding is originated from and the same as its BCR. In addition, a portion of B cells becomes long-lived memory cells. These memory B cells are either retained in the spleen or circulating in blood.

Thymus-dependent (TD) activation

Several factors are required for fully activation of B cells (Vitetta *et al.*, 1987; Burstein *et al.*, 1991). First of all, antigens are captured by BCRs and multimerization of antigen-bound BCRs occur. In addition, CD19 and CD20 molecules play a co-stimulatory role as co-receptors in BCR binding and activation. Subsequently, antigenic interaction activates BCR complex such as Ig-α/ Ig-β which transmit signals to the interior. With the help of receptor-mediated endocytosis, bound antigens are expressed as class II MHC. MHC-antigen complex can then be recognized by thymus derived T cells that in turn are activated. The antigen presentation process is crucial in cellular communication and activation of adaptive immune response (Lopes-Carvalho *et al.*, 2005).

Antigen presentation is bi-directional in its consequences (Figure 1.3) (Noelle *et al.*, 1990). For instances, B cells presenting its MHC II molecule are recognized by TCR on T cells. In addition, CD4 molecule on T cell surface is also capable of binding to Ig on B cells (i.e. BCR). MHC II-TCR communication enhances

cell-cell adherence and induces expression of T cell surface molecules (e.g. CD40) which in turn further stimulate B cells (e.g. CD40L) (O'Garra *et al.*, 2004). On the other hand, activation of TCR triggers the immune responses including cytokines secretion. Cytokines such as interleukin-2 and interleukin-6 fully stimulate B cells. During activation of B cells, they proliferate and differentiate into memory B cells and effector cells. Effector B cells are able to synthesize antibodies and turn class switch recombination mechanism on. As a result, a massive quantity of antibodies with same specificity as original mIg (BCR) will be produced and secreted. It is noted that cytokines secreted by T cells in turn activates itself through an autocrine mechanism.

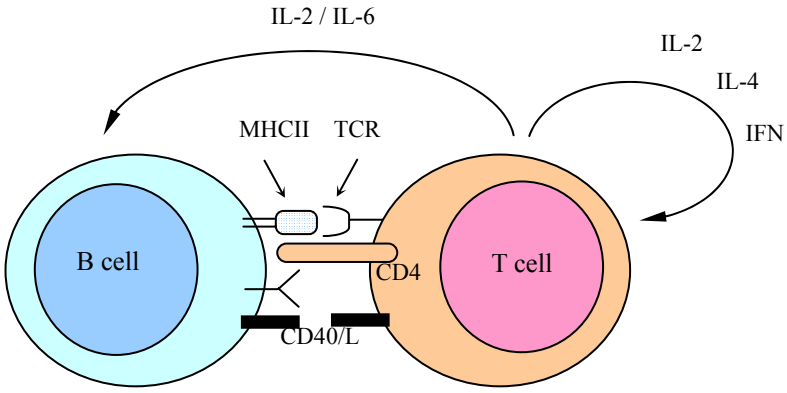


Figure 1.3 – Thymus-dependent B cells activation

1.3.1.4) Effects of cytokines on B cells

Besides producing antibodies, B-cells also produce cytokines for stimulation of other immune cells. Nevertheless, B cells are not considered as the major source of cytokines production although they are able to synthesize many cytokines (Lund *et al.*, 2005). On the other hand, cellular activities of B cells are mostly influenced by cytokines. In addition, different cytokines receptors are present on

B cells surface (Kang *et al.*, 2004). In later sections of this thesis, functions and regulations of cytokines in the immune system will be discussed. The following table summarizes the activities of several cytokines on B cells.

Cytokine	Activities on B cells
Interleukin-1 alpha (IL-1α)	Growth and differentiation
Interleukin-2 (IL-2)	Growth and differentiation
Interleukin-4 (IL-4)	Promotion of immunoglobulin differentiation
Interleukin-6 (IL-6)	Proliferation of activated-B cells
Interleukin-10 (IL-10)	Increase B cell MHC expression
Interferon-gamma (IFN-γ)	Growth factor
Tumor necrosis factor-alpha (TNF-α)	B cell growth and differentiation factor

(Remark: Detailed discussions of each of these cytokines are in later section of the "Literature review")

Table 1.1 – Activities of cytokines on splenic B cells

1.3.2) T lymphocyte (T cells)

T cells are differentiated from the same hematopoietic stem cells as B cells. Further from bone marrow, these precursor cells migrate to the thymus. In the thymus, T cells completed their maturation and enter the bloodstream which drives them to secondary lymphoid organ such as spleen or lymph nodes (Goronzy *et al.*, 2005). Like B cells development, T cells pass through a series of distinct stages which can be identified by differential expression of surface markers, such as T cell receptor (TCR) or cluster of differentiation (CD) molecules (Davis *et al.*, 2003). For instances, CD4 and CD8 are commonly used markers for distinguishing T cells development (Davis *et al.*, 2003). At earliest stage, T cells do express neither CD4 nor CD8. During development and maturation, cellular signals activate the expression of either CD4 or CD8.

On the other hand, various T cells demonstrating diverse functional states and properties are identified by differential surface marker expressions. It had been illustrated that several sub-types of T cells mediate the immunity, contributing a wide variety of functions (Goronzy *et al.*, 2005). For instances, a particular class of T cells ($CD4^+$) responsible for cell-cell interactions is gathered as helper T cells (T_H). Helper T cells are further divided into two subtypes, e.g. type-1 helper T cells (T_H1) and type-2 helper T cells (T_H2). T_H1 cells communicate with phagocytes and help them to destroy pathogens, while T_H2 cells interact with B cells and assist in B cells proliferation, differentiation and antibody production. Therefore, by comparing with B cells, T-cells play a more leading and crucial role in development of the adaptive immune response.

1.3.2.1) Cellular Immunity

As mentioned previously, all T cells subtypes develop and mature in thymus. However, cells that have not encountered their specific antigen are referred as naive T cells. When these naive T cells meet their complementary antigens which are presented by APCs, they will be activated mostly in peripheral lymphoid organs (Cemerski *et al.*, 2006). It is because pathogens or its antigenic components are usually transported to these secondary lymphoid tissues, either through draining of lymph fluid or carried by immune cells (Hommel, 2004).

Cellular immunity is a complicated process involving certain cell-cell interactions and signals transduction (Favero *et al.*, 1998; Li *et al.*, 1999). It refers to any adaptive immune response which is mediated by T cells. Once naive T cells are activated, T cells are driven to antigen-dependent clonal expansion.

Clonal expansion is the process that describes proliferation and differentiation into armed effector T cells. Subsequently, armed effector T cells regulate the immunity by several approaches. Firstly, T cells enhance their adherence ability by expressing various surface molecules. Secondly, cytokines are massively and diversely released for monitoring immune system. It is reminded that, all these responses are initiated after TCR-mediated T cell activation. Moreover, similar to B cells, full activation of naive T cell requires two signals – recognition of antigenic peptide fragments and activation with co-stimulatory molecules.

1.3.2.2) T cell receptor (TCR)

T cell receptor (TCR) is ubiquitously expressed on mature T cells surface and responsible for antigen recognition (Huang *et al.*, 2004; Garcia *et al.*, 2005). TCR is a disulfide bond-linked heterodimer molecule which consists of two transmembrane glycoprotein chains, TCR-α chain and TCR-β chain respectively. Each chain consists of three sections: variable region, constant region and a transmembrane domain (Figure 1.4).

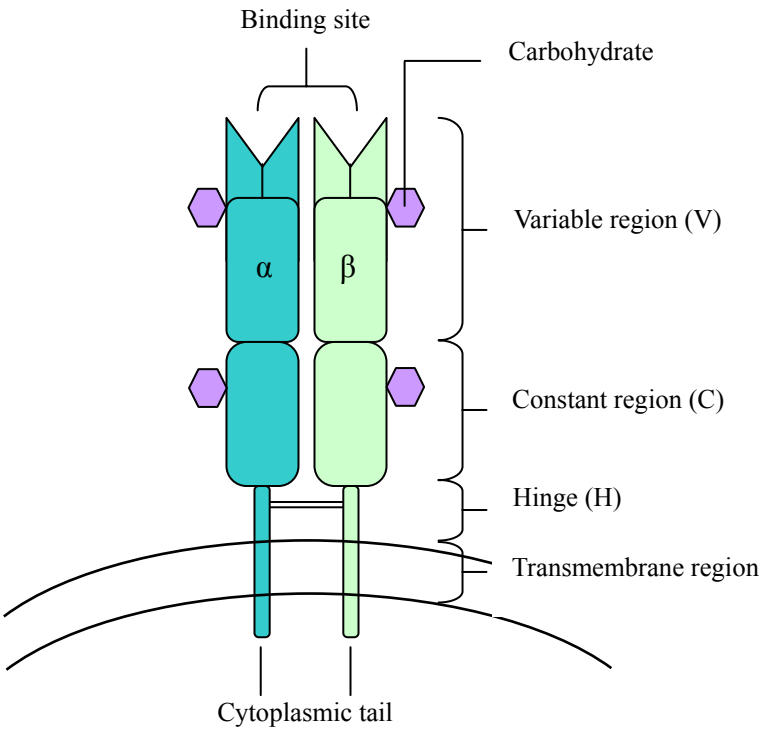


Figure 1.4 – Structure of T cell receptor (TCR)

TCR complex

TCR is responsible for antigen recognition but not for signal initiation. TCR complex is expressed on cell surface and TCR are associated with several additional accessory transmembrane polypeptides (Wilson *et al.*, 1997). In other words, signaling co-receptors are required to complete the entire TCR signaling complex (Koyasu *et al.*, 1992). During T cells development, CD3 molecule is expressed and associated with TCR to form the TCR signaling complex. Total by four CD3 subunits take part in the formation of TCR complex, including CD3γ, CD3δ and two CD3ε chains. All these CD3 proteins resemble the Ig-α and Ig-β chains of BCR, and have an extracellular Ig-like domain as well as ITAM motif within their cytoplasmic tail. In addition, a homodimer of ζ-chain polypeptide also incorporates into the TCR-CD3 molecule to complete the structure of TCR

signaling complex (Figure 1.5). Since TCR complex is indispensable in TCR signaling, hence, CD3 molecule is commonly used as marker of T cells identification. It is noted that, however, both CD3 subunits and ζ-chains do not bind any antigen and their function is solely for producing and transmitting signals from the cell surface receptor to interior cytoplasmic machinery of the cell.

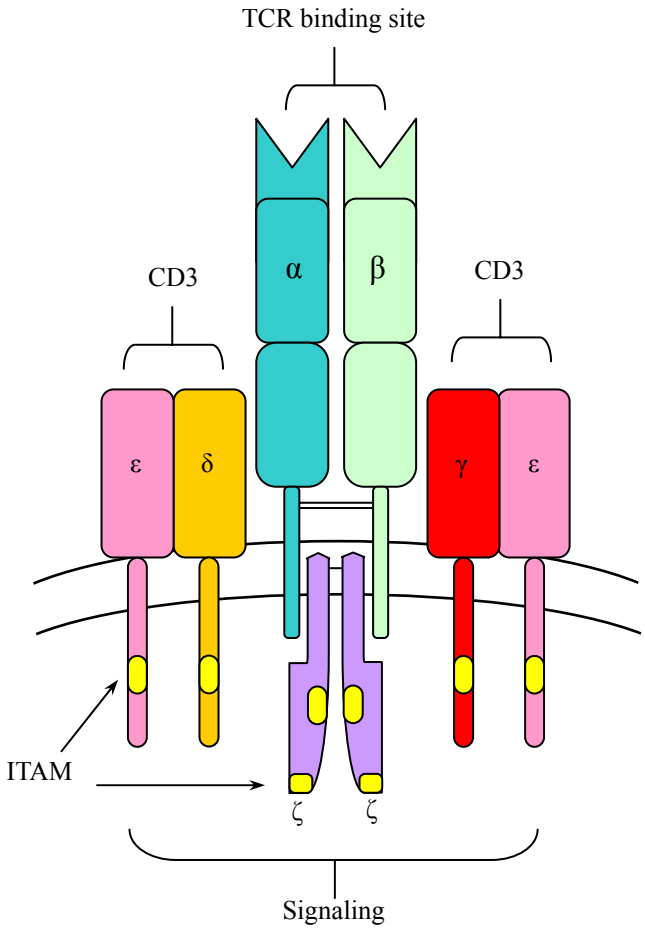


Figure 1.5 – Structure of TCR signaling complex

Major histocompatibility complex (MHC)

In cases of intercellular digestion, macromolecules or microbes will be digested by lysozyme and internalized. Subsequently, these antigens hereafter will bind

with polypeptides and form the major histocompatibility complex (MHC) which will be finally presented at the cell surface. These processes are called antigen processing and antigen presentation (Marks, 1998; Pamer *et al.*, 1998). Cell associates with such activities are collectively called antigen-presenting cells (APCs) (Knight *et al.*, 1993). Antigen processing and presentation contributes significance in activating adaptive immunity that will be discussed in later section.

Antigen can be recognized by TCR with the aid of antigen presenting molecules. Major histocompatibility complex (MHC) plays a major role in antigen presentation and contributes to the activation of TCR (Konig, 2002). Therefore, MHC molecule is an indispensable key in cell-cell mediated acquired immunity. The main function of MHC is to transport those digested fragments or peptides of pathogens to the cell surface. Alternatively, MHC serves a binding site for antigen-dependent TCR recognition (Kumar *et al.*, 2001). Collectively, two classes of MHC had been identified and shown to be different in structure and expression pattern (Fabre, 1991; Rohn *et al.*, 1996). Class I MHC is found in pathogen-infected cells while class II MHC is expressed in APCs such as macrophages and B cells. However, infected APCs can also present class I MHC on their cell surface. Such differential expression of MHC molecules can be recognized by TCR that leads to diverse downstream effector activities.

1.3.2.3) T cell activation – TCR-MHC interaction

With respect to the differential expression of surface markers, several subsets of T cells can be identified. These include $CD4^+$ T helper cells (T_H1 and T_H2 cells)

and $CD8^+$ cytotoxic T cells (T_C cells). CD molecules not only are commonly used for immune cells identification, but also are responsible for MHC recognition. Hence, CD molecules are referred as TCR co-receptors. Distinct MHC molecules are recognized by different T cell subtypes ($CD4^+$ or $CD8^+$) with various specificities (Kumar *et al.*, 2001; Konig, 2002). The following example illustrates the mechanisms of how adaptive immunity can be acquired by cellular interaction via TCR activation.

Activation of $CD4^+$ T helper cells

$CD4^+$ T helper cells recognize class II MHC which is solely expressed on APCs such as B cells (Clark *et al.*, 2004). For instance, antigen is recognized by naive B cell via BCR (e.g. in spleen) that immediate triggers internal signaling cascade. One of the consequences is the induction of class II MHC molecule expression. Binding of TCR and $CD4^+$ to MHC-antigen complex presented on B cell result in activation of T_H cells. Activated T_H cell produces diverse immune signals including cytokines that in turns stimulate B cell. Hence, B cell will be fully activated and further differentiated into memory cells and effector antibody-producing B cells. Therefore, the role of $CD4^+$ T cells is a helper in cell-cell interaction (Konig, 2002). This is usually referred as thymus-dependent B cells activation (Figure 1.3). It is highlighted that MHC-TCR interaction is a dual immune response.

As mentioned before, stimulated $CD4^+$ T cells produce enormous cytokines that not only modify B cell activities, but also regulate $CD4^+$ T cells itself. Distinct cytokines have diverse functions. For instance, IL-2 and TNF-α act as

stimulatory cytokines on T cells while IL-4 and IL-10 exert their inhibitory effects on both B and T cells. As a result, activation of $CD4^+$ T cells involves in development and regulation of B cells and T cells adaptive immune responses.

Second signal for T cell activation

After activation by MHC molecule, T cells become capable of synthesizing and secreting cytokines. In addition, it should be noted that TCR-MHC association does not fully stimulate naive T cells. A second signal is required for full activation of T cells (Grewal *et al.*, 1996). In most cases, the secondary signal comes from recognition of the co-stimulatory molecule which drives the clonal expansion of naive T cells. As briefly mentioned before, it was found that the cluster of differentiation (CD) superfamily acts as co-stimulatory molecules and plays the crucial role in cellular interaction (Grewal *et al.*, 1998; Zola *et al.*, 2005b).

Cluster of differentiation (CD) molecule

These CD molecules (a superfamily of surface marker of leukocytes) were first identified in the early 1980s (Howard *et al.*, 1981; Janossy *et al.*, 1981) and were initially assigned as the human leukocyte differentiation antigens (HLDA). Up to date, more than 339 CD specifications have been found with the use of monoclonal antibodies (mAb) (Ahmed *et al.*, 1983; Zola *et al.*, 2005a; Zola *et al.*, 2005b). Particular set(s) of CD molecules not only perform the role of co-receptors against varies antigens, their presence can also be applied on cell sorting because of its expression unity. For instances, differential expressions of CD molecules are used for identification of different cell type and functional

state. Loken and colleagues had developed a technique that cell sorting can be achieved by probing with two different color emission antibodies (Loken *et al.*, 1977). Up to dated, three-color laser cell sorter had been developed which claimed that 11 fluorescence colors can be measured simultaneously (Herzenberg *et al.*, 2000). Numerous CD surface markers are expressed on lymphoid cell surface, However, anti-CD20-antibodies is still one of the more commonly used antibodies against B cells sorting (Roberts *et al.*, 2002) while anti-CD3-Ab/ anti-CD4-Ab/ anti-CD8-Ab are currently applied in T cell identification (Koyasu *et al.*, 1992; Caraher *et al.*, 2000).

On the other hand, the most crucial role and significance of these CD molecules is their involvement in signaling transduction. For example, CD19/ CD20 compose the BCR signaling co-receptor (Gauld *et al.*, 2002) while CD3 associates with other peptides and form the entire TCR signaling molecule (Call *et al.*, 2004). In addition, co-stimulatory signal transductions rely on cellular interactions between CD surface markers of APCs and T cells. Cell-cell interaction-mediated signals confer full activation of the adaptive cellular immunity. For example, T cell co-stimulatory (COS) ligand B7 protein is expressed on APCs cell surface and subsequently incorporated with CD28 on T cells (Yoshinaga *et al.*, 1999). Moreover, CD40 of APCs and CD154 of T cells demonstrate another co-stimulatory pair that triggers naive T cells to proliferate (Grewal *et al.*, 1998).

To summarize, at the moment in which both binding of TCR-MHC-antigen complex and secondary co-stimulatory molecules coincidently occur, T cells will

then be fully activated and differentiated into effector T cells. In addition to different molecular associations, effector T cells have various activities against different cell types. Nevertheless, the most important activities and functions of activated T cells is its ability to secret signal mediators termed cytokines.

1.4) Cytokine

Cytokines are small soluble proteins or glycoproteins which are produced and secreted by various immune cells in respond to an external stimulus (Cohen *et al.*, 1996; Fitzgerald, 2001). In addition, secreted cytokines also induce responses or activate other cells through binding to respective receptors of cytokines. Bound or activated cytokines receptors are always coupled to intracellular signal transductions that mediate cellular activities. Therefore, cytokines play a regulatory role in host defense system against both pathogens and regulate inflammation inside body (Alexander, 2002; Ozato *et al.*, 2002).

1.4.1) Mode of actions

Cytokines are chemical communicators and responsible for both intercellular and intracellular signal transduction throughout the immune network. They can act in an autocrine manner, affecting the activities of the cell itself that releases that particular cytokine (Foa *et al.*, 1988). Autocrine activity can be achieved via binding of cytokine onto own cell surface receptor. Secondly, cytokines can also modulate adjacent cells in a paracrine mode. It is because adjacent immune cells may bear the respective receptors on their surfaces. Furthermore, several cytokines are stable enough and able to act as endocrine substances that regulate the activities of distant cells.

1.4.2) Properties of Cytokines

Fitzgerald *et al* (2001) had reviewed and summarized the properties of cytokines. Firstly, cytokines are often pleiotropic and redundancy in their actions. Pleiotropism refers to the ability of single cytokine acting on several different cell types. For example, IL-4 induces Ig production from B cells and T cells differentiation; but inhibits macrophage activities. It is because of the presence of cytokine receptors, which is routinely expressed on varies cell types. With this mechanism, cytokines could mediate diverse biological effects in the immune system. Secondly, experiments illustrated the redundancy characteristics that multiple distinct cytokines will bring the same functional effect on same cell type. For instances, several types of interleukins (IL-2, IL-4 and IL-5) can induce proliferation of B cells. Thirdly, in contrast to pleiotropic mechanism, different cytokines may share the same receptor subunits by which the transduction motif generates the same downstream signal consequently. Because of its redundancy properties, application of a single cytokine inhibitor may not resulted in the expected functional consequences. In addition, cytokines and its receptors demonstrate a very high affinity and that makes cytokines potent even in low concentration (Economides *et al.*, 2003). Effective concentrations of cytokines always are mostly in range within femtomolar to nanomolar. Fourthly, cytokines act in a cascade manner (Tilg *et al.*, 1996). For example, tumor necrosis factor (TNF) can activate both the NF-κβ and the MAPK pathways in T cell which in turn induce gene transcription of another cytokine such as IL-2 (Veglianese *et al.*, 2006). IL-2 will be secreted as a secondary signal and exerted effects on other cells. Lastly, immune cell are able to secret both agonist and antagonist against the same cell type. For instances, B cells can either produce interferon (IFN) to

activate macrophage or secret IL-10 to relief its activities (Boger *et al.*, 2001). As a result, immune cells not only modify homoeostasis of the cytokine pool, but also increase complexity of the cytokine network.

1.4.3) Role of cytokines

Cytokines play a central role in monitoring immune cells (Kang *et al.*, 2004). Originally, cytokines are produced in innate immunity for instant inflammatory response. On the other hand, these cytokines are critical for activating and directing subsequent adaptive immune responses. At the moment, hundreds of cytokine species mediated diverse activities in immune response (O'Shea *et al.*, 2002b). Usually, a cocktail of cytokines will be secreted and contributed to various functions. Further, as mentioned before, cytokines are pleiotropic and redundancy. Hence, these properties allow cytokines to be collectively classified according to their effector activities. In addition, the biological background of distinct categories of cytokines will be briefly introduced in the following section. These include growth factor (IL-2), pro-inflammatory (i.e. TNF-α, IFN-γ and IL-1α), inflammatory cytokines (IL-6) and anti-inflammatory cytokines (IL-10).

1.4.3.1) Growth factor – Interleukin-2 (IL-2)

Interleukin-2 (IL-2) was discovered as a growth factor of PHA-stimulated lymphocyte about 30 years ago (Morgan *et al.*, 1976). IL-2 was known to specifically support the growth of activated T cells *in vitro*, therefore it was denoted as T cell growth factor (TCGF) initially.

Structure

Human IL-2 composes of 133 amino acids with molecular weight of 15 kDa and appears as a monomer. IL-2 size heterogeneity is sometimes seen and mainly attributed to the extent of certain post-translational modification (PTM). Glycosylation can be found in the structure of IL-2 but it does not appear to be a pre-requisite for effective IL-2 functions (Benczik *et al.*, 2004).

Source

Several studies had shown that production and secretion of IL-2 is mainly conducted by mature $CD4^+$ T cells upon TCR activation (Habib *et al.*, 2003; Benczik *et al.*, 2004). Antigen-mediated activation on TCR of resting T cells initiates a cascade of signaling pathway that leads to cellular response including differentiation and cytokines production. Subsequently, activated-TCR triggers phospholipase C-dependent pathway which in turn activates transcription factor, e.g. nuclear factor-κβ (NF-κβ) and activated factor-1 (AP-1) and nuclear factor activated T cells (NFAT) etc. In addition, secretion of IL-2 demonstrated a rapid response while IL-2 level can be measured within 4 – 8 hours after stimulation, and maximum production is during 16 – 24 hours upon stimulation (Raju *et al.*, 2001).

Receptor

Three distinct classes of IL-2 receptor (IL-2R) were found and each was found to be composed of different receptor subunits (Lin *et al.*, 1997). These include IL-2Rα, IL-2Rβ and IL-2Rγ_C chain that form the complete IL-2R complex. IL-2R γ_C chain is now identified as the 'common cytokine receptor γ chain' since

it shared a high degree of homology with other IL receptor such as IL-4 etc. In addition, IL-2Rβ chain primarily associates with Jak1 proteins while Jak3 is found to interact with γ_C chain in a dominant fashion. During IL-2 binding, both Jak1 and Jak3 proteins are activated due to close proximities. Activated Jak1 and Jak3 recruit and phosphorylate specific downstream substrates Stat5, a transcription factor Stat family member. A couple of phosphorylated Stat5 form a dimer structure which subsequently translocates into nucleus and monitors target gene expression.

Biological effects

It was found that IL-2 exerts its activities on many cell types through autocrine or paracrine manner. Although T cells are the major source of IL-2, the dominant receiver is shown to be T cells via the T cell growth factor (TCGF) (Sarin *et al.*, 1984; Malek, 2003). For example, IL-2 activates and promotes differentiation of $CD4^+$ and $CD8^+$ T cells. In order to maintain immune homeostasis and autoimmune responses, inhibitory activity of IL-2 confers a negative feedback loop by down-regulating immune responses. It is because IL-2 regulates T cells population and hence indirectly influences the production of all T cell-derived cytokines such as IL-4. These T cell-derived cytokines in turn monitor the activities of T cells.

1.4.3.2) Pro-inflammatory cytokine

1.4.3.2.1) Tumor necrosis factor-alpha (TNF-α)

TNF-α was initially identified as a soluble endotoxin which exerted significant cytotoxic activities on many tumor cell lines, causing tumor necrosis (Carswell *et*

al., 1975). It functions in a wide variety of physiological and pathological aspects, including inflammation etc. TNF superfamily comprises of several well-studied members such as TNF-α, TNF-β (lymphotoxin LT), Fas ligand (FasL) and CD40 ligand (CD40L) etc (Gardnerova *et al.*, 2000). After successful cloning of the human TNF (Shirai *et al.*, 1985; Wang *et al.*, 1985), studies of TNF-α has been increased exponentially.

Structure

TNF-α is expressed in two forms, a transmembrane isoform with 26 kDa in molecular weight and a 17 kDa secreted form (Ware, 2005). Secreted TNF-α is non-covalently associated as a trimer molecule, resulting in a functional 51 kDa TNF-α. It is interesting that both membrane-bound and soluble trimer of TNF-α is biologically functional.

Source

Although the biosynthesis of TNF-α is tightly controlled, it is secreted by numerous cells in respond to an assortment of stimuli (Locksley *et al.*, 2001). For instances, inflammatory stimuli or mitogen are prominent in induction of TNF-α synthesis. Stimuli such as LPS activation, TCR-binding in T cells and cross-linking of surface immunoglobulin (sIg) on B cells are all effective for induction of TNF-α production (Chen *et al.*, 2002). It is also stressed that these multiple signaling pathways have good converge on the NF-κβ transcription factor (Takasuka *et al.*, 1995). Hence, NF-κβ becomes the major regulator in TNF-α. Previous studies also demonstrated that synthesis of TNF-α is secreted in activated cells rapidly, e.g. within 30 minutes (Takeda *et al.*, 2004). This suggests

TNF-α plays a leading and regulatory role in immune responses and the development of immunity.

Receptor

Biological response of TNF-α is mediated by two distinct receptors, 60 kDa TNFR-1 and 80 kDa TNFR-2 respectively (Gardnerova *et al.*, 2000). TNFR-1 is ubiquitously expressed and generally considered to be responsible for the majority of biological actions. Specifically, TNFR-2 is mainly confined to cells related to the immune system. In the extracellular domain, both TNFR-1 and TNFR-2 contain four cysteine-repeat motifs that form an elongated shape and interacts with trimeric ligand of TNF-α, (either as membrane-bound or soluble TNF-α). On the other hand, the intracellular cytoplasmic tail of TNFR-1 shares homology with IL-1R. For example, they carry the same death domain (DD) which is critical in signaling transduction (Chen *et al.*, 2002). In addition, sequence homology has been demonstrated in Toll domain of Toll-like receptor (TLR) (will be further discussed in later section).

Biological functions

As mentioned above, TNF-α was originally found to cause necrosis in tumor and induce apoptosis (Carswell *et al.*, 1975). Subsequently, it has been recognized that TNF-α exerts diverse effects on either lymphoid cells or non-lymphoid cells (Gardnerova *et al.*, 2000). For instances, TNF-α not only regulates cell proliferation, differentiation, but also participates in immune response and inflammation process. Further studies on inflammation showed that TNF-α like IL-1 is a powerful inducer of the inflammatory response. In addition, TNF-α is

identified as the central regulator of innate immune response. TNF-α is found to monitor the activities of T_H1 cells and induce the secretion of second-mediators such as IL-2, IL10 and IFN-γ etc. Biological activities of TNF-α are summarized as following table (Table 1.2).

Immune Cells	Non-immune cells
Macrophages	*Vascular endothelial cells*
Autocrine induction of TNF-α	Enhanced expression of MHC class I
Increase cytotoxicity	Induction of production of NO, IL-1
Induction of IL-1, IFN-γ	Induction of ICAM-1, surface antigen
Chemotaxis	Increase vascular permeability
Lymphocytes	*Fibroblast*
B cell proliferation	Induction of proliferation
T cell proliferation	Induction of IL-1, IL-6
Activation of cytotoxic $CD8^+$ T cells	
Induction of mature and aged T cells	
Induction of cytokines production	

Table 1.2 – Diverse biological functions of TNF-α

1.4.3.2.2) Interferon-gamma (IFN-γ)

Interferon-gamma (IFN- γ) was first identified as a substance that protected cells from viral infection and was initially considered as an antiviral agent (Isaacs *et al.*, 1957). Later on, IFN-**γ** had been found to have diverse activities after being secreted from cells in respond to variety of stimuli (Pestka *et al.*, 2004b; Bonjardim, 2005).

Structure

With bioinformatic analysis, IFN-γ is predicted to have a mature molecule of 143 amino acids with size 17 kDa (Pestka *et al.*, 2004b). However, variable glycosylation occurred during the peptide translation process which results in the production of several apparently mature IFN-γ isoforms. These isoforms are weighted 17 kDa, 20 kDa and 25 kDa respectively. Each IFN-γ monomer is routinely helical in their tertiary structures that mediate formation of IFN dimer. As a result, in physiological status, two glycosylated IFN-γ polypeptides structurally self-assemble to form a noncovalent homodimer protein. The IFN-γ dimer in turn becomes the active form of IFN-γ.

Source

Genetically, every single cell inside our body is able to produce IFN-γ. However, secretion of IFN-γ is restricted by immune cells such as $CD4^+$ T_H1 cells, $CD8^+$ T_C cells and NK cells (Bach *et al.*, 1997). On the other hand, IFN-γ production can be induced by two independent stimuli and had been demonstrated in $CD4^+$ T_H cells. These are either by TCR activation or cytokine activation with IL-12 or IL-18-induced secretion.

Receptor

IFN exerts its effects on various cells and most of these cells express and interact through with the distinct high affinity IFN-γ receptor (IFNGR) (Bach *et al.*, 1997). IFNGR comprised of two co-expressed subunits. IFN-R1 (α-chain) is responsible for ligand association. IFN-R2 (β-chain) is required for IFN-γ signaling and plays a minor role in ligand binding. Notably, both IFN-R1 and

IFN-R2 lack the intrinsic tyrosine kinase domain. IFN-R1s and IFN-γR2s associate constitutively with Jak1 and Jak2 via their cytoplasmic tails respectively (Hemmi *et al.*, 1994; Soh *et al.*, 1994). Upon IFN-binding, Jak1 and Jak2 of corresponding IFN-R1 and IFN-R2 subunits will be brought to close proximity and cross phosphorylate each other. Activated Jak1/ Jak2 hence recruit and phosphorylated Stat1 protein. Subsequently, phosphorylated Stat1 protein detaches from the receptor complex and form a dimeric structure that homodimeric Stat1 then translocates into nucleus. Stat1 dimer interacts with other nuclear factors and accessory proteins to activate IFN-γ-responsive gene expression.

Biological Activities

The pleiotropic features of IFN-γ had been extensively demonstrated. Such pleiotropic effects are mediated by complex patterns of cell type-specific gene regulation (Tau *et al.*, 1999). However, coupling of IFN-γ and JAK/ STAT pathway makes it an ideal system to rapidly coordinate the activation of acute phase responses. Therefore, it is comprehensive that IFN-γ plays an important role in both innate and adaptive immunity that require quick and precise immune activities against invaded pathogens. For instances, production of IFN-γ at early phase of host innate immunity brings about the activation of acute inflammation in macrophages. Induction of inducible nitric oxide synthase (iNOS) is one of the examples of acute inflammation. In addition, IFN-γ-activated T cells are able to secret differential cytokines or cytotoxic substances that manipulate immune responses.

1.4.3.2.3) Interleukin-1 alpha (IL-1α)

Interleukin-1alpha (IL-1α) is classified as a proinflammatory cytokine according to its stimulatory ability on inflammation-related genes expression (Boraschi *et al.*, 1996). IL-1α is first synthesized as its precursor with molecular weight of 31 kDa. It is associated with plasma membrane and cleavage of the 31 kDa IL-1α precursor results in the 17.5 kDa mature form of IL-1α (Braddock *et al.*, 2004).

Receptor

IL-1α receptor (IL-1R) is a heterodimer formed by IL-1RI (IL-1R type 1) and IL-1R-AcP (IL-1 accessory protein receptor) (Liew *et al.*, 2005). It is found that extracellular region of IL-1RI has three immunoglobulin-like domains. Interestingly, the cytoplasmic tail of IL-1RI is unique in that it shares a high homology to Toll receptor domain which is a specific characteristic of Toll-like receptor (TLR) (Cao *et al.*, 1996). Further studies revealed that Toll-homologous domain of IL-1R were responsible for intracellular signal activation. Induction of IL-1R signaling in turn triggers IL-1α- or inflammation-responsive gene expressions. Details of signal initiation and transduction through IL-1R or Toll-homology domain (in addition with TLR) will be discussed in later section (Section 1.5.4).

Biological activities

IL-1α is primarily a pro-inflammatory cytokine because it induces the expression of genes associated with inflammation (Rosenwasser, 1998; Fitzgerald, 2001). For example, IL-1α is able to induce an increase in the expression of adhesion molecules like ICAM-1 on endothelial cells. This is an important property as

infiltration of inflammatory cells into the extra-vascular spaces is strictly controlled by the presence of ICAM-1. Activated macrophages and PMNs will in turn produce other inflammatory cytokines such as TNF-α around the inflammation site. Besides, IL-1α activates expression of inducible nitric oxide synthase (iNOS) which synthesize nitric oxide (NO) which act as inflammatory agent (Dinarello, 2002). Furthermore, IL-1α also stimulates other cell to produce cytokines including IL-2, IL-4 and IL-6 etc, that regulate and develop the adaptive immune responses (Burger *et al.*, 2002).

1.4.3.2.4) Interleukin-6 (IL-6)

Interleukin-6 (IL-6), in the past, was known by a variety of names because of its discovered activities. These include interferon-β2, T cell replacing factor-like factor, B cell differentiation factor and B cell stimulatory factor-2 etc (Simpson *et al.*, 1997). IL-6 protein is always stabilized by post-translational modification (PTM) such as N-/ O-linked glycosylation and phosphorylation. Hence, IL-6 shows molecular size heterogeneity and has ranges a molecular weight from 21 kDa – 28 kDa (Heinrich *et al.*, 1998).

Source

Diverse immune cells, including lymphoid cells and non-lymphoid cells, produce IL-6 upon various behind of stimulation (Eyles *et al.*, 2003). For instances, mitogen-activated T cells initiate secretion of the signaling molecule NF-κβ. Further, one of the transcriptional binding sites of NF-κβ has also been identified within the conserved region of the IL-6 promoter (Ozato *et al.*, 2002). However, at certain extent, T cells are considered as the main source of IL-6.

Receptor

IL-6 receptor (IL-6R) and its signaling pathway is manifested by two interacting molecules, an 80 kDa IL-6 binding protein called IL-6α chain, and a 130 kDa signal transducer glycoprotein β chain termed gp130 (Heinrich *et al.*, 1998). Once IL-6 binds onto its receptor, it induces ligand receptors hexamer formation. IL-6 not only binds onto and link with IL-6α, it also associates with gp130 subunits. Such interaction brings a close spatial conformation between IL-6α and gp130 that contribute to the activation of JAK kinase (Jak1 and Jak3) (Montero-Julian, 2001). Activated Stat3 forms homodimer or heterodimer prior to interacting with others transcription factors. Corporation with other signaling factors initiates various IL-6-responsive genes transcription, and exerts diverse effects of IL-6.

Biological functions

Although responses from IL-6-activated and Stat3-mediated signals had been shown to be growth-related, IL-6 still has diverse functions on various cell types (Naka *et al.*, 2002). Originally, IL-6 was identified as an inducer for plasma B cells differentiation factor (BCDF). Further studies revealed that IL-6 is a growth and differentiation factor on various immune cells, including hematopoietic progenitor and T cells (Barton, 1997).

Similar to TNF-α, IL-6 is also pleiotropic (Helle *et al.*, 1989). For instances, IL-6 could stimulate thymocyte proliferation. With the addition of IL-1, thymocyte produces IL-6 production which in turn activates B cells differentiation. It was proposed that, IL-6 was induced as an essential mediator of the inflammatory

response to a localized inflammation (Naka *et al.*, 2002). Once IL-6 is induced and secreted at the inflammatory sites, it will promote inflammation. Hence, IL-6 is also considered as an inflammatory cytokine.

1.4.3.3) Inhibitory cytokine – Interleukin-10 (IL-10)

Interleukin-10 (IL-10) is identified as a key regulator in both innate and adaptive immunity (Spits *et al.*, 1992). Because of the ability in turning off production of pro-inflammatory cytokines such as IL-1α, TNF-α and IFN-γ, IL-10 was termed the cytokine synthesis inhibitory factor (CSIF) (Conti *et al.*, 2003). For example, activated macrophage secrets inhibitory cytokine IL-10, which in turn suppresses its activities. This demonstrates an autocrine property of IL-10 with negative feedback loop regulation at work.

Structure

Different from other cytokines, IL-10 is neither glycosylated nor modified and has a molecular weight of 18 kDa. In addition, each IL-10 monomer consists of six α-helices and forms a tight but noncovalent homodimer (Saito, 2000).

Source

Originally, IL-10 is secreted by mitogen-activated T cells and was described as CSIF denoting their inhibition on the production of cytokines from other cells. Besides, pathogens can induce macrophages activation and IL-10 production (Hawrylowicz, 2005). Oppositely, IFN-α can induce IL-10 production in T cells.

Receptor

IL-10 receptor composes of two subunits, forming a heterodimer receptor. Subunits are named as IL-10 receptor 1 (IL-10R1) and IL10 receptor 2 (IL-10R2) respectively (Pestka *et al.*, 2004a). IL-10R1 composed of an extracellular domain for IL-10 binding and a cytoplasmic tail for signaling regulation. This cytoplasmic tail is responsible either for inhibition of proliferation, or for induction of anti-inflammatory pathway. While IL-10R1 is able to bind its ligand IL-10 and then possesses Stat recruitment, an additional second accessory receptor chain is required for signal initiation (Mocellin *et al.*, 2003).

Functions

Different groups had investigated the functions of IL-10 and found diverse suppressive effects on a board range of immune cell types (Mosmann *et al.*, 1991; Dumoutier *et al.*, 2002). One of the implications is, IL-10 is the regulator or suppressor of T_H cell subsets. Besides, IL-10 is able to inhibit the production of pro-inflammatory cytokines (e.g. TNF-α) and as well as induce anti-inflammatory cytokines (e.g. IL-1). However, IL-10 is classified as anti-inflammatory cytokines. Ding *et al* had reviewed the biology and immuno-suppressive functions of IL-10 (Ding *et al*., 2003). Their findings are summarized and subscripted in the following table (1.3).

Effective cell types	Inhibitory actions of IL-10
Macrophages	IL-1α, TNF-α, ROS production
Monocytes	IL-6 production, MHC II expression
T1 helper cells	IFN-γ, IL-2, IL-4 synthesis
B lymphocytes	Thymus-dependent activation
Dendritic cell	Antigen presentation
Natural killer cells	IFN-γ production
Endothelial cell	ICAM-1 expression

Table 1.3 – Summary of inhibitory effects of IL-10

1.4.4) Cytokine network

In summary, cytokines acts as chemical signaling messengers which are indispensable in the immune system (Cohen *et al.*, 1996; Fitzgerald, 2001). It is produced, or even secondarily induced for secretion. As discussed previously, distinct cytokines has its specific functions, but they have redundant activities. These pleiotropic and redundancy properties of cytokines not only regulate intra-cellular activities. In addition, it inter-monitors a wide range of cells, especially immune cells and immune system. For instances, both IL-2 and IL-4 are stimulatory for T cells and B cells growth and differentiation. On the other hand, IL-10 is specific for immune response inhibition. Therefore, it is comprehensive in describing that cytokines act in a complex network in which every single cytokine is able to affect many other cytokines and cells. Alternatively, consequence of cytokines is accounted from the presence and the concentration of various cytokines species. Undoubtedly, the presence of immune cell-types is also of importance as well. In the following diagram, a simplified cytokines network is summarized and shown schematically in Figure 1.6.

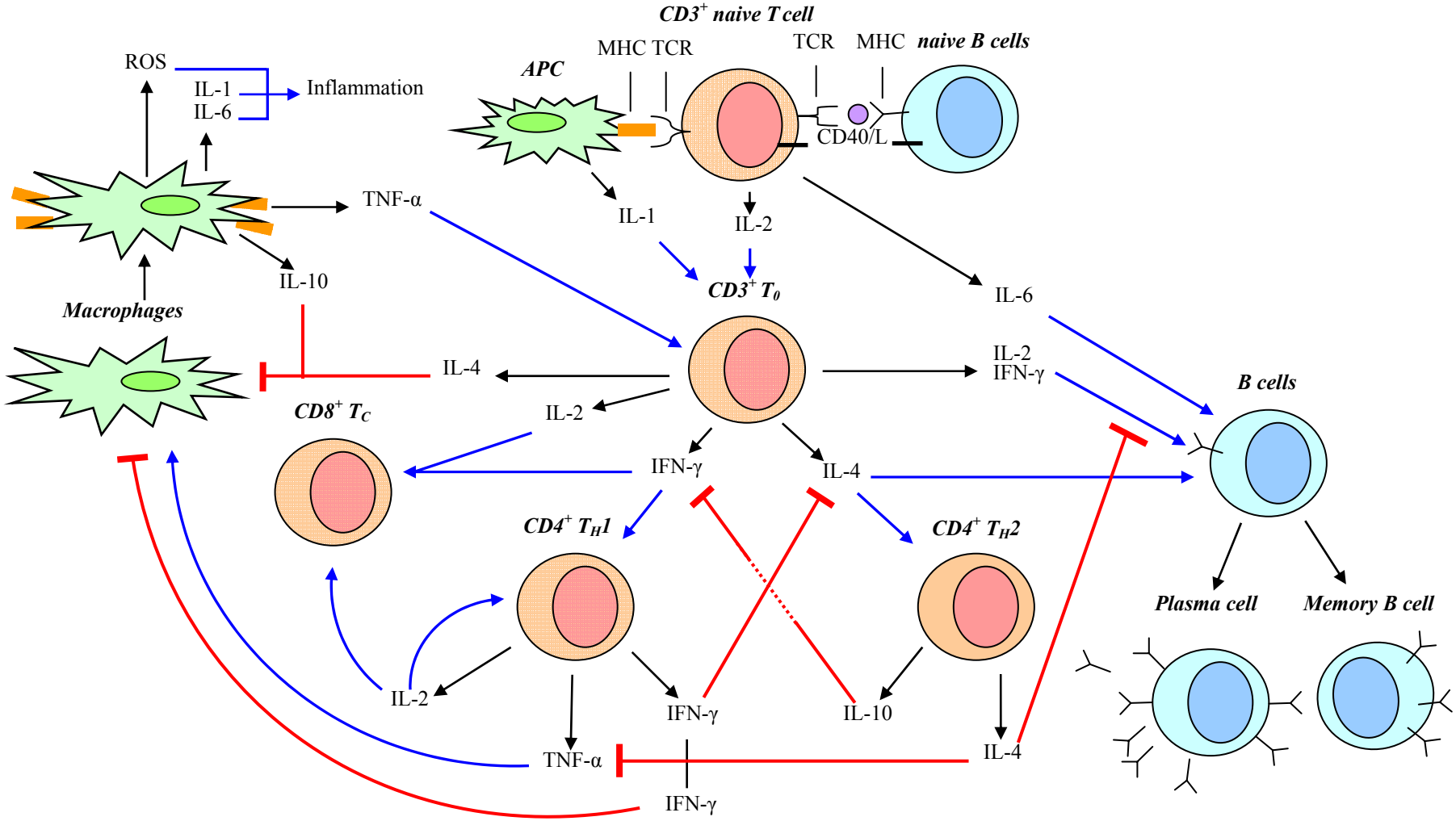


Figure 1.6 – Cytokines networking among B cells, T cells and macrophages.
Cytokine secreted ⟶ : Stimulatory effect ⟶ : Inhibitory effect ⊣

1.5) Advanced Signaling Pathway Studies

In the previous sections, the innate immunity and adaptive immunity was briefly introduced. Also, the fundamental biology of B cells, T cells and cytokines were discussed (Hoebe *et al.*, 2004; Pasare *et al.*, 2005) On the other hand, it is noted that activities of immune cells and their functional effectors depend on both the intracellular and extracellular signaling transduction pathways (Gauld *et al.*, 2002; Huang *et al.*, 2004). Meanwhile, signaling transduction confers the central role of those immune cells and mediators. Therefore, different signaling transduction pathways are going to be discussed in this section. These communication transduction cascades include NF-κβ pathway, BCR pathway, TCR pathway, TLR pathway and JAK-STAT cytokine pathway.

1.5.1) Nuclear factor-κβ (NF-κβ) signaling pathway

Transcription factor nuclear factor-κβ (NF-κβ) plays a central and crucial role in regulating expression of a wide variety of immune-responsive genes (Baeuerle *et al.*, 1994; Li *et al.*, 2002). These genes obviously function in both innate and adaptive immune system only. On the other hand, much studies had been done on BCR signaling (Gugasyan *et al.*, 2000; Weil *et al.*, 2004), TCR signaling (Schmitz *et al.*, 2003; Weil *et al.*, 2004), cytokine signaling (Yasukawa *et al.*, 2000; Alexander *et al.*, 2004) and discovery of TLR (Takeda *et al.*, 2004, 2005). These studies reported that activation of NF-κβ is the consequence of these signaling pathways. Hence, NF-κβ plays an indispensable signaling factor in, and builds up the bridge between both innate immunity and adaptive immunity. Investigation of NF-κβ revealed that NF-κβ signaling cascade involves post-translational modifications (PTMs) (Baeuerle *et al.*, 1994). Cascade of

phosphorylation in turn monitors the signaling transduction. The cascade is composed of upstream kinase and downstream substrates. These signaling intermediates include the regulator IKK, inhibitor I-κβ and effector NF-κβ.

1.5.1.1) Inhibitor of nuclear factor-κβ kinase (IKK)

Inhibitor-κβ-kinase (IKK) is an essential and central regulator of NF-κβ signaling (Takaesu *et al.*, 2003). In the IKK family, it consists of three distinct components which are catalytic members: IKK-α, IKK-β and additional regulatory subunits IKK-γ or called nuclear factor-κβ essential modulator (NEMO) (Piccolella *et al.*, 2003). Activities of IKK are regulated by phosphorylation which is facilitated by a variety of its upstream kinase, such as phospholipids C (PKC) family (Guo *et al.*, 2004) and TAK protein (Takaesu *et al.*, 2003) etc. It is emphasized that IKK acts as a convergent point at which differential signals are mediated into the same effector machine. For instances, activation of either BCR or TCR or TLR always leads to activation of NF-κβ signaling.

Experimental evidences indicated that IKK is not only a kinase and determines the activity level of NF-κβ, it also modulates the quality of NF-κβ-related transcription (Schmitz *et al.*, 2003). For instances, IKK also regulates the composition of NF-κβ dimer, either being homodimer or hetero-dimer. Subsequently, specific dimerization of NF-κβ subunits leads to differential expression of target genes.

1.5.1.2) Inhibitor of nuclear factor-κβ (I-κβ)

Inhibitor of nuclear factor-κβ (I-κβ) contains several family members (Liou, 2002). The most common units are I-κβ-α, I-κβ-β and I-κβ-ε. All these inhibitor molecules are characterized with the presence of repeated amino acid domain called ankyrin. These ankyrin motifs are believed to mask, at least, one of the nuclear localization signals (NLS) of NF-κβ (Blank *et al.*, 1992). Blocking of NLS motifs will result in termination of DNA binding by NF-κβ. I-κβ is originally believed to retain NF-κβ in cytoplasm by masking NLS. However, recent studies had demonstrated that, balancing between active and inactive NF-κβ depends on continuous flux of free I-κβ and NF-κβ across nuclear membrane (Li *et al.*, 2002).

It has been well documented that I-κβ-α monitor transient NF-κβ activation while I-κβ-β is responsible for maintaining a persistent activation of NF-κβ (Liou *et al.*, 1993). Routinely, I-κβ stays in cytoplasm whereas it ubiquitously associates with and inactivates NF-κβ. On the other hand, IKK is responsible for regulating I-κβ activities through phosphorylation on two serine residues (at position 32 and 36) of I-κβ-α. Besides, I-κβ proteins contain specific and conserved serine residues that serve as a degradation signal site. Phosphorylation and ubiqutinylation of I-κβ will lead to its proteolytic degradation.

1.5.1.3) Nuclear factor-κβ (NF-κβ)

Nuclear factor-κβ (NF-κβ) is a ubiquitous by expressed transcription factor family consisting of five member proteins (Li *et al.*, 2002; Liang *et al.*, 2004). These members are p65 (REL-A), REL-B, cytoplasmic c (REL), p50 and p52.

Moreover, they have a structurally conserved amino-terminal domain or called REL homology domain (RHD). RHD is composed of 300 amino acids in which the dimerization domain, DNA-binding domains and the nuclear localization domain which is known as the NLS were identified.

Except REL-B, each NF-κβ member can form homodimer and heterodimer with another member as well. Different combinations of subunits in turn confer differential target gene transcription activities (Liou, 2002). The most common activated form of NF-κβ is the heterodimer of p50 and p65 subunits. In additional, NF-κβ exerts their DNA-binding activities through, or depends on, interactions with other transcription factors and regulators inside the nucleus.

1.5.1.4) Activation cascade of NF-κβ signaling

Once immune cells are stimulated, external signaling is transduced from membrane to cytoplasm via a cascade of kinase transducers (Weil *et al.*, 2004). Actually, several pathways including BCR activation (Gugasyan *et al.*, 2000), TCR activation (Schmitz *et al.*, 2003) and LPS stimulation (Triantafilou *et al.*, 2002) will initiate NF-κβ signaling. From these studies, activations of signaling intermediates of distinct pathways are all able to induce activation of IKK. Consequently, activated IKK began to regulate the NF-κβ pathway through phosphorylation of I-κβ.

To briefly summarize what had been found so far, in resting condition, un-phosphorylated or inactive I-κβ-α/ -β is usually retained in cytoplasm and tightly “lock” with NF-κβ. Once immune cell is stimulated, upstream regulator

IKKs is being activated by other signaling kinase. Subsequently, activated IKK phosphorylates both I-κβ-α/ -β. Phosphorylated I-κβ complex will dissociate from NF-κβ in cytoplasm as a consequence. Phosphorylated-I-κβ is modified and undergone ubiquitin-dependent proteolytic degradation.

In the meantime, NF-κβ is free and translocates into nucleus. NF-κβ dimer interacts with specific transcription co-factors in nucleus and initiates target gene transcription (Baeuerle *et al.*, 1994; Gugasyan *et al.*, 2000; Schmitz *et al.*, 2003). NF-κβ signaling pathway is illustrated and summarized in Figure 1.7. Except these responsive genes are transcribed, it is also found that activated NF-κβ rapidly induces transcription of gene encoding I-κβ-α. Such regulatory mechanism thereby generates high level of I-κβ which binds with NF-κβ in nucleus. As a result, cellular activities are restored to resting state.

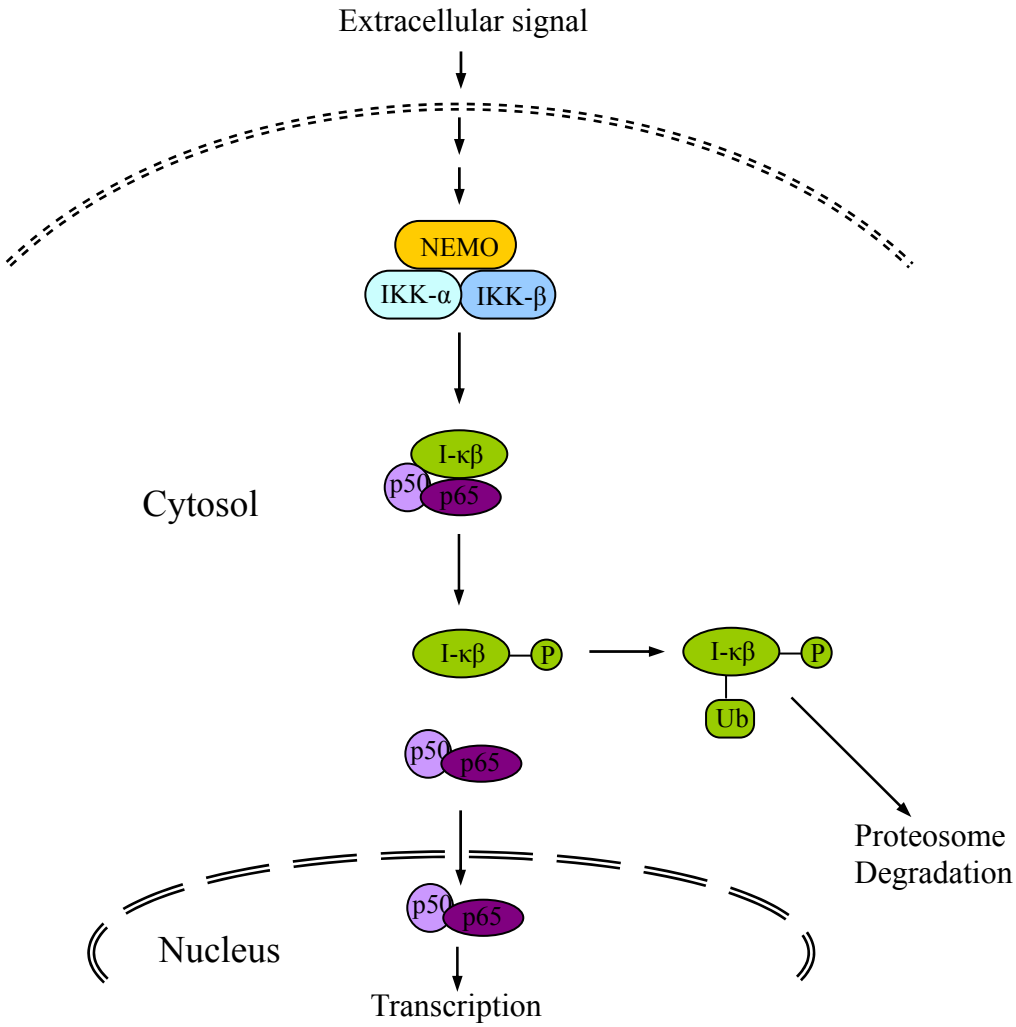


Figure 1.7 – Activation cascade of NF-κβ

Several extracellular stimulations are able to activate NF-κβ pathway. BCR-, TCR-, TLR-signaling are some of the examples. Briefly, extracellular ligands bind onto their respective receptors and trigger recruitment of signal mediators. Receptor signalosome complex transduce the message from plasma membrane into cytosolic space via conformational change and sequential kinase activities. NEMO – central regulator kinase is activated by differential upstream kinases (depends on which receptor is involved) and activates its downstream substrates I-κβ. Phosphorylated I-κβ is detached from the I-κβ/ NF-κβ complex while

NF-κβ dimer is unlocked from its inhibitor. Subsequently, NF-κβ translocates into nucleus whereas regulates receptor-specific genes transcription.

1.5.2) B cell receptor (BCR) & T cell receptor (TCR) signaling

In previous sections (Section 1.3.1.3 & 1.3.2.5), we have briefly reviewed the development and activation of B cell and T cell respectively. Moreover, it is stressed that their surface receptors play an important role in cellular development and immunity as well (Kumar *et al.*, 2001; Siemasko *et al.*, 2001).

1.5.2.1) BCR & TCR & NF-κβ

Past studies on BCR and TCR signaling had revealed an important event. That is, either antigen-mediated BCR oligomerization or MHC-coordinated TCR stimulation leads to activation of transcription factor NF-κβ (Gugasyan *et al.*, 2000; Schmitz *et al.*, 2003). In the immune system, functions of NF-κβ include regulation on cell growth, differentiation, apoptosis, cell surface marker expression and cytokine production etc. For instances, it is known that NF-κβ controlled the lymphocyte development (Siebenlist *et al.*, 2005). Therefore, it is not surprising that antigen binding onto either BCR or TCR are able to activate the NF-κβ cascade, which in turn monitor immune response as a consequence. However, signal transduction from either BCR or TCR to intracellular cytosol involves a large set of components. Exact details of many of the steps are unclear and more research is needed.

1.5.2.2) B cell receptor (BCR)-mediated signaling

In earlier sections, BCR had been described as antigen recognition receptors (Gauld *et al.*, 2002). Moreover, BCRs are able to bind to a large pool of antigen patterns because of somatic DNA rearrangements (Manis *et al.*, 2002). Besides, BCR are associated with co-receptors (Ig-α/ CD79α and Ig-β/ CD79β) which are essential for signaling cascade initiation (Matsuuchi *et al.*, 2001). Interestingly, BCR plays a dual role in immune response. Firstly, antigenic stimulation of BCR triggers signal transduction that regulates a wide variety of target gene expressions. Secondly, acting as APCs, BCR directs the uptake of antigen and the internalization of antigen-bound BCR. Although these two processes seem to be interdependent, little is known about details of such differential signaling mechanisms (Gauld *et al.*, 2002).

Antigen Binding

In resting B cells, BCR is constitutively expressed on B cell surface that are apart from lipid rafts (Cheng *et al.*, 2001). Upon antigens bind onto recognition sites on BCR, this stimulates both transmembrane region and cytoplasmic tail of BCR and its co-receptors as well. Subsequently, BCR acquired affinity and is shuttered to the lipid rafts (see later section and Figure 1.8) at where it permits signaling adaptors or linkers recruitment and orientation. However, translocation of antigen-bound BCR to rafts is a transient process (Pierce, 2002). According to Aman *et al*, BCR can be detected within rafts in minutes after antigen binding. Nevertheless, 15 – 30 minutes after activation, BCR is no longer be detectable (Aman *et al.*, 2000). On the other hand, lipid rafts is involved in antigen internalization and trafficking process.

Oligomerization

It is known that, multivalent antigen promotes cross-linking or oligomerization between BCRs. For instances, repeated antigenic patterns on the same molecule can be bound by several BCRs at the same time. Such interactions result in the formation of cross-linked of BCR through the presence of a single antigenic molecule (Matsuuchi *et al.*, 2001). Oligomerization of BCR not only enhances BCR activation status, but also induces the clustering of lipid rafts. Fusion of lipid rafts provides a large platform for signaling transduction.

Lyn activation

Biochemical studies had revealed that Src-family protein tyrosine kinase (SFK), such as Lyn, is expressed in spleen B cells. In addition, Lyn is the resident within lipid rafts. Condensation of BCRs pulls SFK members in close proximity. Meanwhile, association between Lyn kinases and ITAM motifs allows rapid activation of Lyn and phosphorylation of ITAM. Phosphorylation of ITAM motifs within the cytoplasmic tails of Ig-α and Ig-β are crucial for initiating downstream signaling molecules recruitment (Gauld *et al.*, 2002). In addition, it had been proved that signalosome recruitment is Lyn-determine/ -dependent process. It is because absence of Lyn kinase abrogates BCR signaling completely. Hence, Lyn is critically involved in the early signaling process of BCR activation.

Although what make inactive Lyn become active is not yet defined, several studies found that Lyn kinases weakly associates with non-phosphorylated ITAM motifs in resting B cells (Gauld *et al.*, 2004). This non-covalent interaction

attributes ITAM motifs to maintain an inactive to partially active state, or vice versa. On the other hand, antigen-elicited cross-linking of BCR will bring Lyn kinases close together. Therefore, it is predicted that close spatial proximity is able to induce trans-phosphorylation within the catalytic domain of Lyn molecules. Subsequently, phosphorylated Lyn attains their kinase activities. Nevertheless, this hypothesis needs to be validated by further work.

Syk recruitment

It is stressed that phosphorylated ITAM motifs play an important role in signal transduction (Isakov, 1997). Once conserved tyrosine residues within ITAM motifs are phosphorylated, they served as docking sites for SH2-domain-containing adaptors. In B cells, another kinase family or spleen tyrosine kinase (Syk) participates in this step. SH2-domain-containing Syk is recruited and transported to the lipid rafts where it associates with phosphorylated ITAMs. In addition, dual phosphorylations of ITAMs are essential for Syk binding and activation (Monroe, 2006). Subsequently, Syk is phosphorylated by Lyn kinase before triggering downstream signal cascade. Activated Syk enrolls and interacts with another signaling linker, called BLNK (B-cell linker protein). BLNK plays a positive role in BCR signaling pathway. Binding of BLNK bases on the phosphorylated tyrosine residue on Syk, that provides a docking site for BLNK. Association between Syk and BLNK induces BLNK protein phosphorylation that is a Syk-dependent reaction.

Nearer to the lipid rafts, BCR cross-linking triggers another signaling pathway – the PI3K (phosphoinositide 3-kinase) pathway (Glassford *et al.*, 2005). Although

the mechanism of BCR-induced PI3K activation is under investigation, it is known that activation of PI3K pathway generates a product called PI-3,4,5-P_3 (phophatidyl-inositol-3,4,5-triphosphate). PI-3,4,5-P_3 are able to interact with protein carrying a PH domain (Pleckstrin homology domain), such as that from Btk (Burton's tyrosin kinase). Similar to Syk, Btk also possesses a conserved tyrosine residue within its activation loop. Phosphorylation of Btk seems to be Lyn-dependent phosphorylation and is required for the signaling cascade (Kurosaki, 2000).

PLC-γ2 recruitment

In order to cascading the BCR signal, signaling linker from membrane to cytosol is necessary. Several groups had provided evidence that in BCR signaling, phospholipids C (PLC) isoform PLC-γ2 take part in this signal intermediate. In addition, it had been found that level of PLC was elevated after BCR stimulation and BLNK phosphorylation, suggesting that PLC is the downstream signaling molecule (Kurosaki *et al.*, 2000).

In addition, presence of phosphorylated Syk, Btk and BLNK are pre-requisites for the translocation of PLC. Genetic and biochemical studies provided several clues that recruitment and activation of PLC-γ2 within lipid rafts needs a few criteria. Firstly, both PH-domain and SH-2 domain on Btk are involved in recruitment of PLC-γ2. Secondly, unphosphorylated or inactive BLNK protein is unable to orientate PLC-γ2. Thirdly, lack of either Syk kinase or Btk kinase will result in malfunction of PLC-γ2. Taken overall, Kurosaki suggested that Syk-mediated phosphorylation of BLNK generates phosphorylated tyrosine

which is recognized by SH-2 domain of PLC-γ2 (Kurosaki *et al*, 2002). BLNK associates with PLC-γ2 which is subsequently phosphorylated by Syk. Phosphorylated PLC-γ2 in turn provides a tyrosine site for Btk. PH-domain of Btk kinase then interacts with PLC-γ2. Meanwhile, proximity allows Btk to further phosphorylate specific tyrosine residue(s) on PLC-γ2. This dual tyrosine phosphorylation was found to be critical for full PLC-γ2 functionality and BCR signaling cascade.

PKCβ activation

In B cells, various PLC members are expressed. However, PLCγ2 is expressed in majority and found to be involved in BCR signaling. PLCγ2 recruitment and activation had been briefly explained previously. On the other hand, activation of PLCγ2 resulted in the hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) and the synthesis of IP3 (inositol-1,4,5-triphosphate) and DAG (diacylglycerol). IP3 migrates to the endoplasmic reticulum and bind onto its receptor, leading to the release of calcium (Ca^{2+}) ions. DAG is a well-known regulator of PKC, act as the second messenger. As a result, PKC is transported and localized within membrane rafts region, via PLCγ2-elicited recruitment.

Among its family members, classical isoform PKCβ is dominantly expressed in B cells (Shinohara *et al.*, 2005). In the presence of DAG, PKCβ is transported to BCR signalosome and undergoes conformational changes. Structural alternation exposes its tyrosine residues that were followed by Syk-induced phosphorylation. Many studies had elucidated that phosphorylation of effector PKCβ resulted in BCR-dependent IKK and NF-κβ activation (Gugasyan *et al.*, 2000). However,

signaling substrates and linkers in between PKCβ and NF-κβ upstream regulator IKK remains an unclear issue. Figure 1.8 summarized our current understanding of BCR activated downstream to the protection of NF-κβ.

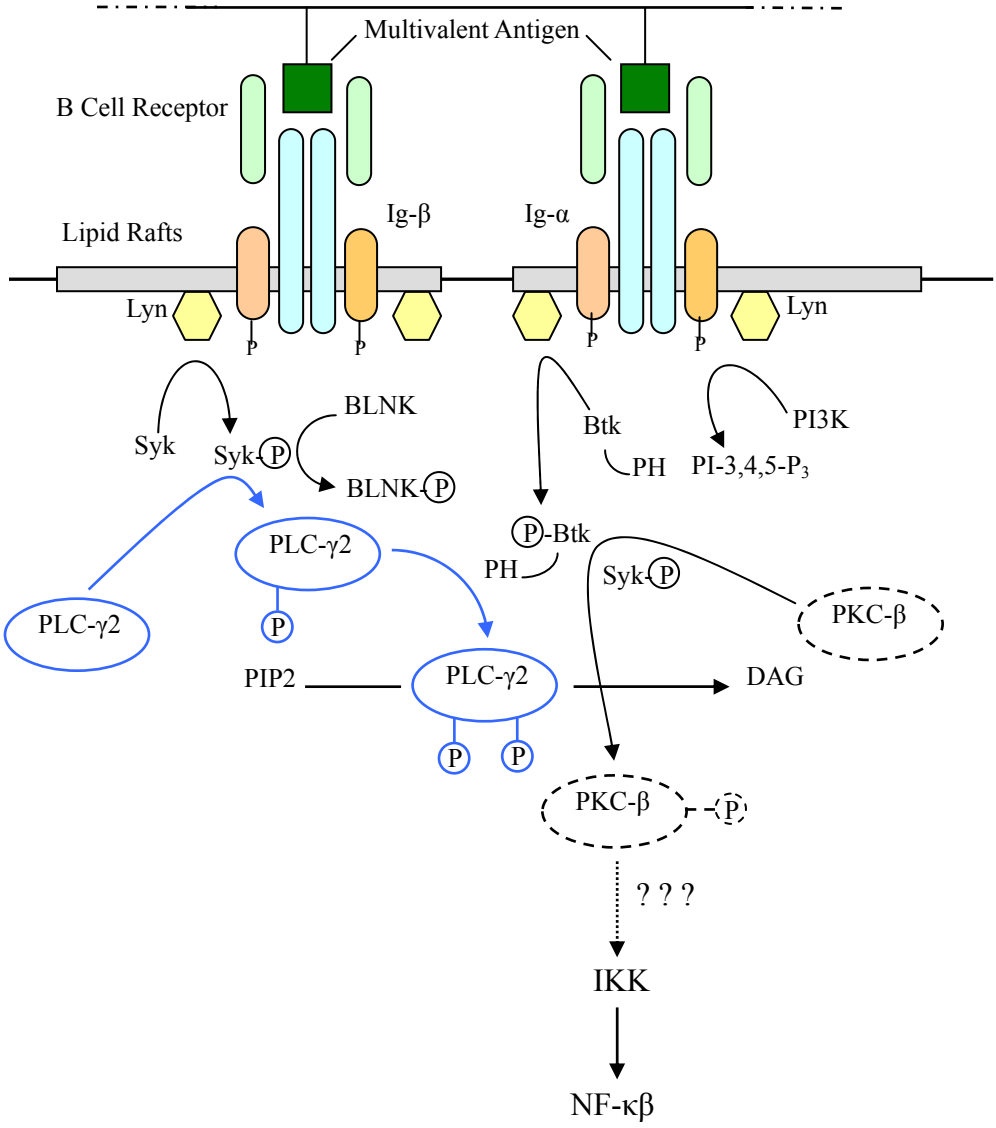


Figure 1.8 – Schematic illustration of BCR signaling to NF-κβ

In B cell, BCR is able to recognize multivalent antigen. Binding of ligands causes dimerization of BCRs in lipid rafts. Lyn kinases then auto-phosphorylate itself and serve a docking site for several signaling mediators such as Syk, Btk

and PI3K. After recruitment of these mediators, which are phosphorylated by Lyn kinase, protein adaptors BLNK and PLC-γ2 is activated by phosphorylated-Syk/ -Btk respectively. BCR signalosome associated with PKC-β and caused it to phosphorylate. Subsequently, activated PKC-β was translocated to the cytoplasm to activate the IKK family and the NF-κβ pathway finally.

1.5.2.3) T cell receptor (TCR)-mediated signaling

Formation of SMAC

As mentioned previously, T cell could be activated through interactions between TCR and class I/ II MHC (Konig, 2002). During cellular contact, multiple surface components on T cell will be temporarily and spatially reorganized. The area where receptor reorganization and cellular interaction occurs is termed the immunological synapse (Wilson *et al.*, 1997). In addition, a large active multi-components complex or supramolecular activation cluster (SMAC) is recruited to the immunologic synapse. SMAC associates with TCR to manifest signaling transduction. Molecules directly interact with TCR are classified as components of the central SMAC (cSMAC) while components localizing and encircling the cSMAC is termed peripheral SMAC (pSMAC) (Purtic *et al.*, 2005).

Recruitment of ZAP-70, SLP-76

In resting state, TCR-CD3 signaling complex is distal to the lipid raft (Simons *et al.*, 1997; Miceli *et al.*, 2001; Call *et al.*, 2004). Upon MHC binding, mobility of TCR-complex is enhanced and immunological synapse forms (Purtic *et al.*, 2005). In T cells, Src-family kinase Lck and Fyn are constitutive residents of the

lipid raft and they interacted with the TCR complex. In addition, early actions of kinase lead to the phosphorylation of substrate proteins. Phosphorylation of substrates contributes to the activation of their intrinsic enzyme activities and formation of signaling complexes (Schmitz *et al.*, 2003). Subsequently, ZAP-70 (zata-chain-associated protein 70), a homologous protein tyrosine kinase of the spleen tyrosine kinase (Syk) family, is inducibly transported to TCR complex after stimulation. It is suggested that ZAP-70 interacts with the ζ-chain of TCR complex and is the key step in TCR signaling. ZAP-70 influences downstream signaling by phosphorylating respective signaling linker proteins (Purtic *et al.*, 2005). These include SLP-76 (SH2-domain-containing leukocyte protein) and LAT (linker of activation of T cells). SLP-76 and LAT lack intrinsic enzymatic activities and are substrates of ZAP-70. Presence of SLP-76 and LAT are significant in recruitment of, and correct organization of signaling complex (Call *et al.*, 2004).

Recruitment of Vav-1& PLCγ1

Co-stimulation of CD28-binding induces accumulation of Vav-1 protein. In resting state, Vav-1 is suggested to be a lymphocyte-specific protein and constitutively associates with PKCθ (Dienz *et al.*, 2000). However, Piccollella (2003) provided contradictory results that showed Vav-1 associated with IKK. They showed that once TCR was stimulated, Vav-1 was released from its precursor before being recruited to the TCR complex or lipid raft. Subsequently, Vav-1 underwent rapid tyrosine phosphorylation. The phosphorylated SLP-76 interacted with the partner phosphorylated Vav-1 and formed a stable complex which eventually mediated downstream signaling. Hence, Vav-1 is considered as

a signal integrator for TCR-derived signaling. Furthermore, phosphorylated Vav-1 is responsible for recruitment of another adaptor protein, PLCγ1 (phospholipase Cγ1). PLCγ1 interacts with LAT in lipid raft before being phosphorylated (Wange, 2000). Experiments revealed that PLCγ1 converted substrate PIP2 (phosphatidylinositol 4'5-bisphosphate) into IP3 (linositol 1,4,5-triphosphate) and DAG (diacylglcerol). In most cases, metabolite IP3 regulates intracellular Ca^{2+} flux while DAG monitors the activity of PKC family members (Altman *et al.*, 2003).

PKCθ activation

PKCθ was discovered as another SMAC and has significance in TCR signaling transduction pathway (Dienz *et al.*, 2000). PKCθ belongs to the serine/ threonine protein kinase family. Interestingly, PKCθ is an alternative isoform or subfamily of housekeeping PKC family. Moreover, PKCθ is strictly and specifically expressed in T cell, thymocyte and muscular cell only. Different from other classical PKC family, PKCθ is Ca^{2+}-independent (Aman *et al.*, 2000).

On the other hand, since Vav-1 is suggested to be interacting with PKCθ while PLCγ1 metabolite DAG is able to regulate PKC family members (Altman *et al.*, 2003). However, presence of Vav-1 and PLCγ1 in synapse and recruitment of PKCθ to raft did not demonstrate important consistency. In addition, it is proposed that other pathways, such as PI3K (phosphatidylinositol 3-kinase), mediate PKCθ recruitment. Co-stimulation of CD28 on T cells triggers PI3K signaling pathway in which downstream signaling protein Akt is activated. Activated Akt in turn cooperates with PKCθ for proper translocation (Khoshnan

et al., 2000). However, up to date, no defined mechanism can be concluded as regard to PKCθ recruitment.

Regardless to upstream regulation, massive data had illustrated that PKCθ is inducibly translocated to cSMAC upon TCR activation. Besides, PKCθ is associated with Lck kinase. Although there is no direct relationship between Lck kinase and PKCθ phosphorylation, PKCθ activity is regulated by tyrosine residue (Tyr90) phosphorylation. Furthermore, studies had revealed that defects in either PKCθ expression or activity would result in NF-κβ inactivation (Khoshnan *et al.*, 2000). Hence, it suggested that PKCθ recruitment is required and indispensable for TCR-mediated NF-κβ activation. Furthermore, undoubtedly, activities on IKK are strictly relied on PKCθ kinase activity. However, what substrates (either directly or indirectly interact) or what consequence that makes the IKK becomes active is still in doubt. The following figure illustrated our current understanding of the TCR signaling pathway.

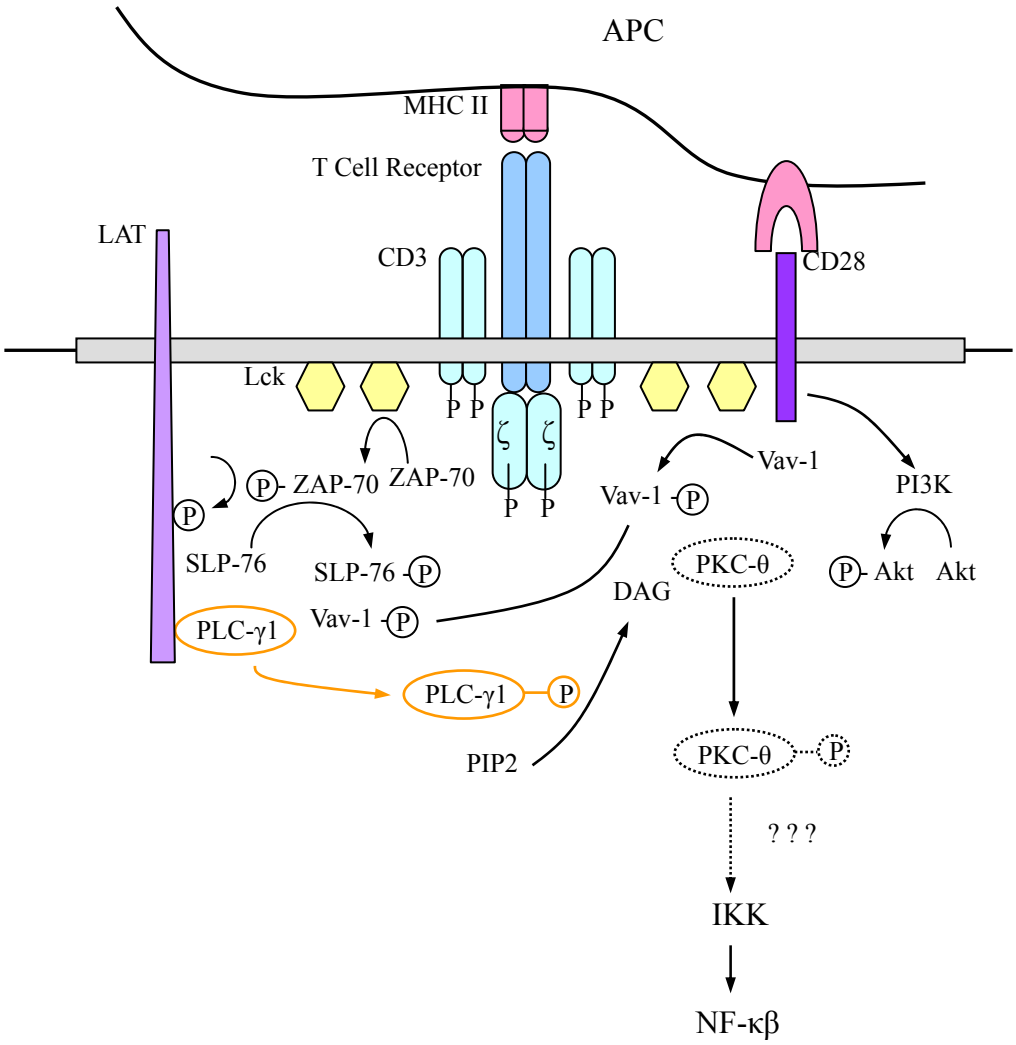


Figure 1.9 – Schematic illustration of TCR signaling to NF-κβ

MHC molecule is recognized by TCR before being translocated to the lipid raft. Once MHC interacts with TCR, conformational change of receptor brings Lck kinase to close proximity whereas CD3 subunits are first phosphorylated. Phosphorylated signaling CD3ζ subunits served a docking site for several adaptor proteins, including ZAP-70. ZAP-70 is phosphorylated by Lck kinase. Activated ZAP-70 in turn activates LAT, SLP-76 etc. In addition, Lck kinase phosphorylates signaling mediators such as Vav-1. After recruitment and

phosphorylation of PLC-γ1, PKC-θ is brought to the TCR signalosome complex whereas PKC-θ is phosphorylated. Subsequently, signal cascade is preceded from PKC-θ to IKK.

1.5.2.4) Downstream of PKC – Role of Bcl10, CARMA1 and MALT1

In BCR and TCR signaling, their primitive function is to initiate various immune-related genes activations (Weil *et al.*, 2004). Recent studies had illustrated that activation of either BCR or TCR are able to activate NF-κβ pathway. Previous studies showed that both the BCR and TCR signal transduction cascades their signal to the activation of PKCβ and PCKθ respectively (Dienz *et al.*, 2000; Guo *et al.*, 2004). As a consequence, activation of PKCβ and PCKθ activates IKK family that in turn regulates NF-κβ activities (Khoshnan *et al.*, 2000). However, there is a critical but missing link between PKC activation to IKK stimulation. It is not defined that what mediator(s) is involved in the signaling transduction from upstream active PKC to downstream inactive IKK. Recently, discovery of Bcl10 (B cell lymphoma 10), Carma1 (CARD–MAGUK1) and MALT1 (musoca-associated lymphoid tissue 1), provides some clues to connect PKC and IKK (Ruland *et al.*, 2001; Egawa *et al.*, 2003; Hara *et al.*, 2003; Jun *et al.*, 2003).

Bcl10 was identified as an anti-apoptotic protein and involved in lymphoma tumorigenesis. It carries a caspase recruitment domain (CARD) which is homologous to that of several apoptotic molecules (Willis *et al.*, 1999; Zhang *et al.*, 1999). Ruland *et al* was first to propose Bcl10 acts as key mediator-bridge on NF-κβ activation. Furthermore, these authors suggested that Bcl10 has a crucial

role in BCR-/ TCR-dependent NF-κβ activation (Ruland *et al.*, 2001). They also found that overexpression of Bcl10 will result in potent NF-κβ activation. However, the linker between Bcl10 and IKK has been poorly identified.

CARAM-1: Linker between PKC and Bcl10

Applying bioinformatic analysis, another family of CARD-containing membrane-associated guanylate kinase (MAGUK) had been discovered and characterized (Jun *et al.*, 2003). These molecules are suggested to be the signaling adaptor of Bcl10. Members of this novel CARMA (CARMA-MAGUK) family includes Carma1, Carma2 and Carma3. All these members are able to interact with Bcl10 via their N-terminal CARD motif (Hara *et al.*, 2003).

Nevertheless, among these Carma members, only Carma1 takes part in Bcl10-dependent NF-κβ pathway (Ruland *et al.*, 2001; Guo *et al.*, 2004). Several lines of experimental evidences support this. Firstly, Carma1 are predominantly expressed in lymphocytes while Carma2 and Carma3 are expressed in other tissues. In addition, although the expression and contribution of Carma1 has not been completely identified, both Bcl10 and Carma1 associate with TCR complex during signalosome recruitment. Thirdly, TCR-induced NF-κβ activation is blocked in Carma1-knockout mice. Expression of PKCθ does not activate NF-κβ in Carma1-deficient cells. Besides, overexpression of Carma1 induces constitutive Bcl10 phosphorylation and robust NF-κβ activation.

According previous bioinformatic studies, Carma1 was found to interact with Bcl10 via the CARD domain (Egawa *et al.*, 2003). Moreover, Carma1 not only

cooperates with Bcl10, but also recruit MALT cells in an unknown mechanism. Further investigation indicated that Carma1 is ubiquitously located at lipid rafts in resting T cells. Upon TCR stimulation and signalosome recruitment, Carma1 was associated with TCR complex prior to Bcl10 functioning (Shinohara *et al.*, 2005). Such observation suggested that Carma1 may play the role as scaffold protein which integrates the orientation of signalosome within BCR and TCR. Subsequently, Carma1 coordinates Bcl10 binding and activation. Furthermore, phosphorylation of Bcl10 is a Carma1-dependnt reaction. Therefore, all these findings elucidate that Carma1 is the downstream signal transducer of PKCθ, but upstream coordinator of Bcl10.

MALT1: Linker between Bcl10 and IKK – MALT1

In 1999, human paracaspase MALT1 (mucosa-associated lymphoid tissue 1) was characterized (Zhang *et al.*, 1999). Nevertheless, its connecting role between Bcl10 and IKK complex is demonstrated by several experiments (Willis *et al.*, 1999). For instance, yeast-two-hybrid system demonstrated that Bcl10-MALT-binding is required for synergistic activation of NF-κβ. In the meantime, several findings fulfill the requirements of another signaling model.

Since Bcl10 has no intrinsic catalytic activities and is necessary to recruit other factors for signal transduction. Bcl10 serves a CARD domain which can be recognized by Ig-like domain within MALT1 (Ruland *et al.*, 2001; Ruland *et al.*, 2003). Such interactions between Bcl10 and MALT1 are essential for Bcl10-dependent NF-κβ signaling (Egawa *et al.*, 2003). In addition, caspase-like domain of MALT1 shares proteolytic activities that are able to cleave unknown

substrates. These substrates or mediators then directly or indirectly interfere with the IKK complex. Experiments on mutation of conserved cysteine within caspase-like domain of MALT1 greatly reduced IKK activities and NF-κβ activation. Therefore, it is speculated that MALT1 is the downstream effector of Bcl10 (Schmitz *et al.*, 2003; Guo *et al.*, 2004).

1.5.3) JAK/ STAT cytokine signaling

As discussed before, several interleukin receptors transduce their signal by sharing a common pathway called JAK/ STAT pathway (Leonard *et al.*, 1998; Takeda *et al.*, 2000). JAK/ STAT signaling comprises of two components. These are upstream enzyme JAK kinase family and downstream STAT substrate. Incorporation between JAK and STAT allows rapid and accurate signal cascade. These signals in turn regulate immune responses.

1.5.3.1) Expression, structure and functions of Jak family protein

JAK (Janus kinase) belongs to the protein tyrosine kinase (PTK) family (Shuai *et al.*, 2003). JAK proteins was first identified in IFN signaling studies (Darnell *et al.*, 1994). Up to date, mammalian JAK family includes four members: Jak1, Jak2, Jak3 and Tyk2 respectively. Jak are relatively large kinase molecule with molecular sizes from 120 – 130 kDa. Among these member, Jak1, Jak2 and Tyk2 are constitutively expressed. In contrast, Jak3 are strictly expressed and regulated in specific tissue cell such as vascular smooth muscle cell and endothelial cell (Hebenstreit *et al.*, 2005).

Consistent with their conserved genomic organizations, sequence homology is also found in all Jak kinases (Rawlings *et al.*, 2004). For example, several tandem kinase-like Jak homology (JH) domains are found in the sequence. Moreover, total seven JH domains are structured in Jak kinase, designated as JH1 – JH7 (Ivashkiv, 2000; Shuai *et al.*, 2003; O'Shea *et al.*, 2004). Starting from the C-terminal, JH1 domain is identified as the catalytic kinase region and features with a protein tyrosine kinase (PTK) domain. Furthermore, tyrosine residue in the activation loop of PTK domain or JH1 domain plays a critical role in regulating catalytic activity. Phosphorylated tyrosine within activation loop greatly enhances accessibility of other substrates. Experiments also demonstrated that multiple auto-phosphorylations occurred in Jak proteins.

JH2 is usually classified as pseudo-kinase domain because it lacks of any kinase activities. Although they have most structural properties as kinase, some critical amino acid residues are missing or replaced; that make the domain malfunction. Hence, exact function of JH2 has not been determined. However, several studies indicated that JH2 somehow regulated the catalytic activity of JH1 domain. Furthermore, JH2 is postulated to serve as a docking site for other signaling molecules. On the other hand, JH6 and JH7 are located at the N-terminal and defined with their binding activities. Domain JH6 and JH7 are important in association with receptors subunits. Although the precise region of receptor that bind to Jak had been characterized, binding mechanism between the receptor and Jak proteins remains unclear. Specifically, for those domains that are located in the middle, such as JH 3 – 5, their functions remain obscure.

1.5.3.2) STAT molecule

In the JAK/ STAT signaling pathway, STAT (signal transducer and activator of transcription) family proteins are the substrates of Jak kinase proteins (Schindler, 1999; Rawlings *et al.*, 2004). Hence, downstream STAT substrates act as a threshold molecule that mediate signaling transduction from membrane into cytosol and then into nucleus.

Up to date, seven members are included in mammalian STAT family (Imada *et al.*, 2000). These are Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6. Their molecular sizes range from 75 kDa to 85 kDa. Takeda *et al* had reviewed the biology of STAT family and Ihle had further discussed about the role of each member in STAT family in details (Takeda *et al.*, 2000; Ihle, 2001). Similar to the Jak kinase proteins, Stat proteins are highly homologous in terms of amino acid sequence.

For example, Stat proteins have a conserved C-terminal tyrosine residue which is indispensable in dimer formation and signaling transduction (Schindler, 1999; O'Shea *et al.*, 2004). This tyrosine is the substrate of Jak kinase upon binding onto cytokines receptor. Following phosphorylation on tyrosine, Stat is dissociated from receptor and forms the dimmer. Dimer interaction is mediated by phosphorylated tyrosine and dimerization domain of another Stat. STAT dimerization domain interacts with phosphorylated tyrosine residue of another Stat molecule, forming a Stat dimer. Near to the tyrosine residue, a SH2 (SRC homology 2) domain can be identified. Although Stat proteins demonstrate homology in SH2 domain, different SH2 domain recognize phosphorylated motif

differentially. In addition, this SH2 domain is responsible for dimer formation. Within the polypeptide, there is a DNA-binding domain which is responsible for binding onto target DNA inside nucleus.

1.5.3.3) Mode of JAK/ STAT signaling pathway activation

In the past decade, JAK/ STAT signaling had been exploited (Liu *et al.*, 1998; Schindler, 1999; Imada *et al.*, 2000). Besides finding of JAK and STAT family members, it was also found that activities of STAT members are strictly regulated by distinct JAK kinase. Moreover, it is reminded that, different cytokine receptors are associated with different JAK members. Leonard *et al* had summarized the specificity among cytokine type, JAK kinase and STAT molecule (Leonard *et al.*, 1998). Table 1.4 below summarized his finding-

Cytokine	JAK kinase	STAT family
Type I cytokine		
Cytokine receptor with γ_C chain subunit		
IL-2	Jak1, Jak3	Stat3, Stat5a, Stat5b
IL-4	Jak1. Jak3	Stat6
Cytokine receptor with gp130 subunit		
IL-6	Jak1, Jak2, Tyk2	Stat3
Types II cytokine		
IFN-γ	Jak1, Jak2	Stat1
IL-10	Jak1, Jak2	Stat3

Table 1.4 – Specificity between cytokines, JAK kinase and STAT member

From the table, there were several points to note. Although Jak kinases are shared with various receptors, for example, Jak1 kinase is associated with all cytokine receptors. Specific Jak kinase is only recruited by its respective cytokines receptor, such as Jak3 against IL-2R or IL-4R, and Tyk2 against IL-6R etc. Similar cases occurred in terms of specificity between JAK kinases and its substrates STAT members. Further, Stat3 is able to share with type I and type II cytokine such as IL-6 and IL-10. In contrast, Stat6 are specific downstream molecule of IL-4R, or Stat1 is incorporated with IFN-γ receptor only. Therefore, specificity seems not to be an important mechanism in signaling transduction since the roles of Jak kinases and Stat members are redundant (Kile *et al.*, 2001; Leonard, 2001). On the other hand, this raises our attention in signaling transduction in that interaction between different and several JAK/ STAT pathways must be present. These interactions influence the resultant effect of JAK/ STAT activation. In addition, cross talk between JAK/ STAT and other signaling pathway must take place so that precise and responsive gene can be activated in nucleus.

On the other hand, JAK/ STAT signaling satisfy a couple of criteria which is necessary for correct signaling in respond to extracellular stimuli (Takeda *et al.*, 2000; Hebenstreit *et al.*, 2005). First, physically, Jak kinases are constitutively associated with receptor subunits and are able to trigger a rapid signaling event intracellularly. Secondly, JAK/ STAT is an essential signaling component which build up, at least, part of the signaling network that is required for correct and appropriate signaling against stimuli.

Cytokines are able to form in dimer or trimer that is functionally active during ligand-receptor binding (Burger *et al.*, 2002; Benczik *et al.*, 2004). Upon cytokine binding, cytokine receptor subunits associate as homo-dimer or hetero-dimer. Dimerization of receptor subunits is a pre-requisite for signaling initiation while Jak kinases consistently associate with the cytoplasmic tail of subunits. Because of proximity alternation, Jak kinases are brought to be activated. It is noted that Jak kinases activation is independent and that lack of any Jak kinase will not fail the signaling activation (Heinrich *et al.*, 1998). For instances, IL-6R signaling is composed of three Jak kinases (Jak1, Jak2 and Tyk2) that absence of any one Jak will not interfere with functions of the others.

Activated Jak kinases in turn phosphorylate appropriate and specific tyrosine residues of receptor subunits. These phosphorylated tyrosine sites serve as a docking site for binding proteins that carry phosphotyrosine binding domain. Stat proteins are one of the examples (Liu *et al.*, 1998; O'Shea *et al.*, 2002a). After recruitment of Stat molecules to its receptors, Stat molecules are phosphorylated at the conserved C-terminal tyrosine residue by Jak kinases. Activated Stat transducers are detached from receptor. Since phosphorylated tyrosine are able to associate with dimerization domain on another Stat protein. With the aid of itself SH2 domain, a couple of phosphorylated Stat molecules counteracts each other and form either homodimer (Stat1 and Stat1) or heterodimer (Stat1 and Stat3). Stat dimer is instructed by chaperone or cytosolic anchoring protein, and translocates into nucleus. Inside the nucleus, Stat dimer is able to bind DNA directly. Nevertheless, it usually incorporates with other accessory or transcription factors. Differential combination of transcriptome regulates

responsive gene(s) activation. Probably, cross-talk of JAK/ STAT signaling with other pathways occurs inside the nucleus (Alexander *et al.*, 2004). It is because, other signal transduction like NF-κβ pathway or MAPK pathway activates itself respective transcription factor (e.g. NF-κβ or c-Jun). These transcription molecules are able to interact with Stat dimers inside the nucleus (Heinrich *et al.*, 1998; Benczik *et al.*, 2004; Pestka *et al.*, 2004a). The following diagram (Figure 1.10) illustrates the activation pathway of JAK/ STAT and signaling transduction.

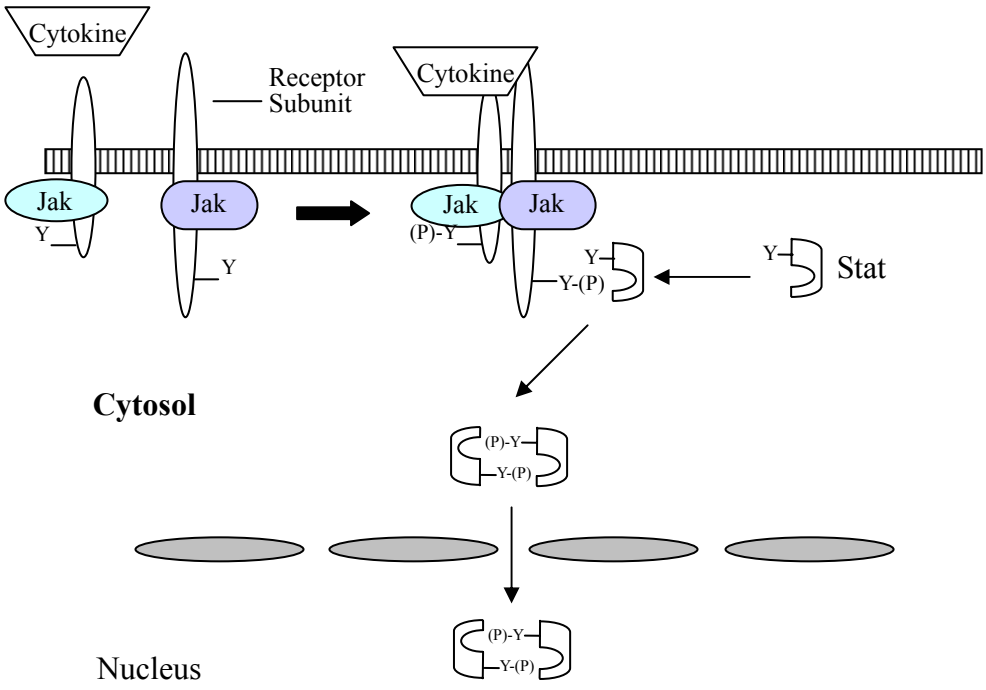


Figure 1.10 – Schematic illustration of the JAK/ STAT pathway activation

1.5.4) Toll-like receptor (TLR) signaling

Toll protein (Toll) is originated from flies *Drosophila* and has been shown to induce immune response in adult *Drosophila*. In 1997, Medzhitiv and colleagues had a breakthrough in finding the human homologue of *Drosophila* Toll protein (Medzhitov *et al.*, 1997). This homology also provides implications on their involvement in immunological functions in human. Afterwards, discovery of Toll and its receptor Too-like receptor (TLR) brought a new era in studies of innate immunology (Applequist *et al.*, 2002). Previous studies found that TLR is an important member of the PRRs family. Furthermore, this family plays a crucial role in early host defense against invading pathogens. Meanwhile, the TLR superfamily currently has 11 different TLR species in mammals (Takeda *et al.*, 2005). Due to its significance in innate and adaptive immunity, TLR is also the focus of many studies.

TLRs are transmembrane protein which is composed of an extracellular region and a cytoplasmic tail (Barton *et al.*, 2002; Akira *et al.*, 2004). The extracellular region is for ligand binding and the cytoplasmic tail is responsible for signaling transduction. Interestingly, cytoplasmic regions of TLRs and IL-1R are homologous in which a stretch of ~200 amino acids is conserved (Liew *et al.*, 2005). This highly homological motif is designated as TLR/IL-1 receptor domain (TIR) domain. Moreover, due to this structurally characteristics, binding of TLRs or IL-1R with respective ligands will result in similar signaling cascade (O'Neill, 2002). Despite similarity of cytoplasmic region between TLR and IL-1R is identified, their extracellular regions differ markedly. Extracellular region of TLRs contains high content of leucine or leucine-rich repeat (LRR), hence is

identified as LRR motif. The LRR domain is implicated in the recognition of various pathogens directly. Despite sequence homology among distinct TLRs LRR domains are identified, different TLRs are still able to recognize several structurally unrelated ligands (Takeda *et al.*, 2004). Such structural properties aim to attain high diversity of ligands binding. Instead of LRR domain, three Ig-like extracellular domains is identified in IL-1R.

Different TLR members share similar structure. However, it is noticed that not all TLRs are expressed on cell surface (Barton *et al.*, 2002). Some of them are retained in cytoplasm or at endoplasmic reticulum etc. Stimulation of different TLRs induces distinct patterns of genes expression. Particular member recognizes specific molecule which are conservatively present in bacteria or microorganisms. As for TLR4, Poltorak and co-workers (Poltorak *et al.*, 1998) had demonstrated that mutation in *Tlr4* gene resulted in defective respond to LPS. Hence, it illustrated that LPS is the ligand to the TLR4. On the other hand, neither TLRs nor IL-1R possess intrinsic kinase activity, they need to recruit other molecules or adaptor proteins for signaling transduction (Medzhitov *et al.*, 1998).

1.5.4.1) TLR signaling components

MyD88

Myeloid differentiation primary-response protein-88 (MyD88) is a universal adaptor protein for all TLRs (Takeuchi *et al.*, 2002). MyD88 consists of an N-terminal death domain (DD) and a C-terminal TIR domain. Hence, C-terminal domain of MyD88 associates with TIR domain of TLRs by TIR-TIR interaction.

Death domain of MyD88 recruits IRAK and interacts with DD domain of IRAK. This is followed by auto-phosphorylation of IRAK (Wesche *et al.*, 1997). In addition, Wesche *et al* had demonstrated that MyD88 react only with un-phosphorylated IRAK. They also suggested that MyD88 is the linker between TLR and signal transducer IRAK. Moreover, MyD88 knock-out mice did not produced inflammatory cytokine IL-6 in respond to LPS (Kawai *et al.*, 1999). This also inferred that MyD88 is an indispensable molecule in MyD88-dependent TLR4 signaling pathway.

IRAK

Interleukin-1 receptor-associated kinase (IRAK) is originally discovered as a signal transducer for the production of pro-inflammatory cytokine IL-1 (Croston *et al.*, 1995; Cao *et al.*, 1996). After the discovery of TLR, IRAK was further implicated as a signaling kinase involved in the activation of TLR pathway. Janssens had discussed the functional diversity and regulation of IRAK in detail (Janssens *et al.*, 2003). Four members were identified in human and they are IRAK1, IRAK2, IRAK4 and IRAK-M. At the moment, IRAKs are characterized as multidomain proteins which are composed of an N-terminal DD domain and a serine/ threonine-specific kinase domain. The N-terminal DD domain interacts with MyD88. Obviously, kinase domain is responsible for specific serine/ threonine residues phosphorylation. However, only IRAK1 and IRAK4 illustrate intrinsic kinase activities upon TLR stimulation. In addition, IRAK1 was shown to be the direct substrate of IRAK4, not vice versa. Similar to MyD88, IRAK4-deficient mice showed impairment in the response to TLR4 ligand such as LPS (Suzuki *et al.*, 2002).

TRAF

The tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family constitutes a group of adaptor proteins and a total of six members were found (Ye *et al.*, 2002). TRAF protein consists of two important domains. They are the coil-coil N-terminal domain and a conserved C-terminal or called TRAF-N and TRAF-C domain respectively. TRAF-N domain contains of a RING-/ Zinc-finger which is essential for TLR downstream signaling. On the other hand, the coil-coil-terminal domain mediates the association with upstream signaling adaptors such as IRAK.

Among the TRAF family members, TRAF6 has been shown to be involved in TLR signaling pathway (Medzhitov *et al.*, 1998). Experiments demonstrated that, upon TLR activation, TRAF6 was recruited to the TLR receptor complex and incorporated with IRAK molecule (O'Neill, 2002). Subsequently, TRAF-6 dissociated from IRAK/ TRAF6 complex. The dissociated TRAF6 interacted and form another complex with other downstream signaling molecules, such as TGF-β-activated kinase 1 (TAK-1) and TAK-binding proteins (TAB-1 and TAB-2). TRAF-6/ TAK-1/ TAB-1/-2 signaling complex migrated to the cytoplasm whereas TAK-1 is self-activated (Asehnoune *et al.*, 2005).

TAK-1

TAK-1 is a member of mitogen-activated protein kinase kinase kinase (MAPKKK) family. In addition, TAK-1 had been demonstrated to be essential in LPS-induced NF-κβ pathway (Takaesu *et al.*, 2003). TAB-1 expression and interaction with TAK-1 obviously enhanced TAK-1 kinase activities. Hence,

TAB-1 acts as regulator of TAK-1. In contrast, TAB-2 has no effect on TAK-1 kinase activities but it regulated molecular interaction instead. TAB-2 functioned as an adaptor which linked between TRAF-6 and TAK-1. Thereby, TAB-2 facilitates the orientation of TAB-1-mediated TAK-1 activation. Beside involves in NF-κβ activation, TAK-1 also activates MAPK pathway as well (Shinohara *et al.*, 2005).

1.5.4.2) Activation cascade of TLR pathway

In the circulation system, LPS-binding protein (LBP) is present in blood and act as a transporter of LPS (Triantafilou *et al.*, 2002). Binding of LPS to LBP forms a complex which is circulated to secondary lymphoid organ such as the spleen. LPS-LBP complex interacts with cell surface receptor CD14 of monocytes and macrophages (Jiang *et al.*, 2005). Once LPS binds onto CD14, it is transferred across the membrane to the transmembrane receptor TLR4. Moreover, it is highlighted that, LPS can still be directly recognized by TLR4 without LBP (Poltorak *et al.*, 1998). After stimulation of TLR4, TLR4 dimerizes and undergoes conformational change for recruitment of downstream signaling molecules or adaptor proteins.

Upon TLR4 dimerization, MyD88 adaptor protein is recruited and its C-terminal TIR domain interacted with TIR domain of TLR4. Next, signaling factor IRAK4 begin to bind to MyD88, followed by interaction betwen IRAK1 to IRAK4 (Takeuchi *et al.*, 2002). Once interaction between IRAK4 and IRAK1 occurs, IRAK1 is phosphorylated by IRAK4 instantly. Phosphorylation of IRAK1 induces its own intrinsic kinase activity that in turn auto-phosphorylate at its

N-terminal (Janssens *et al.*, 2003). After IRAK1 is phosphorylated, another linker protein TRAF6 is recruited and associated with activated IRAK1 (Ye *et al.*, 2002). Subsequently, IRAK1-TRAF6 complex is released from the receptor complex and interacted with plasma membrane. Pre-formed complex of TAK-1, TAB-1 and TAB-2 is attracted to the IRAK1-TRAF6 unit. Once the complex is formed, IRAK1 is dissociated from this signaling complex and remained in plasma membrane followed by degradation (Takaesu *et al.*, 2003).

On the other hand, signaling complex (consists of TRAF6, TAK-1, TAB-1 and TAB-2) is released and translocated to cytosol. Upon TRAF6 is ubiquitinylated, three signaling molecules (TAK-1, TAB-1 and TAB-2) are also phosphorylated. Besides, TAK-1 is further ubiquitinylated. Modified TAK-1 then regulated the activation of both IKK and MAPK pathways. TAB-1 and TAB-2 are responsible for the NF-κβ signaling (Li *et al.*, 2002; Denkers *et al.*, 2004; Asehnoune *et al.*, 2005). The TLR4 signaling pathway is outlined schematically in Figure 1.11.

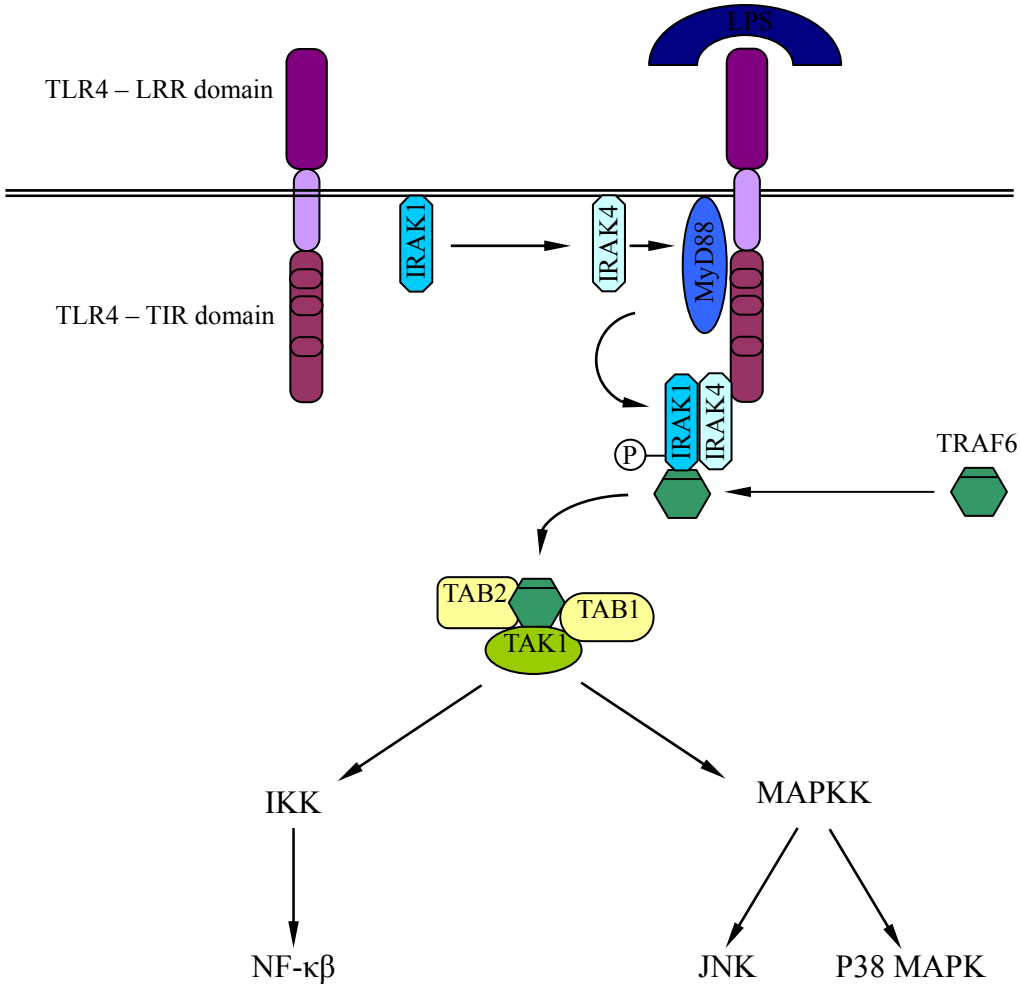


Figure 1.11 – TLR4 signaling pathway

1.5.5) Mitogen-activated protein (MAP) kinase (MAPK) pathway

TAK-1 activates not only the IKKs family members such as IKK-1 and IKK-2, but also MAPKK (Takaesu *et al.*, 2003). Basically, TAK-1 is one of the members of MAPKKK family. TAK-1 stimulates another kinase family called MAPKK. In turns, MAPKK is the upstream regulator of MAPK pathway. TAK-1 modifies and phosphorylates MKK3, MKK4 and MKK6 that lead to activation of p38 and

JNK MAPK pathway (Denkers *et al.*, 2004). During MAPK activation, transcription factor such as AP-1 and c-Jun will be activated for gene transcription and mRNA stabilization.

Beside NF-κβ signaling, MAPK is another crucial signal transduction pathway in cell. MAP kinase is involved in many aspects of immune responses, e.g. proinflammatory and inflammatory cytokines production, growth responses etc (Chang *et al.*, 2001). Different upstream signals, including cytokines, can lead to the activation of MAPK. However, LPS is found to be a potent activator of the MAPK pathways in lymphocytes (Guha *et al.*, 2001).

Three major groups of MAPK are classified as (i) extracellular signal-regulated (ERK) protein kinase, (ii) c-Jun NH2-terminal kinase (JNK) and (iii) p38 MAP kinase (Dong *et al.*, 2002). All three MAP kinase cascades are composed of a series of phosphorylation MAPK-regulating kinases. These kinases, according to its upstream level, include MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK). ERK MAP kinase is activated by its upstream regulator MAP kinase kinase-1 (MKK-1); p38 MAP kinase is phosphorylated by MKK-3, MKK-4 and MKK-6; JNK kinases are activated by MKK-4 and MKK-7. Further, these MKK are phosphorylated and activated by several different upstream kinases MAPKKK, such as TAK-1 (Chang *et al.*, 2001). It is noted that inducer such as inflammatory cytokine, e.g. TNF-α can activate that MAP kinase signal pathway. Wysk *et al* reported that p38 activation was inhibited by its upstream kinase *MKK3*$^{-/-}$ mutant in respond to TNF-α, but not for IL-1 (Wysk *et al.*, 1999). However, Allen and colleagues demonstrated

that IL-1-induced IL-6 production was greatly affected in fibroblasts *p38*$^{-/-}$ mutant (Allen *et al.*, 2000). Although it is clear that MKK is responsible for the activation of p38 signal transduction, exact interactions between MKK and p38 MAP is no yet defined.

On the other hand, JNK activities are also strongly induced by TNF-α and IL-1. Dong *et al* had reviewed that activation of JNK depends on its regulatory kinase MKK-4 and MKK-7 (Dong *et al.*, 2002). However, Tournier *et al* examined either MKK4- or MKK-7-deficient or MKK-4/ MKK-7-deificient fibroblasts, and found that MKK-7 was solely required for JNK activation in respond to inflammatory cytokines (Tournier *et al.*, 2001). Therefore, activation of JNK MAP kinase remains a debatable aspect. On the other hand, it is suggested that functions of MKKs may be pleiotropic or redundant, or both.

As discussed above, inflammatory cytokines triggered MAPK responses. Hence, it is clear that there is a close relationship between inflammation and MAPK pathway activation (Schieven, 2005). In order to restrict excessive inflammation and autoimmunity, MAPK is also important in activating cell-death mechanism. It can be achieved by lowering number of activated lymphocytes in bodies. Studies on MAPK revealed that p38 kinase regulates population of T-cells subsets. It was found that activation of p38 MAP kinase induces apoptosis in $CD8^+$ T-cells, but not $CD4^+$ T-cells. In addition, JNK played a role in thymocyte negative selection and apoptosis of T-cells.

1.6) Spleen – Studies and importance of primary spleen culture

Spleen is the largest secondary lymphoid organ that combines the innate and adaptive immune system in a unique structure (Dailey, 2002). Due to its complex and well-organized lymphoid compartment, spleen is the key organ in removal of blood-borne microorganisms. For instance, spleen provides distinct compartments that house specialized white cells types. Moreover, these compartments allow cell-cell interactions that are essential for the initiation of immune response (Mebius *et al.*, 2005). In this study, white cells were collected from spleen for various types of experiments. Therefore, a brief discussion is devoted to the importance of primary spleen culture for screening purposes (Brown, 1992; Van *et al.*, 1992). It is aimed to illustrate that primary spleen cultures cells are better than transformed cell lines in reflecting the "true" conditions *in vivo*.

According to its functional aspect, spleen consists of enormous and diverse immune cell types. In addition, spleen cells are always used for studies in immunological interactions and functions. Previously, researchers had demonstrated that immune response could be monitored by several aspects. For instances, (1) immunoglobulin production by B cells (Takasugi *et al.*, 2001); (2) oxidative stress or NO production by macrophage (Lee *et al.*, 2005); (3) cytokines secretion (Eyles *et al.*, 2003; Yu *et al.*, 2004); (4) cell-cell contact/ interactions (Nakaya *et al.*, 2003); and the most central and critical parameter (5) the presence of immune cell types (Wu-Hsieh *et al.*, 1998; Hwang *et al.*, 2004), all five parameters were able to regulate immune responses.

In order to understand how immune responses, it is not surprising that cultivation of immune cells is necessary. Primary spleen cells and immune cell lines are alternatives. When compared to primary cells cultures, immune cell lines (e.g. B cell hybridoma, CTLL-2 or RAW 264.7 etc) conferred predominant flexibility in cell preparation and the cultivation process. Hsieh and Plavec had highlighted a few pros and cons of using cell lines (Hsieh *et al.*, 2002; Plavec *et al.*, 2004). Firstly, cell lines have been transformed that most of tumor cells carry unidentified defects with uncertain pathway consequence. For example, cell cycle control or checkpoints regulation must be defective in transformed cell lines. Secondly, mixture or concurrent culture of several cell lines violates the systematic circumstance definitely. Such factors precondition the cell lines to be either unresponsiveness or hyper-responsiveness against stimulus, and interfere with the results as artifact consequence.

In contrast, primary spleen cells compose of a variety of immune cells that none of each are dispensable in the immunity (Winfield, 1971; Eloranta *et al.*, 1999; Lopes-Carvalho *et al.*, 2005). It is stressed that complete network of the immune cells circuits is required for proper functions of the immune system. Otherwise, sensitivity and response could be diminished or even inhibited. In addition, primary spleen culture mimics the *in vivo* condition inside the body. Thirdly, lymphocytes in primary culture are naive and quiescent. This is pre-requisite prior to characterization of each stage of immune response upon stimulus. Fourthly, because of the involvement of distinct immunological components from cytokines to cell, analyze a small set of biologically parameters are more informative instead of measuring target expression of single cells. Moreover,

expression profiles of these parameters provide a chance to examine the direct effects of stimulus on single target. Further, it supports indirect evidences on interaction among those immuno-competence cells, allowing further biological and functional characterization of the system context.

Taken overall, whole spleen cells culture bears many advantages by providing an entire framework of the immune responses. It, however, involves different cell types and components that may in turn complicate the interpretation of the experimental results. Hence, verification and standardization of spleen cells culture, for example presence of diversity of cell types (B cells, T cells and macrophages) and their relevant populations etc, are necessary.

1.7) Ginseng – A Traditional Chinese Medicine (TCM)

In the past thousands of years, herbal medicine is the most popular medicine in the Oriental culture. Practice of the use of herbal medicine had collectively been referred to as Traditional Chinese Medicines (TCMs) (Wilasrusmee *et al.*, 2002). Today, TCM were applied for a wide variety of disease treatments in Eastern countries such as China, Korea and Japan etc. However, practice of TCMs is considered unreliable and inefficient in the Western countries. It is because most TCMs lack experimental proofs, detailed clinical records, formulation of application dosages and functional mechanisms etc (Wang *et al.*, 2003). In the past few decades, some researchers had recognized the enormous potentials of TCMs. In addition, they had proceeded to study the functions and mechanisms of herbal medicines. As more and more results were accumulated, this will bring about modernization of the TCM.

1.7.1) Ginseng and its components

Ginseng is the most commonly used herbal medicines among several hundreds of TCMs (Attele *et al.*, 1999). Hence, it is called "The King of Herbs". White *Panax Ginseng* C.A. Meyer *(Panax Araliacea)* and American red ginseng (*Panax quinquefolium*) are the two major categories of ginseng (Wang *et al.*, 2003). Siberian ginseng, (*eleutherococcus senticocus*) is another ginseng species which represents a cultivated form of ginseng. Root section of ginseng, instead of the whole plant, is routinely applied for its medicinal functions (Figure 1.12).

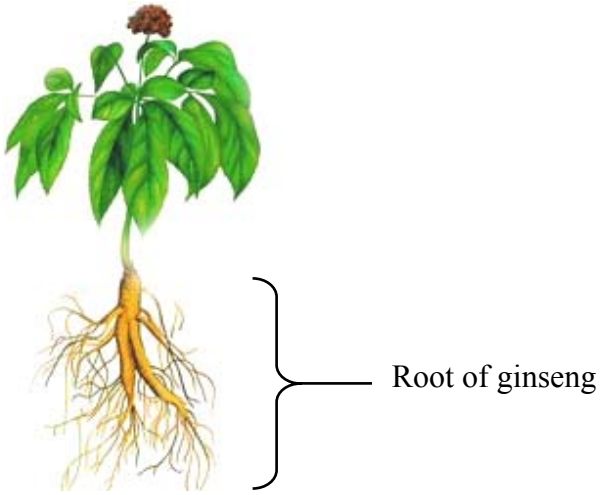


Figure 1.12 – Ginseng and its root section

1.7.2) Identification of ginsenosides

In the early 1960s, chemists began to isolate and characterize the components of ginseng. Fujita and Shibata were the pioneers in investigating the chemical characteristics of *Panax Ginseng* (Fujita *et al.*, 1962; Shibata *et al.*, 1963a; Shibata *et al.*, 1963b; Shibata *et al.*, 1965; Shibata *et al.*, 1966b). In these studies, the authors proposed the presence of glucose derivatives in root of ginseng and that these glucose derivatives represented the functions of ginseng. At around the same time, chemical structures of those acid hydrolysis products of glucose

derivatives had been characterized by Shibata, and termed as panaxadiol and panaxatriol (Shibata *et al.*, 1965). With the aid of thin-layer chromatography (TLC), different species of panaxadiol and panaxatriol were then further isolated and identified as ginseng sapogenin glycosides and were first collectively termed as ginsenosides (Shibata *et al.*, 1966a).

Because of the success in isolation of ginsenosides by TLC and silica gel chromatography, individual ginsenoside was purified to homogeneity and its chemical structure and properties was identified and studied. Sanada and Shibata had summarized all chemical findings of ginsenosides studies (Sanada *et al.*, 1978). According to these authors, ginsenosides were chemically classified into three categories: (1) oleanolic oligoglycoside acid, (2) 20(S)-protopanaxadiol and (3) 20(S)-protopanaxa-triol. Furthermore, species of ginsenosides at various part of ginseng (other than root) such as leaf, seed and juice, were identified and analyzed by high pressure liquid chromatography (HPLC) (Yip *et al.*, 1985a; Yip *et al.*, 1985b; Kasai *et al.*, 1987). Shibata claimed that more than 25 ginsenosides had been identified according to position of the glycosides moiety (Shibata, 2001).

1.7.3) Structure of ginsenosides

From Shibata's studies, core structures of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol were illustrated in the following figures respectively (Figure 1.13 & 1.15). Different ginsenoside species was designated by the position of its respective glycosides residues. Table 1.5 has summarized the structural properties of several representative ginsenosides.

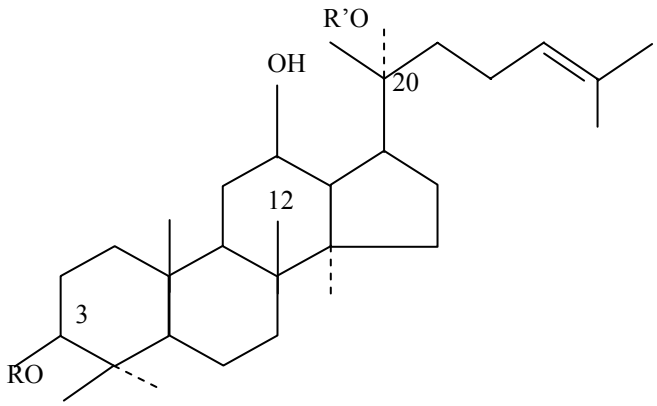


Figure 1.13 – Core structure of 20(S)-protopanaxadiol

20(S)-protopanaxadiol

		R	R'
Ginsenoside	Rb-1	-Glc^{2}-Glc	-Glc^{6}-Glc
	Rb-2	-Glc^{2}-Glc	-Glc^{6}-Ara (pyranosyl)
	Rc	-Glc^{2}-Glc	-Glc^{6}-Ara (furanpsyl)
	Rg-3	-Glc^{2}-Glc	-H
	Rh	-Glc	-H

Table 1.5 – Glycoside position of 20(S)-protopanaxadiol and identification of ginsenosides species [-Glc (Glucopyranoside)]

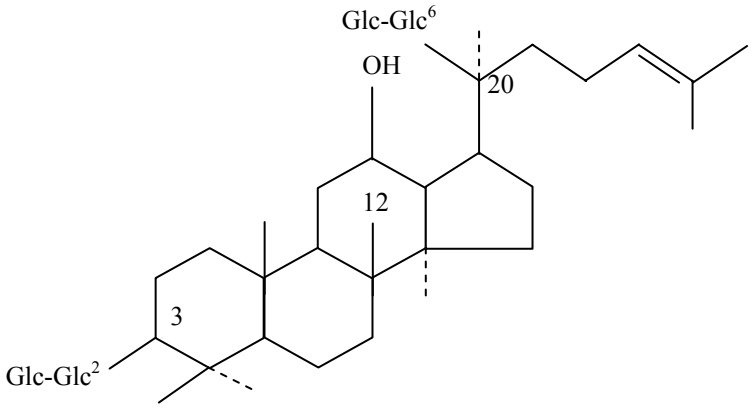


Figure 1.14 – Structure of ginsenosides Rb-1

20(S)-protopanaxatriol

		R	R’
Ginsenoside	Re	-Rha	-Glc
	Rf	-Glc	-H
	Rg-1	-H	-Glc
	Rg-2	-Rha	-H
	Rh-1	-H	-H

Table 1.6 – Glycoside position of 20(S)-protopanaxatriol and identification of ginsenosides species [-Glc (Glucopyranoside)]

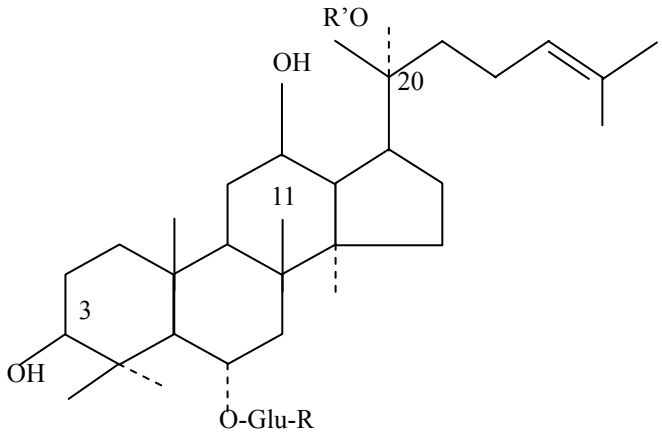


Figure 1.15 – Core structure of 20(S)-protopanaxatriol

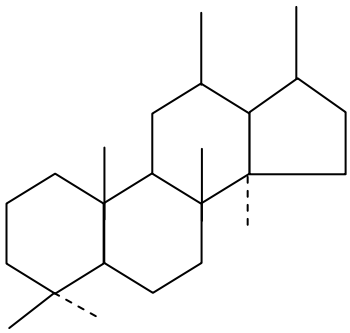


Figure 1.16 – Structure of dammarane-type tetracyclic triterpene

It is highlighted that the fundamental skeleton of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol demonstrated a structural similarity. That is, they both carry a dammarane-type tetracyclic triterpene which is presented as follows (Figure 1.16). In addition, chemists found that the steroid class hormone – glucocorticoid (GC) have the same core structure as 20(S)-protopanaxadiol and 20(S)-protopanaxatriol, showing in Figure 1.17:

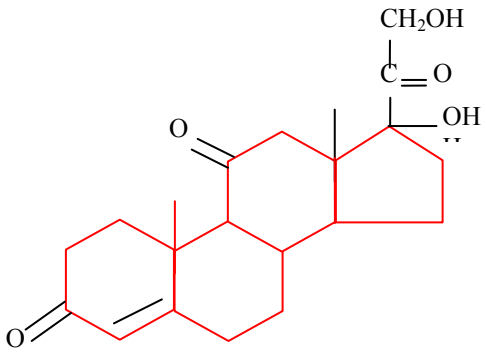


Figure 1.17 – Structure of glucocorticoid (Red-color highlighted the common structure common skeleton among 20(S)-protopanaxadiol, 20(S)-protopanaxatriol and glucocorticoid).

1.7.4) Ginsenosides content

By methanol extraction, aqueous ginsenoside sample was subjected to column chromatography (Sanada *et al.*, 1978). Ginsenosides fraction could be obtained after removal of un-wanted saccharides and amino acid, followed by washing with water. Ginseng fractions were further purified by either TLC or HPLC. In order to get a higher homogeneity of ginsenoside, silica gel column was used to perform a better separation of these ginsenosides (Kasai *et al.*, 1987). Shibata had summarized and compared the ginsenoside content from various ginseng

species among White ginseng, American red ginseng and Siberian ginseng (Shibata, 2001). Ginsenoside Rg-1 and Rb-1 was found to be the most abundant ginsenoside in 20(S)-protopanaxatriol and 20(S)-protopanaxadiol category respectively (Table 1.7).

		White Ginseng	Red Ginseng	Siberian Ginseng
20(S)-protopanaxatriol	Re	0.2	0.2	1.0
Ginsenoside	Rf	0.05	0.07	/
	Rg-1	0.2	0.3	1.9
	Rg-2	0.014	0.01	0.008
	Rh-1	0.0015	0.007	/
20(S)-protopanaxadiol	Rb-1	0.5	0.4	1.8
Ginsenoside	Rb-2	0.2	0.2	0.03
	Rc	0.3	0.1	0.3
	Rd	0.3	0.036	0.5
	Rg-3	0.0003	0.014	/
Oleanolic acid	Ro	0.02	0.045	0.07

Table 1.7 – Average yields (%) of ginsenosides from different ginsengs species

1.7.5) Pharmacokinetic studies of ginsenoside

Due to its abundance, Rg-1 and Rb-1 had been considered as the principal components that manifested pharmacological effects of ginseng. There are increasing numbers of reports regarding the pharmacological studies of ginseng. Takino's and co-workers spent ten years and conducted a detail study in the absorption, distribution, excretion and metabolism of ginsenoside Rb-1 and Rg-1 (Takino *et al.*, 1982; Odani *et al.*, 1983a, 1983b, 1983c; Karikura *et al.*, 1990;

Karikura *et al.*, 1991b, 1991a; Karikura *et al.*, 1992). By using reverse-phase TLC dual-wavelength system, recovery level and amount of different ginsenosides in biological samples (serum, urine), tissues (liver, kidney, brain etc) and digestive tract (stomach, large intestine) were quantified. In summary, Takino's experiments demonstrated that the metabolic performance of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol did not show any significant differences. Firstly, both Rg-1 and Rb-1 could be quantified by TLC from biological samples, e.g. serum and urine, and more than 90 % of ginsenosides could be recovered. Secondly, these authors found that ginsenosides level in serum (~20 %) was lower than that found in urine (~80 %), deducing the absorption efficiency of ginsenosides in digestive tract was extremely low. However, the retention time of Rb-1 was found to be longer than Rg-1. It was proposed that diol-containing molecule had a higher plasma protein affinity than triol molecule. Moreover, ginsenosides was not detected in the tissue such as liver, lung, heart and especially the brain. On the other hand, these authors had assayed the digestive tract samples and found that ginsenosides Rb-1 and Rg-1 was decomposed in stomach and intestine. In the stomach, they found that Rb-1 was hydrolyzed into several unidentified products while 6 chemical species were found from the decomposition of Rg-1. In the case of the intestines, Rb-1 was decomposed into ginsenoside Rd and two unidentified compounds. For Rg-1, ginsenoside Rd and ginsenoside F2 was found as the catabolic products. Later on, Kakikura *et al* found that compound K was one of the products of Rb-1 degradation (Karikura *et al.*, 1992).

Moreover, traditional techniques such as TLC and HPLC were applied in Takino's studies and in analyzing ginsenosides levels (Takino *et al.*, 1982). However, newly developed methods were utilized for a rapid and accurate detection of ginsenosides. Among them, mass spectrometry (MS) is the most successful example. In 1993, combination of gas chromatography (GC) with MS was first reported to determine the ginsenoside content in ginseng samples (Cui *et al.*, 1993). Moreover, Ji *et al* demonstrated the feasibility and compatibility of MS in ginsenosides determination from human plasma sample (Ji *et al.*, 2004). At around the same time, Luchtefeld's group published their results in the isolation and determination of ginsenosides using negative electrospray ion liquid chromatography – mass spectrometry (ESI-LC-MS) (Luchtefeld *et al.*, 2004).

1.7.6) Functions of ginseng

Ginseng has been used as a tonic supplement in the Oriental culture for thousands of years. Starting from 1950, Butturlin had studied the clinical applications of whole ginseng extract (whole root extract) (Buturlin, 1950). In the past half century, increasing evidences and experimental proofs illustrated the pharmaceutical effects of ginseng or ginseng extract. In immunology, especially, researchers provided numerous data showing that whole ginseng extract exerted a certain level of immuno-modulatory effect on immune system.

Jie and co-workers (Jie *et al.*, 1984) prepared a ginseng extract by water boiling extraction while the extract was analyzed by HPLC. The results indicated that Rb-1 and Rg-1 was the most abundant ginsenoside in extract. Such data was

comparable with that of Shibata and Tanaka (Shibata *et al.*, 1966b; Tanaka *et al.*, 1966). Furthermore, ginseng extract was administrated into mouse orally and immunologic tests were performed. Experimental data illustrated that ginseng extract could increase both immunoglobulin G (IgG) and immunoglobulin M (IgM) production, together with secretion of interferon (Mita *et al.*, 1979; Jie *et al.*, 1984). However, activity of LPS-induced splenocytes proliferation was suppressed significantly (Smolinski *et al.*, 2003).

Later, Kim *et al* and Liou *et al* had conducted similar experiments with intraperitoneal injections and similar results were obtained (Kim *et al.*, 1990; Liou *et al.*, 2004). For instances, NK cells activity were not only stimulated but also resumed after cyclophosphamide suppression. Kim and co-workers had postulated that ginseng has some immunomodulatory properties in vivo, which is primarily associated with NK cell activities. Further, serum levels of cytokines (IL-2, IL-4, IL-10 and IFN-γ) were found to be elevated after ginseng injection for three days (Liou *et al.*, 2004). Respective cytokines mRNA expressions were also analyzed and mRNA levels were found to be increased.

Several groups (Song *et al.*, 2002; Wang *et al.*, 2003; Wang *et al.*, 2004) also provided evidences which illustrated that whole ginseng extract was capable in induction or enhancement of concanvalin-A induced cytokines production in macrophages and spleen cells. However, contradictory results were reported by Smonlinski and Rhule (Smolinski *et al.*, 2003; Rhule *et al.*, 2006). These authors demonstrated that neither ginsenosides nor ginseng extract induced cytokines secretions, but they significantly suppressed the LPS-induced pro-inflammatory

cytokines production. In addition, experiments indicated that varies components of ginseng extract would bring diverse effects. For example, whole extract of ginseng could suppress LPS-induced tumor necrosis factor-alpha (TNF-α) and interlekin-6 (IL-6) production significantly. However, Rb-1 alone partially inhibited or had no inhibitory effect on secretion of TNF-α or IL-6 respectively. Hence, these authors speculated that individual ginsenoside alone or in combination would exert diverse effects in immune system.

Beside immunomodulatory activities of ginseng, ginseng extract was found to have a wide variety of pharmaceutical activities. Shibata had summarized that functions of ginseng or ginsenosides were diverse and its activities were specific to different cells or tissues (Shibata, 2001) (Table 1.8)

	Ginseng Content/ Ginsenosides
Anti-platelet aggregation	R-0, Rg-1, Rg-2
Fibrinolytic action	R-0, Rb-1, Rb-3, Rc, Re, Rg-1, Rg-2
Stimulation of pahgocytic action	R-0, Rb-1, Rb-2, Rc, Re, Rg-2, Rg-3, Rh-1, Rh-2
Vasodilation action	Rb-1, Rd, Rg-1
Stimulation of ACTH, corticosterone secretion	Rb-1, Rb-2, Rc, Re
Stimulation of RNA polymerase, protein synthesis	Rb-1, Rc, Rg-1
Protection against ulcer	R2, Rb-1

Table 1.8 – Pharmacological actions of ginseng content/ ginsenosides

1.7.6.1) Steroid-like functions of ginseng extract

Upon ginsenoside isolation and structural characterization in the 1960s, ginseng ingredients were found to have high structural homology with that of steroid class hormones (as described before in Section 1.6.3). Hence, researchers proposed that the whole ginseng extract could carry steroid-like activities. Hiai had studied the relationship between injection of whole ginseng extract and corticosterone secretion (a product after activation of corticoid receptor in brain) (Hiai *et al.*, 1979). In that study, no measurable corticosterone was secreted into blood. However, Pearce and co-workers had demonstrated that entire ginseng fraction bound to steroid receptors, particularly showing high affinity binding to the glucocorticoid (GC) receptor (GCR) (Pearce *et al.*, 1982).

These authors postulated that ginseng extract exert GC-like effects by direct ligand binding of ginsenosides onto receptors. With the tremendous development in isolation and purification techniques of ginseng during 1970s, physical and chemical properties of ginsenoside species were characterized specifically. Moreover, Rg-1 and Rb-1 had been identified as the most abundant ginsenoside constitutes and were considered being the active and functional ingredients in ginseng. Therefore, steroid-like activities and functions of ginsenosides Rg-1 and Rb-1 were being of great interests. Besides, other pharmacological functions of ginsenosides Rg-1 and Rb-1 had been exploited specifically.

1.7.6.2) Estrogen-like activities of Rb-1

In the case of Rb-1, Lee and colleagues revealed the estrogenic activities of ginsenoside Rb-1 (Lee *et al.*, 2003). By luciferase reported plasmid, Rb-1

showed a weak ligand binding activity on estrogen response element (ERE), producing a very low signal. Rb-1 was assayed for its binding affinity on GCR and AR, but Rb-1 did not elicit any receptor binding activities on neither GCR nor AR. Although Rb-1 shared structural similarity with estrogen, no positive evidence was reported on its steroid-like functions. However, Cho and co-workers reported detailed but contradictory results against reports from Lee and co-workers (2003). Cho's and co-workers reported that Rb-1 activated estrogen receptor-α/ -β (ER-α/ -β) in a ligand binding-independent manner (Cho *et al.*, 2004). Their data demonstrated that Rb-1 could significantly activate both ER-α and ER-β, resulting in the trans-activation of estrogen-responsive gene. In addition, western blot studies revealed the activation of ER-responsive genes by Rb-1 through the activation of transcription factor-1 (AP-1) in an estrogen receptor element (ERE)-dependent manner. Moreover, activation of ER by Rb-1 was inhibited by ER antagonist ICI. Furthermore, it was found that Rb-1 failed to compete with estrogen binding to ER from ER competency assay. Hence, these authors suggested that Rb-1 activates ER via a mechanism different from the classical, hormone-mediated activation.

1.7.6.3) Proliferation and differentiation activities

T-cells are involved in the cell-mediated immune system, especially in host inflammation. Upon activation and proliferation of T cells, various signals are produced which regulate other immune cells. Hence, T cells population definitely affects the extent of immune responses. Cho and co-workers reported that Rb-1 stimulated splenic T cells proliferation, and did not inhibit IL-2-induced growth of CTLL-2 cell line (Cho *et al.*, 2002). In the same studies, nevertheless, the

authors demonstrated that other ginsenosides (e.g. Rh-2) exerted an inhibitory effect on IL-2-induced growth of CTLL-2 cell. Hence, the authors suggested that different ginsenosides modulated mitogen-induced lymphocytes proliferation selectively and differentially.

1.7.6.4) Cytokines production

Cytokines are secreted by immune cells in order to communicate with other immune cells and to regulate their activities. Types and amounts of secreted cytokines directly control the immune system responses. In addition, inflammatory mediators such as TNF-α and IL-6 play an important and indispensable role in acute or chronic inflammation responses. Smolinski reported that Rb-1 could significantly inhibit the lipopolysaccharides (LPS)-induced TNF-α and IL-6 production *in vivo* and *in vitro* (macrophage cell line RAW 264.7) (Smolinski *et al.*, 2003). In addition, their data indicated that the inhibitory effect was Rb-1 dose-dependent. For instances, at dosage of 10 μg/ml Rb-1, secretion of both TNF-α and IL-6 was suppressed to 50 % of original level while cytokines productions were totally suppressed with the highest dosage of Rb-1 used which was at 100 μg/ml.

1.7.6.5) Oxidative stress

Past studies pointed out that oxidative stress, mainly nitric oxide (NO), was involved in lymphocyte functions (Zhou *et al.*, 2000; Gordon *et al.*, 2001). Nitric oxide (NO) not only serves as a facilitator to the immune system promoting survival of effector lymphocytes, it also inhibits abnormal lymphocytes proliferation which balances the lymphocytes population. Song and colleagues

reported that ginseng extract consist of 70% of ginsenoside Rb-1 could induce peritoneal macrophages NO production (Song *et al.*, 2002). With verification using RT-PCR, expression level of inducible nitric oxide synthase (iNOS) was up-regulated by ten-folds. This result directly accounted for the increment of NO production in cell culture supernatant.

However, Park provided opposite results and reported that Rb-1 inhibited the LPS-induced iNOS expression (Park *et al.*, 2005). In their studies, Kakikura and colleagues reported that iNOS expression was greatly suppressed with the presence of compound K, a metabolite of Rb-1 (Karikura *et al.*, 1991b). Moreover, these authors were the first group to hypothesize that inhibitory mechanism of Rb-1 or compound K was manipulated by regulation of nuclear factor-κβ (NF-κβ) pathway. Western blot results further showed that the expression of NF-κβ was suppressed by Rb-1 in a dose-dependent behavior.

1.7.6.6) Transcription factor inactivation

Nuclear factor-κβ (NF-κβ) pathway is crucial in the control of the host immune and inflammatory response. Furthermore, NF-κβ activities regulate the expression of a wide variety of immune-responsive genes, including inflammatory cytokines. Constitutive activation of NF-κβ pathway not only is responsible for basis cellular functions, but also associated with acute inflammation response. Oh and colleagues correlated the immune response, iNOS expression and NF-κβ pathway with experimental proof (Oh *et al.*, 2004). First of all, their data showed that ginsenoside metabolites suppressed both iNOS expression and nitrite production from LPS-stimulated macrophages. Secondly,

expression level of iNOS was decreased against increasing dosages of ginsenoside. Thirdly, electrophoretic mobility shift assay (EMSA) results supported that the translocation of NF-κβ in nuclear extract was relieved with the presence of ginsenoside metabolite. Lastly, western blot results revealed that ginsenoside metabolite inhibited phosphorylation of inhibitor of NF-κβ (I-κβ) which regulated both NF-κβ activities and iNOS expression. Again, these authors speculated that many ginsenosides interacted with plasma membrane and possessed a certain level of steroid-like activities. This hypothesis was comparable and similar to what Cho had proposed (Cho *et al.*, 2004). However, further mechanistic studies are necessary for conformation of the hypothesis.

1.7.6.7) Diverse functions of ginsenosides Rb-1

Many studies and report found that Rb-1 was the most abundant ginsenosides in ginseng extract (Table 1.3). Hence, Rb-1 was usually considered as the active and functional ingredient of ginseng. The previous section had discussed about the estrogen-like activity of Rb-1, however, little investigation had been reported. On the other hand, studies of Rb-1 demonstrated diverse activities of Rb-1 on various cell-types and tissues that were summarized in the following Table 1.10.

Activities	Author
Enhancement of antibody production	(Mita *et al.*, 1979)
Stimulation of RNA metobilsm and RNA polymerase	(Iijima *et al.*, 1979)
Induction of central cholinergic metabolism	(Benishin *et al.*, 1991; Benishin, 1992)
Regulation of calcium flux	(Liu *et al.*, 1995)
Induction of lipoprotein lipase secretion	(Masuno *et al.*, 1996)
Regulation of SOD gene transcription activation	(Kim *et al.*, 1996; Chang *et al.*, 1999)
Anti-ulcer activities	(Jeong *et al.*, 2003)
Stimulation of peripheral nerve regeneration	(Tsai Cc *et al.*, 2003)
Stimulation of secretion of luteinizing hormone	(Tsai Sc *et al.*, 2003)

Table 1.10– Activities of ginsenoside Rb-1

To summarize the studies of Rb-1 in respect to immunological activities, it could be categorized into (1) proliferation and differentiation of immune cells; (2) cytokines production; (3) moderation of oxidative stress and (4) regulation of transcription factors or activation.

1.8) Aims & Objectives

As ginseng is in safe use as an health tonic and immunomodulator for thousands of years in the Oriental culture and Rb-1 is the most abundant saponins. It is hypothesized that Rb-1 has an immunomodulatory activities in the body. This project aims to discover if Rb-1 had any effects on the immune system in the rat model. Several different approaches were adopted. These approaches were designed to examine the effects of Rb-1 on immune cell proliferation regulation, cytokines production, protein phosphorylation activities and protein expression.

Firstly, the mitogenic property of Rb-1 was investigated. With the aid of the MTT colorimetric assay, the ability of Rb-1 to induce proliferative activities on primary splenocytes culture was tested. Oppositely, immunotoxicity of Rb-1 was also examined by the addition of various concentrations of Rb-1 (0 – 100 μg/ml) to LPS-stimulated splenocytes. Secondly, as cytokine played a central and crucial role in immunity communication, the ability of Rb-1 in affecting cytokines production was tested. A total of six cytokines were analyzed. Pro-inflammation and inflammation cytokines included interferon-gamma (IFN-γ), interleukin-1alpha (IL-1α), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). Production of inteleukin-2 (IL-2) (controlling growth and differentiation) and interleukin-10 (IL-10) (an anti-inflammatory cytokine) were also measured. Cytokines productions were measured under two different conditions: (a) cytokines secretion by splenocytes with Rb-1 incubation alone were measured by enzyme-linked immunosorbent assay (ELISA); (b) cytokines secretion from Rb-1 incubated LPS-stimulated splenocyte were also measured.

Thirdly, the mode of action of Rb-1 was also investigated. As discussed before, transcription factor NF-κβ played a regulatory role in immune responses such as cytokine synthesis. On the other hand, NF-κβ was strictly regulated by its inhibitors I-κβ. It was hypothesized that Rb-1 control cytokine production through regulating activities or phosphorylation of I-κβ. By application of Bio-plex™ suspension array system, total protein expression and phosphorylation level of I-κβ supplement with Rb-1 (100 μg/ml) was measured. In addition, protein amount and respective phosphorylation activities of other signaling transducers were also examined. These comprised of JNK, ATF-2, p38MAPK, Akt and p90RSK. Translocation of transcription factor NF-κβ was also investigated. After priming with Rb-1 and LPS, cytosolic fraction and nuclear extract of splenocytes culture were prepared. These fractions were then subjected to western blot analysis in that NF-κβ was probed with anti-p65-NF-κβ monoclonal antibodies.

Lastly, in order to exploit possible novel pathway mediated by Rb-1, proteome expression of splenocytes with or without Rb-1 was analyzed. It was achieved by establishment of 2D-maps of both cytosolic and nuclear fraction from Rb-1/ LPS-added splenocyte culture. Hence, differential proteins expression under the influences of Rb-1 were compared and found. Differential expressed protein spots were then identified by matrix-assisted laser desorption/ ionization – time of flight (MALDI-TOF) mass spectrometry (MS).

Chapter 2) Materials and Methods

2.1) Sprague-Dawley rat (SD rat)

Six-week-old male SD rats were used in the whole experiment. Rats were purchased from the Centralized Animal Facility (CAF) of The Hong Kong Polytechnic University, Hong Kong (HKPU, HK). Rats were kept and cared under conditions which fully met the requirements of the Procedures for the Care of Laboratory Animals or Animals (Control of Experiments) Regulations Chapter 340 (Department of Health, HKSAR). Ethics approval (ASESC No.04/9) had been obtained from The Animal Subjects Ethics Subcommittee of The Hong Kong Polytechnic University.

2.2) Preparation of splenocytes

2.2.1) Removal of the spleen

Rat was scarified by cerebral dislocation. Spleen was removed as described previously (Nakaya et al, 2003). Briefly, surface of abdomen cavity was sterilized with 70% ethanol spray. Spleen was removed aseptically and washed with spleen washing medium (Appendix I – Solution preparation). Spleen was minced into small pieces which are transferred onto a sterile fine steel sieve. 10 ml of splenocyte preparation medium (Appendix I) was added. Crude spleen

single cell suspension could be prepared by gentle grinding of spleen pieces with a sterile syringe plunger.

2.2.2) Enrichment of splenocytes

Splenocyte (B lymphocytes and T lymphocytes) from crude spleen single cell suspension was prepared by usage of Ficoll-Paque® Plus lymphocyte isolation kit (Amersham Biosciences, US). Protocol was described in the manufacturer's user manual (Cat. 71-7167-00). 4.5 ml of Ficoll-Paque® Plus solution was added into 15 ml clear polystyrene (PS) centrifuge tube (Iwaki, Japan) and 6 ml of crude spleen cell suspension was added slowly onto the top of Ficoll-Paque® Plus layer (Figure 3.1). Vigorous mixing or disturbance between two liquid layers was avoided.

Two-phase "cell suspension-Ficoll-Paque®" sample was subjected to density gradient centrifugation by using Allegra™21R Centrifuge (rotor S4180, Beckman Coulter™, Germany). Parameters of density gradient centrifugation were set at 400 x g for 30 minutes with minimal acceleration and deceleration degree 1 at constant temperature 16℃.

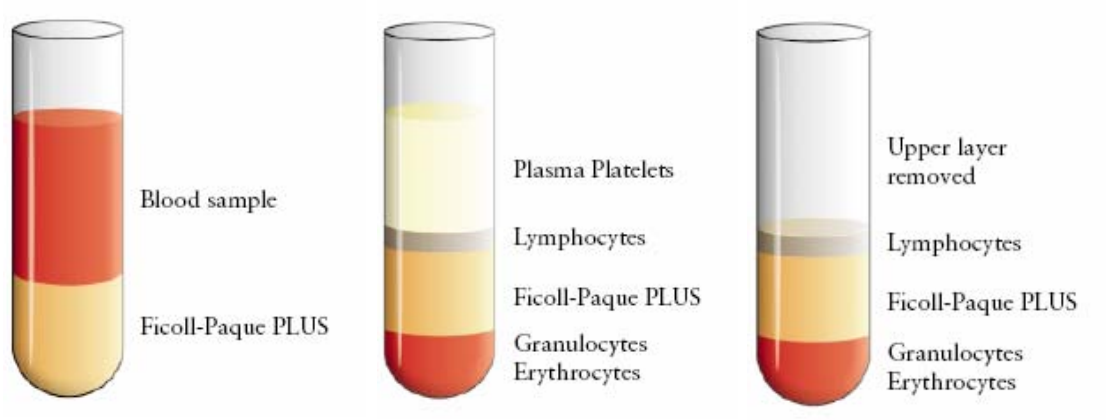


Figure 2.1a Figure 2.1b Figure 2.1c

Figure 2.1 – Steps of Ficoll-Paque density gradient centrifugation

Three distinct phases/ layers were separated after centrifugation. Upper layer was the cell-free medium, splenocyte was concentrated in the middle interphase while the Ficoll-Paque® solution was remained at the bottom phase. Large debris, granulocytes and erythrocytes were centrifuged as pellet.

Upper cell-free medium phase was pipetted off carefully without disturbance of interphase layer. Splenocyte layer (~ 2ml) was transferred into another sterile 15 ml centrifuge tube. 4 volumes or 8 ml of pre-cold (4℃) erythrocyte lysing solution (Appendix I) was added and incubated for 10 minutes for lysis of residual erythrocytes (Zimecki *et al.*, 2003). Splenocyte was recovered by centrifugation at 1000 x g (Beckman, Germany) for 5 minutes. Cell pellet was

washed twice with 10 ml pre-warm 37℃ sterile phosphate buffer saline (PBS) (Appendix I), in order to remove residual platelet and Ficoll-Paque® solution. Splenocytes were resuspended in appropriate volume of complete RPMI 1640 medium (Appendix I).

2.2.3) Viability staining and cell counting

Splenocyte was resuspended in complete RPMI 1640 medium and splenocyte population was calculated. 50 µl of splenocyte was stained with equal volume (i.e. 50 µl) of 0.4% Trypan blue exclusion dye (Appendix I). Splenocyte was incubated and stained for 5 minutes, followed by sample counting with the aid of haemocytometer. Both non-stained cells (viable) and stained cells (unviable) were counted. Viability and population of splenocyte could be calculated. Afterwards, splenocyte was diluted to a working concentration 5 x 10^6 cells/ml in the following experiments, except those stated particularly.

2.3) Characterization and identification of splenocytes

2.3.1) Giemsa-Wright morphological stain

Sterile glass slide was coated with 0.01% poly-L-lysine (Sigma, US) for 10 minutes at room temperature and allowed to air-dry after discard of lysine

solution. 100 µl of splenocyte culture was added onto the lysine-coated slide. Splenocyte was cultured in 37℃ cell incubator with 80% humidity and 5% CO_2 atmosphere for 30 minutes.

Giemsa-Wright staining protocol was according to BD Biosciences online protocol (http://www.bdbioscience.com/pharmingen/protocols). Briefly, medium was aspirated and glass slide was rinsed with PBS twice. Cells were immediately fixed in 100% methanol for 1 minute. Slide was submerged into 0.3% Wright stain (Appendix I) for 4 minutes, followed by sensitization with Sorensen-Wright staining working buffer (Appendix I) for 4 minutes. Slide was further stained by 0.75 % Giemsa working stain (Appendix I) for 4 minutes. Slide was washed with plenty of distilled water and allowed to air-dry. Differential morphology of splenocytes was examined under microscope (Eclipse E400, Nikon). Cell images were captured by digital camera (CMOS-PRO, Sound Vision) and processed by Image Ready and Photoshop 5.5 software.

2.3.2) Fluorescent activated cell sorter (FACS) analysis

Splenocyte was mainly composed of B lymphocyte and T lymphocyte. Populations of B cells and T cells were identified and quantified by fluorescent activated cell sorter (FACS) respectively. Protocol for fluorescent activated cell sorter (FACS) analysis was described in Current Protocols Online (http://www3.interscience.wiley.com).

Briefly, splenocytes was washed with FACS cell blocking buffer (Appendix I) for 15 minutes. Blocking buffer was removed after centrifugation. Anti-CD16/32 immunoglobulin blocking antibodies (Pharmingen, US) prepared in blocking buffer was added to abolish the cross-reactions between fluorescein-conjugated antibodies and immunoglobulins present on B cells. Cells were incubated on ice for 15 minutes and kept in dark with occasional shaking. Subsequent to removal of blocking antibodies after centrifugation, phycoerythrin (PE)-conjugated anti-CD20 (Santa Cruz, US) and fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Pharmingen, US) primary antibodies were used against B- and T-cells correspondingly. 200ng of fluorescein-conjugated antibodies (PE-CD20 & FITC-CD3) against B-cells and T-cells were added to the CD16/32-blocked splenocytes respectively. Cells were left on ice and in dark for incubation with 30

minutes. Excess antibodies were washed out and labeled cells were resuspended and subjected to fluorescent activated cell sorter (Coulter model EPICS® ELITE ESP).

2.4) Biological characteristics of Rb-1

2.4.1) Proliferation activities of Rb-1

Proliferation activities of ginsenoside Rb-1 on splenocytes was measured by colorimetric assay as described by Mosmann *et al* (1983). 5 x 10^5 cells/ 200 µl / well of splenocytes were seed into 96-well flat-bottom plate (Falcon, US). Different concentrations (i.e. control [medium vehicle], 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml) of gensinoside Rb-1 (Sigma, US) were added into splenocytes culture. Splenocytes were cultured for 24-hour in 37℃ incubator with 80% humidity and 5% CO_2 atmosphere. On the next day, one-tenth of culture volume (i.e. 20 µl) of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, US) stock solution was added into each well. After 4-hour incubation, insoluble form of MTT formazan salt was dissolved by 0.04N iso-propanol acid. Purple color established was measured by microplate reader (ELx 800UV, Bio-Tek Instrument Inc, US) using absorbance wavelength 570nm.

2.4.2) Immunotoxicity of Rb-1

5 x 10^5 cells/ 200µl /well of splenocyte were seed into 96-well flat-bottom plate (Falcon, US). Various concentrations (i.e. control, 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml) of Rb-1 (Sigma, US) were added into corresponding wells. Besides, 1.25 µg/ml suboptimal concentration of lipopolysaccharides (LPS) (Sigma, US) and concanvalin-A (Con-A) (Sigma, US) was also added into each well and the culture was incubated for 24-hour and/ or 48-hour. Again, one-tenth of culture volume (i.e. 20 µl) of 5 mg/ml stock solution of MTT (Sigma, US) was added into each well. After 4 hours of incubation, insoluble form of MTT formazan salt was dissolved by addition of 100 µl of 0.04N iso-propanol acid. Purple color of each well was measured by microplate reader (ELx 800UV, Bio-Tek Instrument Inc., US) using absorbance wavelength 570nm.

2.5) Immunomodulatory effects of Rb-1 on cytokine production

2.5.1) Rb-1 challenge – Effects as the production of tumor necrosis factor-alpha and interferon-gamma activities

Total six SD rats were used and experiments were repeated two times. Splenocytes was prepared as described (Section 2.2.2) and 5 x 10^6 cells/ml was cultured in flat-bottom 24 well (Iwaki, Japan). Various concentrations of Rb-1

(control, 1 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml and 100 μg/ml) were added into culture and incubated for 24 hours and 48 hours. After incubation, splenocyte cultures were harvested and centrifuged at 13000 x g at 4℃ for 5 minutes. Supernatants were collected and stored in -80℃ refrigerator until need, e.g. determination of TNF-α and IFN-γ level. Secretory level of TNF-α and IFN-γ was determined by enzyme-linked immunosorbent assays (ELISAs). Rat TNF-α ELISA kit (BioSource, US) and Rat IFN-γ ELISA kit (PBL Biomedical Laboratories, US) were used to quantify the extracellular TNF-α and IFN-γ respectively. Protocols were according to instruction manual. Once substrate reaction was stopped by addition of stop solution, colorimetric reading was acquired by measuring absorbance at wavelength 450 nm and using microplate reader (ELx 800UV, Bio-Tek Instrument Inc., US) within 15 minutes.

2.5.2) Pre-incubation of Rb-1 prior to LPS/ Con-A stimulation.

In this study on pre-incubation of Rb-1, four rats were used and splenocytes were prepared. Splenocytes 5 x 10^6 cells/ml was cultured in flat-bottom 24 well-plate (IWAKI, Japan). Different concentrations of Rb-1 (medium control, 1 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml and 100 μg/ml) were pre-added into splenocytes culture for 0-hour (without pre-incubation), 24-hour and 48-hour. After

pre-incubation of Rb1 with splenocytes, 1.25 μg/ml of LPS and 1.25 μg/ml Con-A were added to culture as a late stimulant. Splenocytes were further cultured for 16 hours. Supernatants were collected as described before and stored in -80℃ refrigerator. Amount of TNF-α and IFN-γ was determined by ELISA kits.

2.5.3) Immunomodulatory effect of Rb-1 on LPS/ Con-A-induced cytokines production

A total eight spleens were utilized and splenocytes were prepared as previously described (Section 2.2.2). Splenocytes 5 x 10^6 cells/ml was cultured in flat-bottom 24 well (Iwaki, Japan). Again, different concentrations of Rb-1 (control, 1 μg/ml, 10 μg/ml and 100 μg/ml) were primed with culture. In addition, 1.25 μg/ml of LPS and 1.25 μg/ml Con-A were added to culture as a mitogen inducer, which activated splenocytes and induced the production and secretion of cytokines. Splenocytes were cultured in 37℃ incubator with 80% humidity and 5% CO_2 atmosphere for 0-hour, 8-hour, 16-hour and 24-hour. At specific incubation time, supernatants were collected as mentioned in previous section 2.4.1. Extracellular levels of cytokines (interleukin-1 alpha [IL-1α], interleukin-2 [IL-2], interleukin-6 [IL-6], interleukin-10 [IL-10], interferon-gamma [IFN-γ]

and tumor necrosis factor-alpha [TNF-α]) were assayed by ELISA kits (BioSource, US). Each sample was measured in duplicates. By plotting the standard curve of each cytokine (Appendix III – VIII), absolute amount of each cytokine secreted by splenocytes could be quantified. Data was presented as mean ± S.E.M. and analyzed by one-way ANOVA pair t-test (Prism v 4.02).

2.6) Inducible nitric oxide synthase (iNOS) activities

Activity of inducible nitric oxide synthase (iNOS) could be studied by measuring the amount of its by-product nitrite. Supernatants collected from previous experiment (Section 2.5.3) not only were used for measurement of extracellular cytokines, but also for quantification of nitrite (NO_2^-). 100 µl of nitrite standard (0 – 10 µM) was added in 96-well plate in triplicate and applied as positive control. Moreover, 100 µl of supernatant was pipetted into 96-well plate. Equal volume of 100 µl Griess Reagent (Sigma, US) was mixed with both nitrite standard and supernatant samples. Mixture of supernatant and Griess solution was incubated for 30 minutes in dark with occasional mixing. Absorbance 540nm was read by microplate reader (ELx 800UV, Bio-Tek Instrument Inc.). Absolute amount of nitrite in supernatant samples was calculated by plotting standard curve of nitrite (Appendix IX).

2.7) Signaling pathway studies

2.7.1) Bio-plex suspension array system –

Phosphorylation activities of signaling factor and transcription factor

2.7.1.1) Sample preparation

Eight six-week-old SD rats (n=8) were used in each experiment and splenocytes were prepared as described in previous section (Section 2.2.2). Two individual sets of experiment were conducted totally (Total n=16). 5 x 10^6 cells/ml splenocytes were seed in 24-well-plate. RPMI 1640 medium was added as vehicle negative control. Except wells of the negative control group, sub-optimal concentrations of 1.25 μg/ml LPS and 1.25 μg/ml Con-A were added in wells as an external stimulant, while 100 μg/ml Rb-1 alone was added for the treatment group. In the first set experiment, cells were harvested at 0 minutes, 30 minutes, 60 minutes and 120 minutes after LPS stimulation. In case of the second set of experiment, since a later phase of signaling interval was studied, cells were harvested at 0 minute, 120 minutes and 240 minutes following LPS addition.

Once splenocytes were harvested at specific time mentioned above, cells were washed with specific wash buffer provided in the Bio-plex Phosphoprotein Cell Lysing Kit (Bio-Rad, US) and collected after centrifugation. Cell pellets were

immediately lyzed with appropriate volume of, e.g. 300 µl, lysing buffer provided in the kit. Lysate was vortex vigorously for 5 minutes and then centrifuged at 13000 x g for 5 minutes at 4℃. Supernatant was collected and protein concentration of lysate was determined by Bradford protein assay (Bio-Rad, US) instantly. Protein concentration of lysate was adjusted to 500 µg/ml (suggested range: 200 – 900 µg/ml) with lysing buffer. Afterwards, lysate was further diluted with the phospho assay buffer provided (from the assay kit) in ratio 1:1 (i.e. 100 µl lysate + 100 µl assay buffer). Diluted lysates were stored in -20℃ until measurement.

2.7.1.2) Bio-plex suspension assay

With the aid of Bio-plex suspension array system (Bio-Rad, US), total six signaling factors were studied in terms of their total protein amount and its phosphorylation activities. Six targets tested were p38MAPK, JNK, ATF-2, p90RSK, Akt and I-κβ respectively. Detection procedures of total protein and phosphorylated protein of six targets were according to the Bio-plex Phosphoprotein Testing instruction manual. Briefly, 96-well filter plate was wet by wash buffer, and (i) mixture of 6-plex coupled-beads against total p38MAPK, JNK, ATF-2, p90RSK, Akt and I-κβ; (ii) mixture of 6-plex coupled-beads against

phosphorylated p38MAPK, JNK, ATF-2, p90RSK, Akt and I-κβ; were added into different and corresponding 96-well plate respectively, the following diagram showed the platform of assay:

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	9	9	17	17	25	25	33	33	41	41
B	2	2	10	10	18	18	26	26	34	34	42	42
C	3	3	11	11	19	19	27	27	35	35	43	43
D	4	4	12	12	20	20	28	28	36	36	44	44
E	5	5	13	13	21	21	29	29	37	37	45	45
F	6	6	14	14	22	22	30	30	38	38	46	46
G	7	7	15	15	23	23	31	31	39	39	47	47
H	8	8	16	16	24	24	32	32	40	40	48	48

Figure 2.2 – Platform of Bio-plex analysis [Sample A – H (Rat 1 – 8)]

– Red: 6-plex coupled beads against total target proteins detection of negative control lysate (RPMI 1640 medium Control)

– Green: 6-plex coupled beads against total target proteins detection of positive control lysate (LPS & Con-A)

– Blue: 6-plex coupled beads against total target proteins detection of treatment lysate (LPS & Con-A + Rb-1 100 μg/ml)

– Pink: 6-plex coupled beads against phosphorylated target proteins of negative control lysate (RPMI 1640 medium Control)

– Greenish Blue: 6-plex coupled beads against phosphorylated target proteins of positive control lysate (LPS & Con-A)

– Purple: 6-plex coupled beads against phosphorylated target proteins of treatment lysate (LPS & Con-A + Rb-1 100 μg/ml)

50 µl of protein lysate (500 µg/ml) or 25 µg of lysate were added into each well and incubated for 16-hour at room temperature (e.g. 22℃) in dark with continuous shaking. Next day, lysate samples were removed by vacuum suction and plate was washed three times with washing buffer provided. 25 µl of corresponding 6-plex detection antibodies ([i] detection antibody against total target beads – Red, Green & Blue or [ii] detection antibody against phospho target beads – Pink, Greenish blue & Purple) was added into its respective wells. Plate was incubated and shaked for 30 minutes at room temperature in dark. Unbound antibody was removed by vacuum suction followed by washing three times.

After washing, 50 µl of streptavidine-PE substrate was added into each well and incubated for 10 minutes in dark with shaking. Excess substrate was removed by vacuum suction. Each well was then rinsed with resuspension buffer provided for three times before the buffer was removed. 125 µl of resuspension buffer was added into wells in order to resuspend bead molecules by 30 seconds shaking. Data was acquired by Bio-Plex Suspension Array System and was analyzed by Bio-Plex Manager software (Bio-Rad, US).

2.7.2) Activation of nuclear factor-κβ (NF-κβ) subunit p65 (NF-κβ p65)

Splenocytes were prepared and 5 x 10^6 cells/ml splenocyte were seed in 24-well plate. Sub-optimal concentration of 1.25 μg/ml LPS and Con-A was added as stimulant. Again, RPMI 1640 medium was used as negative control while 100 μg/ml of Rb-1 was added as treatment. At 0-hour and 4-hour after LPS stimulation, splenocyte culture was harvested.

2.7.2.1) Preparation of cytosolic fraction and nuclear extract

Splenocyte was harvested at specific time and subjected to subcellular fractionation. Cytosolic fraction and nuclear extract was prepared according to Antalis and Dyer with some modifications (Antalis *et al.*, 1991; Dyer *et al.*, 1995). Splenocyte was washed with hypotonic buffer (Appendix I) and cell pellet was resuspended in 500 μl 37℃ pre-warm PBS buffer. 0.1% Nonident-P 40 (NP-40) was added into splenocyte suspension and mixed by several gentle inversions. Nucleus was immediately collected by centrifugation with 13000 x g for 30 seconds. Supernatant was pooled as cytosolic fraction. Nucleus was washed with 100 μl nucleus isotonic buffer (Appendix I) and spun down. Lysis buffer (Appendix I) was applied for complete solubilization of both nuclear membrane protein and nuclear extract. Lysate was incubated on ice for 30

minutes with occasional vortexing, subsequent to 13000 x g centrifugation at 4℃ for 10 minutes. Supernatant was collected as nuclear extract. Protein concentrations of the cytosolic and nuclear fractions were determined by the Bradford protein assay (Bio-Rad, US).

2.7.2.2) Western blot

Proteins from the cytosolic fraction and nuclear extract (5 – 30 μg) were heated with 4X SDS sample buffer (Appendix I). Denatured protein samples were resolved by 7.5% sodium dodecyl sulphate (SDS) polyacrylamide separating gel (Appendix I) using Mini Protean II system (Bio-Rad, US). Protein marker SDS-7B (Sigma, US) was loaded as a molecular weight reference. After SDS-PAGE separation, protein samples were transferred onto 0.45 μm nitrocellulose (NC) membranes (Amersham Biosciences, US) using a Hoefer Transphor Unit. 100 μA was used as the transfer current for 60 minutes. Subsequently, on the membranes were blocked by the membrane blocking buffer (Appendix I) at 4℃ overnight. Subsequently, on the next day, membrane was washed with 20 mM TBST (Appendix I) for 30 minutes (5minutes per cycles). Membranes was incubated with mouse-origin anti-mouse NF-κβ p65 (BD Sciences, US) primary antibodies with dilution (1:2000) in antibody buffer

(Appendix I) for 60 minutes. Membrane was washed with TBST for 30 minutes. Afterwards horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies (Santa Cruz, US) with dilution (1:2000) in antibody buffer was added onto the membrane and allowed to incubate for 60 minutes. After thoroughly washing with TBST for 30 minutes, blots were developed by using SuperSignal® chemiluminescent substrate detection kit (Pierce, US). If needed, membrane was stripped with membrane stripping buffer (Appendix I) at 60℃ for 15 minutes. After removing the β-mercaptoethanol, membranes would be re-probed against antibodies to α-tubulin. Rabbit-origin anti-α-tubulin primary antibodies (Santa Cruz, US) with dilution 1:1000 and HRP-conjugated anti rabbit secondary antibodies (Pierce, US) with dilution 1:20000 were used for priming. Positive binding on the membrane was detected with the aid of substrate detection kit.

2.8) Proteome studies of splenocyte cytosolic fraction and nuclear extract analysis

A total of eight SD rats were used in each experiment and experiment was repeated. A total of 16 rats were used in this proteomics studies (n = 16). In addition, Group I is the control; Group II is the LPS-stimulated splenocytes; Group III is the Rb-1-stimulated splenocytes; Group IV is the Rb-1/

LPS-stimulated splenocytes. Splenocyte 5 x 10^6 cells/ml was cultured in 24-well plate. Complete RPMI 1640 was used as normal vehicle control (Group I). 1.25 μg/ml of LPS and Con-A was added into each well (Group II), and 100 μg/ml of Rb-1 was supplementary to splenocyte culture as treatment (Group III). 4-hour and 24-hour after LPS-stimulation, three groups of cultures were harvested. Cytosolic fractions and nuclear extracts were fractionated as described before (Section 3.6.2.1). Protein concentration was determined by protein assay (Bio-Rad, US). In order to study the proteome of splenocyte, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used for this purpose. 2D-PAGE consisted of two dimensions: first dimension isoelectric focusing (IEF) and second dimension SDS-electrophoresis.

2.8.1) Isoelectric focusing (IEF)

Isoelectric focusing (IEF) and subsequent second dimensional gel electrophoresis (SDS-PAGE) was performed according to Gorg (Gorg *et al.*, 2004). IEF immobilized pH gradient (IPG) strips of appropriate pH ranges (e.g. pH 5 – 8 for cytosolic and pH 7 – 10 for nuclear extract) (Amersham Biosciences, US) were rehydrated passively or without electric current application. 320 μl of rehydration buffer (Appendix I) with 1% IPG buffer of corresponding pH range (Amersham

Biosciences, US) was pipetted in the rehydration tray evenly. Protective plastic covers of the strips (e.g. 18 cm) were removed before being placed into the tray in an upside down orientation. Mineral oil was used to cover the strips to prevent drying. Strip was rehydrated in constant temperature (e.g. 20℃) for 12 hours.

Strip was then place into the Bio-Rad cup loading strip holder (Bio-Rad, US). Mobile electrodes were placed at both ends of the gel while sample cup was placed near the anode electrode. 30 μg of protein sample (cytosolic fraction or nuclear extract) of splenocyte lysate was loaded into the cup and the strip holder was placed in the Protean IEF Cell (Bio-Rad, US). IEF protocols for cytosolic fraction and nuclear extract were shown in Table 2.1 and 2.2 respectively.

Step	Mode	Voltage (V)	Time (hr)	kVh
1	Gradient	500	2:00	0.5
2	Gradient	1000	4:00	2
3	Gradient	4000	2:00	4
4	Gradient	8000	2:00	8
5	Step-n-hold	8000	10:00	80
6	Step-n-hold	500	10:00	5.0
			Total kVh	100

Table 2.1 – Setting of isoelectric focusing for cytosolic fraction (pH 5 – 8)

Step	Mode	Voltage (V)	Time (hr)	kVh
1	Gradient	500	3:00	0.75
2	Gradient	1000	6:00	3.0
3	Gradient	4000	1:00	2
4	Gradient	8000	1:00	4
5	Step-n-hold	8000	5:00	40
6	Step-n-hold	500	10:00	5.0
			Total kVh	50

Table 2.2 – Setting of isoelectric focusing for nuclear extract (pH 7 – 10)

2.8.2) Second dimension gel electrophoresis

Equilibration of IPG strip

After IEF, the strip was taken out and subjected to equilibration. Either 100 mg of dithiothretol (DTT) or 250 mg of iodoacetamide (IAA) were freshly prepared in 10 ml equilibration buffer (Appendix I) to form the reduction or alkylation buffer respectively. Strip was first submerged in the equilibration buffer containing DTT (reduction) for 15 minutes with continuous shaking, and substituted with equilibration buffer containing IAA (alkylation) for further 15 minutes with shaking

SDS-PAGE separation of proteins

Equilibrated strip was rinsed with water and placed on the top of the 10% SDS-polyacrylamide separating gel (Figure 2.3a & 2.3b). Molten 0.5% agarose sealing gel (Appendix I) was added on the top of separating gel in order to seal the strip with connection to the gel (Figure 2.3c). Protein marker SDS-7B was also loaded as molecular weight references. Gel electrophoresis was initially conducted with constant current at 15 mA/gel for 15 minutes. Later on, the current was increased to 35 mA/gel until the blue dye front reached the bottom of the gel.

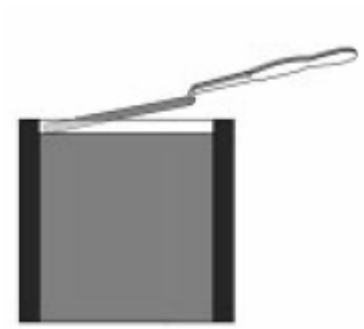


Figure 2.3a

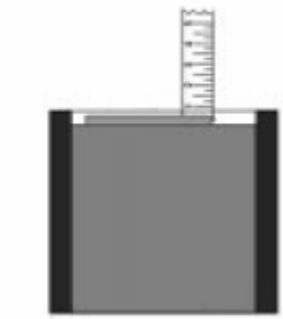


Figure 2.3b

Figure 2.3c

Figure 2.3 – Preparation of 2D SDS-PAGE

Gel images development and analysis

Proteins in the 2D-map were stained by either Commassive Blue (Appendix I) or mass-spectrometry compatible silver staining (Appendix X). Upon gel images had developed, images were scanned by scanner (OpticPro S28, Plustek,

Germany) and the images were saved as tiff format. Images were then analyzed by 2D-gel analysis software Melanie version 4.0 (Gene-bio, Sweden).

2.8.3) Protein identification

Comparison among control group, positive group (LPS-activated) and treatment group (Rb-1-added/ LPS-stimulated) was done and those differentiated expressed spots under Rb-1 influences were identified. Gel spots of interest would be cut and saved in eppendrof vials for identification at a later stage.

Washing of gel pieces

In-gel protein trypsin-digestion was according to the manual "Proteomic protocol for mass spectrometry" from the manufacturer of Bruker Daltonik, Germany. Briefly, spots gel were cut and washed with gel washing solution (Appendix I) for 15 minutes. 200 µl of silver destain solution (Appendix I) was added for destaining for 10 minutes. 25mM NH_4HCO_3 solution was added for repeated washing until the gel pieces became colorless. l50 µl of acetonitrile (ACN) was added to dehydrate the gel piece. Once the gel pieces shrunk, ACN was removed and the gel pieces were allowed to air-dry.

Reduction and alkylation

10 mM dithiotreitol (DTT) in 25 mM NH_4HCO_3 was freshly prepared. 200 µl of 10 mM DTT solution was added to swell gel pieces before being incubated at 56℃ for 45 minutes. Subsequently, eppendrof tube containing the gel pieces were chilled to room temperature before the DTT solution was removed. Equal volume (i.e. 200 µl) of 55 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3 (freshly prepared) was added and allowed to incubate with the gel pieces at room temperature for 30 minutes in dark. Gels were washed with gel washing solution for 15 minutes. 200 µl of ACN was added to dehydrate gel pieces again. ACN was subsequently removed and gel pieces were left to air-dry.

In-gel trypsin digestion

20 ng/ul of trypsin (Promega, US) was freshly diluted and prepared in 25 mM NH_4HCO_3 solution. Appropriate volume (~ 3 µl) of enzyme solution was added to gel pieces before being incubated at 56℃ for 90 minutes or 37℃ overnight.

Peptides extraction

3 µl of peptide extraction solution (Appendix I) was added to the gel. The tubes were sonicated for 10 minutes and supernatant were recovered. Sonication with

extraction solution was repeated three times. Supernatant for different extraction were pooled and subjected to speed vacuum drying. 2 – 3μl of TA solution (Appendix I) was added to resuspend the peptides.

Peptide mass fingerprint (PMF) analysis

Preparation of matrix and peptide sample was according to the manual "AnchorChip™ Technology Revision 2.0" of the manufacturer Bruker Daltonik, Germany. 2 mg/ml matrix of α-cyano-4-hydroxycinnamic acid (HCCA) (Sigma, US) in peptide extraction solution and 5 mg/ml 2, 5-dihydeoxybenzoic acid (DHB) in TA solution was prepared. 0.5 μl of HCCA and DHB matrix solution was dropped onto the anchor-chip™ target plate and allowed to air-dry 0.5 μl of resuspended peptides sample was dropped onto the matrix after the matrix droplet was air-dried.

Once the droplet became dried, the target plate was placed into the Autoflex matrix assist laser desorption and ionization-time of flight mass spectrometry (MALDI-TOF MS) machine (Bruker Daltonik, Germany) for protein analysis. Peptide mass fingerprint (PMF) spectrum was generated and submitted to an in-house NCBI database (Rodentia taxonomy) for protein matching. Fixed

carbamidomethyl modification, variable oxidation modification, one mis-cleavage limitation and 200ppm of molecular weight tolerance were set as searching criteria.

CHAPTER 3) Preparation of splenocytes

3.1) Isolation of splenocytes

Spleen cells mainly contained B lymphocytes, T lymphocytes and macrophages. Responses of these cells to exogenously added drug candidate reflected possible responses of the immune system (Mebius *et al.*, 2005). In order to examine the immunomodulatory effects of Rb-1, rat splenocytes were used in this project.

Initially, it is necessary to enrich the B cells and T cells population from the minced spleen first. Moreover, it is necessary to remove cell debris and unwanted cell types such as epithelial cells. Ficoll-Paque® Plus lymphocyte isolation kit was adapted to this situation (Romeu *et al.*, 1992). By providing the proper density, lymphocytes were concentrated and retained at the interphase. Ficoll-Paque procedures were performed as described in the Materials and Methods section (Section 2.2). The following figures showed the results.

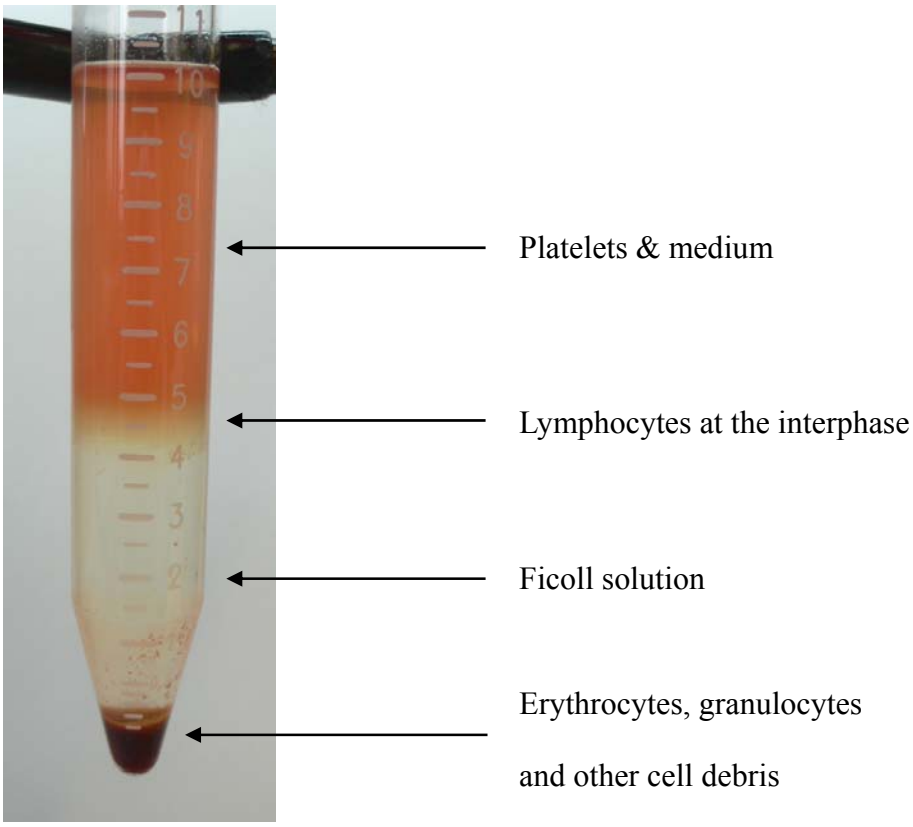


Figure 3.1a – Formation of different liquid phases after Ficoll-Paque gradient centrifugation

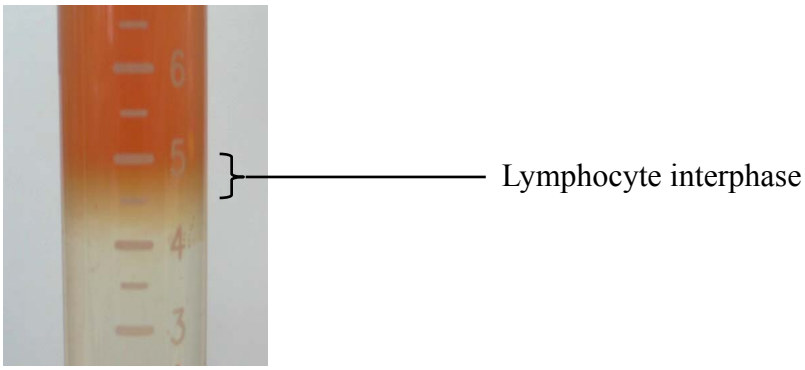


Figure 3.1b – Magnified section of lymphocytes interphase

Figure 3.1a & b – Isolation of lymphocytes by using Ficoll-Paque gradient centrifugation.

In Figure 3.1, it was found that several liquid phases were formed. Erythrocytes and large cell debris were collected at the bottom. Although lymphocytes were concentrated at the interphase, it did not form a very sharp band. After removal of the upper medium phase, the lymphocytes phase (1.5 – 2 ml) was collected. Subsequently, lymphocytes were incubated with 155 mM ammonium chloride (NH_4Cl)-PBS solution for lysis of residual erythrocytes. Lymphocytes were collected by centrifugation at 1000 x g for 5 minutes. Lastly, lymphocytes were washed with pre-warm (37℃) PBS twice. Splenocytes pellet was resuspended in 2 ml complete RPMI 1640 medium. Viability assay and differential morphology staining were performed subsequently to assess quality of the splenocytes prepared.

3.2) Viability assay

Once splenocytes was resuspended, cells population and viability was examined. Trypan blue exclusion dye staining is permeable to cell membrane and routinely used for testing cell viability (Zimecki *et al.*, 2003). Viable cell actively pumped the dye out while dead cell lost such function. Hence, viable cells appeared transparent but dead cells were stained blue. Under light microscope (shown in Figure 3.2), viable cells could be counted.

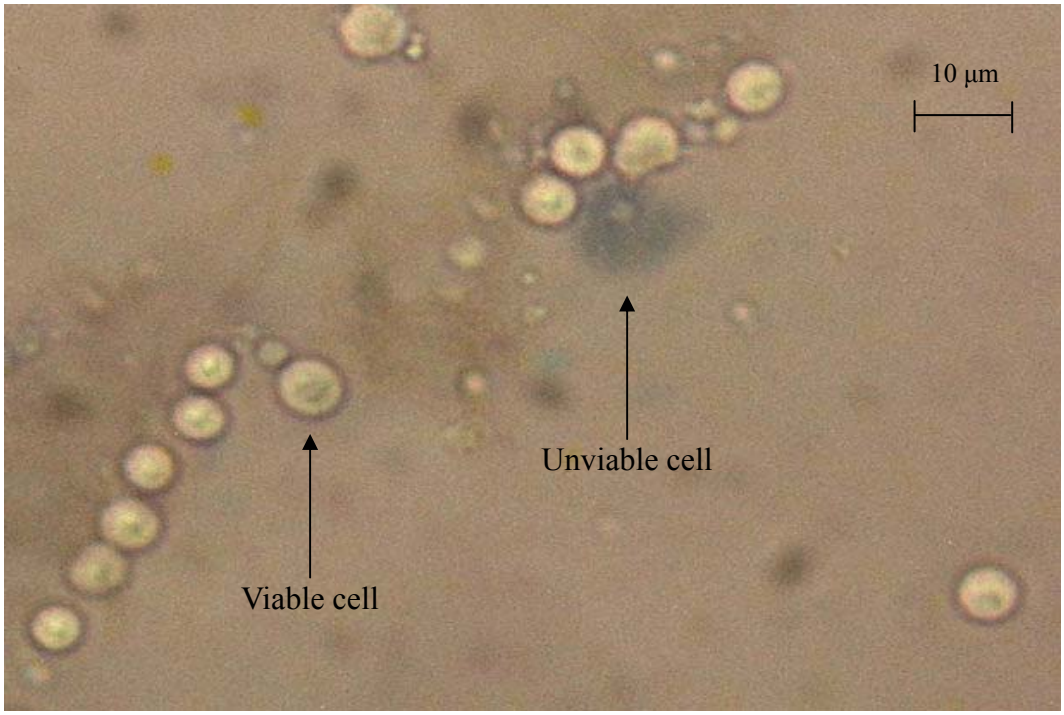


Figure 3.2 – Microscopic images (X100) of Trypan blue exclusion staining of viable and unviable splenocytes

In Figure 3.2, more than 95% of lymphocytes were shown as transparent. Several other cells were stained blue. In the Ficoll gradient operation manual, it declared that viable lymphocytes expectation after Ficoll gradient centrifugation was 95 $\pm$ 5%. Figure 3.2 illustrated that more than 95% of cells were viable and less than 5% were dead. Such results conformed to what instruction manual described and expected. Hence, these results illustrated that our procedures are reasonable and could produce viable lymphocytes compared to established methodology. Subsequently, stained splenocytes were subjected to cell counting with the aid of

a hematocytometer with the standard procedure. In this study, a range of 5 x 10^7 – 8 x 10^7 cells per spleen was obtained. Except stated experiment, 5 x 10^6 cells/ml of working cell concentration was used.

3.3) Wright-Giemsa staining

Once lymphocytes were prepared, it was necessary to clarify which type of lymphocyte the splenocytes contained. It was known that different immune cells had unique cellular morphologies. As shown in Figure 3.2, most of the splenocytes prepared were round in shape which fitted the characteristics of both B lymphocyte and T lymphocyte. Splenocytes were further stained with Wright-Giemsa stain and their cell morphologies were studied. The following figures showed representative microscopic images of the stained cells.

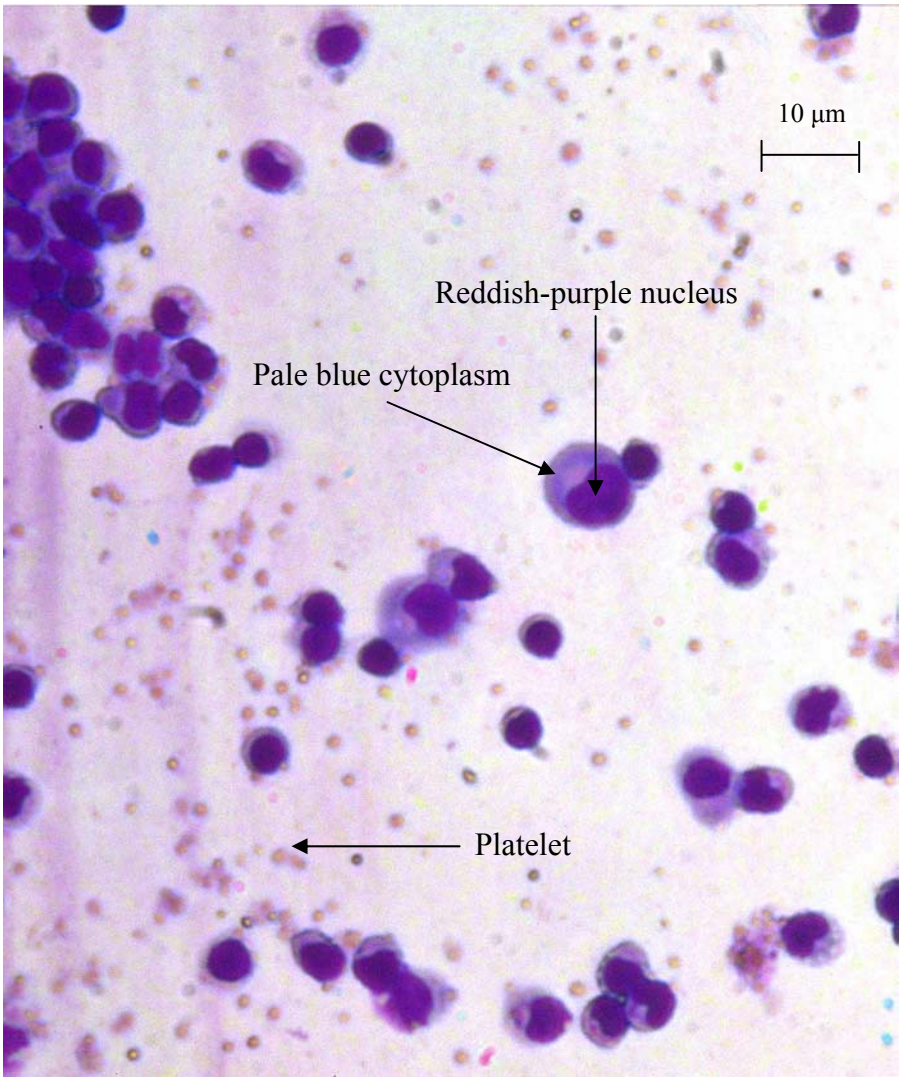


Figure 3.3a – Microscopic image (X400) of splenocytes stained with Giemsa-Wright dye

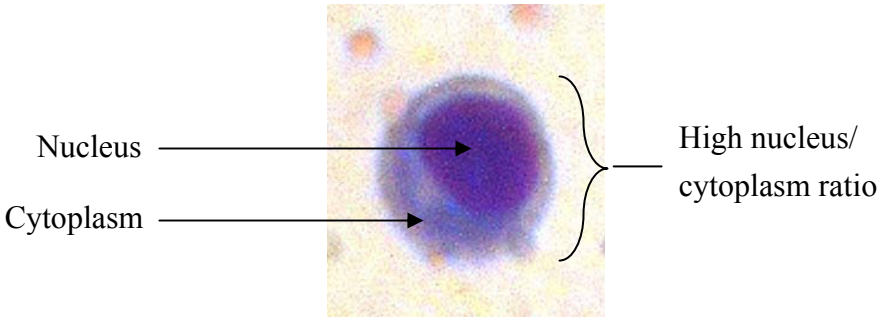
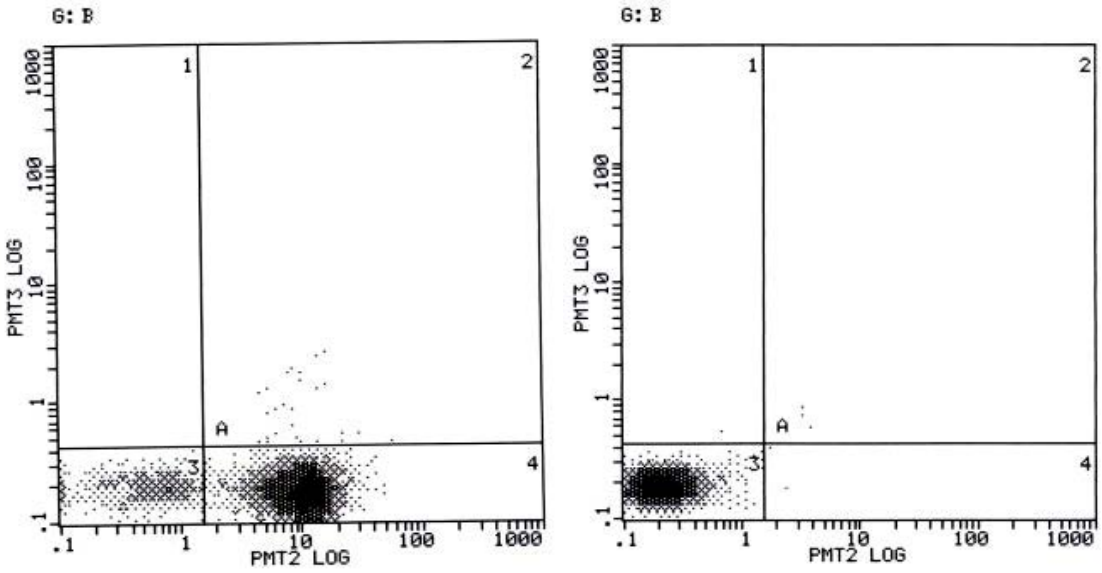


Figure 3.3b – Microscopic image (X1000) of a representative splenocyte (showing a high nucleus to cytoplasm ratio)

Under microscopic examination (X400) (Figure 3.3a), more than 95% of isolated splenocytes were seen and having a single, round, reddish-purple nucleus. Moreover, high power magnification (Figure 3.3b) showed a high ratio of nuclei to cytoplasm. This high nuclei-cytoplasm ratio represented another unique cellular morphology of lymphocytes. Therefore, splenocytes prepared were either B lymphocytes or T lymphocytes.

3.4) Enumeration of B cells and T cells – FACS analysis

As described above, no information about the relative population of B lymphocytes and T lymphocytes in the splenocytes prepared was obtained. In the meantime, the information of B cells and T cells population is important in understanding our splenocytes system. Since CD 20 and CD3 are specific surface markers of B cells and T cells respectively. Therefore, anti-CD20-PE-conjugated and anti-CD3-FITC-conjugated antibodies were used for priming B cells and T cells in splenocytes respectively. Respective populations were obtained by passing the probed splenocytes preparation through a fluorescent activated cell sorter (FACS) machine. Results obtained were shown in the following figures.



3.4a) B cells – CD20-PE 3.4b) T cells – CD3-FITC

Figure 3.4a & b – Population analysis of B cells and T cells by FACS machine

In this experiment, two separate cytometric runs were performed because of the application of two different fluorochromes. PE had emission wavelength of 520 nm while FITC emitted at 585 nm. Wavelength emission and detection was conducted by FACS machine automatically. In each run, 5000 cells or splenocytes were sorted and measured. After calculation and averaging the numbers of 2 runs, 45% of cells were found to emit at wavelength of 585 nm and were taken as B cells. On the other hand, 57% of cells were detected by a 520 nm filter and were taken as T cells. As most FACS machine has an accuracy limit 1 – 3%, therefore, it could be summarized that the splenocytes preparation contained a majority of T cells and a minority of B cells. Macrophages population was negligible in splenocytes prepared in our experiments.

CHAPTER 4) Biological and immunomodulatory activities of Rb-1

4.1) Biological characteristics of Rb-1

Before studying the immunomodulatory aspects of Rb-1, it was necessary to assay its pharmacological effects. Previous studies had reported that ginseng extract enhanced cytokines production (Smolinski *et al.*, 2003; Wang *et al.*, 2003). However, actions of purified Rb-1 on cytokines production in splenocytes are unknown. Therefore, proliferation and immunotoxicity action of Rb-1 were investigated first.

4.1.1) Proliferative activities of Rb-1

Various concentrations of Rb-1 were directly added into splenocytes culture. After 24-hour incubation, cell population was examined by MTT colorimetric assay. The experiment was performed using 8 rats each time and the experiment was repeated once. Therefore, a total of 16 rats were used (n=16). Samples were measured in duplicates and presented as mean $\pm$ S.E.M. Possible significant difference was analyzed by student's t-test. A standard curve of MTT absorbance at 570 nm against various cell numbers of splenocytes was plotted. The standard curve was shown in Appendix II. The results were presented in Figure 4.1 below.

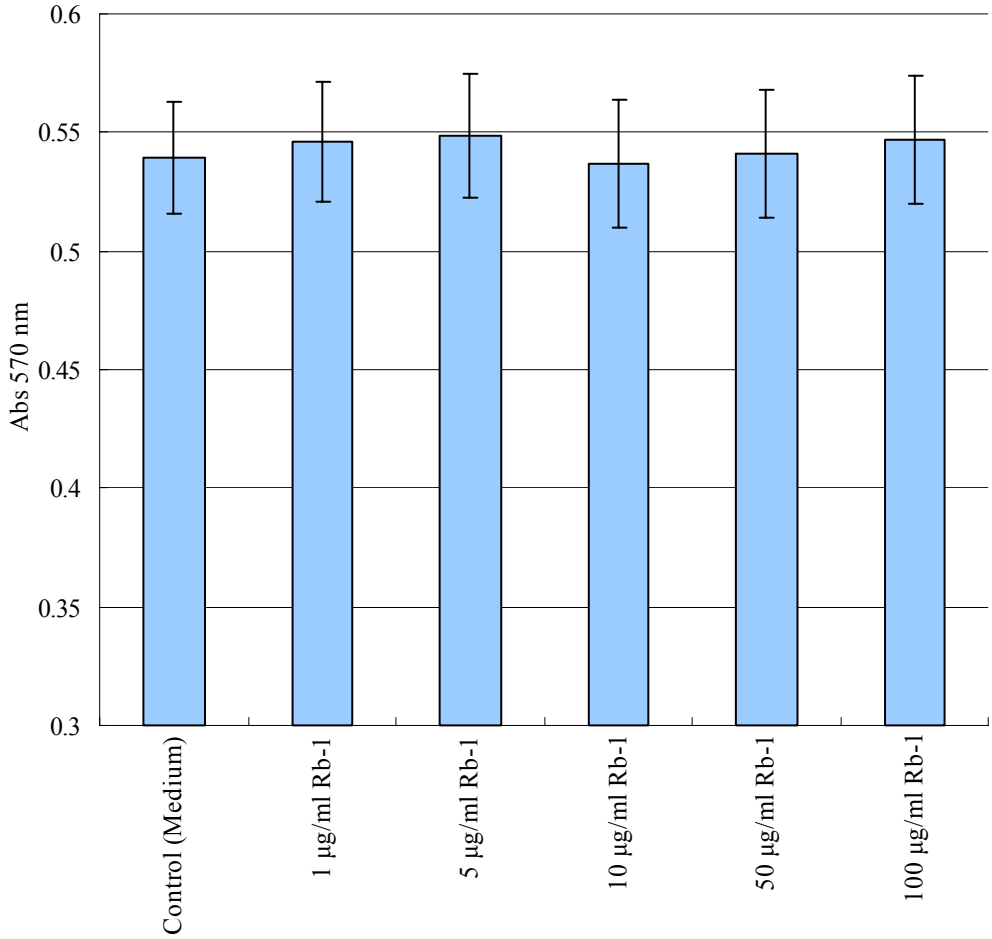


Figure 4.1 – Absorbance at 570 nm of the splenocytes culture with the addition of various concentrations of Rb-1 (1 – 100 µg/ml) after 24 hours of incubation. Mean ± S.E.M (n=16)

Our results showed that Rb-1 did not induce splenocytes proliferation after 24-hour of incubation. This was supported by the fact that absorbance at 570 nm of splenocytes cultures was not changed significantly against various concentration of Rb-1. If Rb-1 had any proliferative effects, splenocytes should be induced to grow. Proliferating splenocytes synthesized more mitochondria which would increase the absorbance of the culture at 570 nm.

4.1.2) Immunotoxicity of Rb-1

Since Rb-1 alone did not induce proliferation of splenocytes, it was of interest to understand if Rb-1 is immunotoxic. LPS is a well-known and strong mitogen which induce lymphocytes to proliferate. Therefore, Rb-1 was supplement with LPS before their addition to the splenocytes culture. If Rb-1 is immunotoxic, it will not only inhibit the growth of splenocytes but will also slow down the conversion of soluble MTT into insoluble MTT formazan.

In this study, the experiment (n = 8) was repeated once and a total of 16 rats (n=16) were used. The experimental set-up was the same as the previous Rb-1 proliferation assay. Further, 1.25 μg/ml LPS was added into splenocytes culture as mitogen. Each sample was measured in duplicates. Data was presented in mean $\pm$ S.E.M and statistical significance was analyzed by student's t-test. The results were shown as follows.

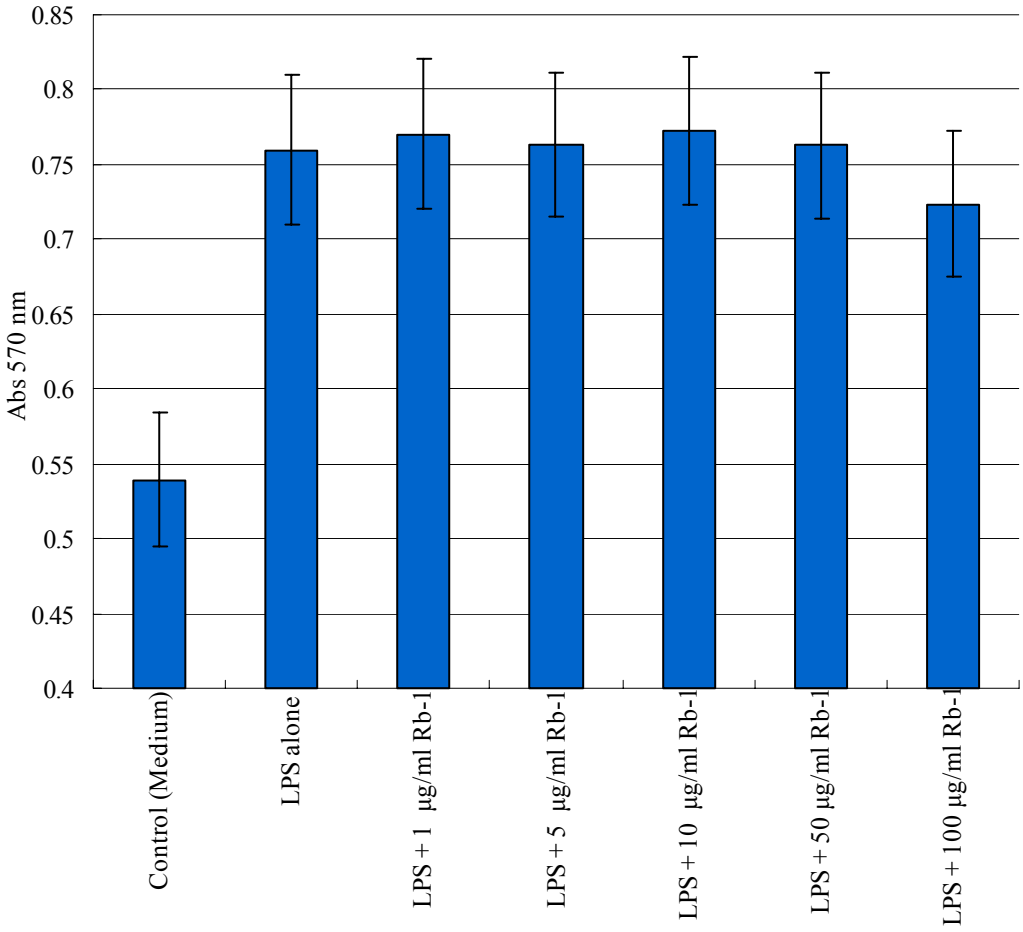


Figure 4.2 – Absorbance at 570 nm of LPS-stimulated splenocytes culture with the addition of various concentrations of Rb-1 (1 – 100 µg/ml) for 24-hour incubation. Mean ± S.E.M. (n=16)

There are several observations from the above results. First of all, absorbance at 570 nm of LPS-stimulated splenocytes was increased when compared with un-stimulated splenocytes. In particular, absorbance was increased from 0.54 (un-stimulated) to 0.76 (stimulated). With reference to the standard curve (Appendix II), absorbance of 0.76 represented nearly a doubling of cell number.

This reflected that LPS activated splenocytes to proliferate and hence more mitochondrial dehydrogenase was produced. Newly synthesized dehydrogenase in turn converted more soluble MTT salt to insoluble MTT formazan, yielding a higher absorbance as a result. On the other hand, there was no significant difference among varies dosages of Rb-1 applied in the culture. Therefore, it is unlikely that Rb-1 is immunotoxic to splenocytes, even at a high dosage of 100 μg/ml.

4.2) Immunomodulatory effects of Rb-1 on cytokines production

4.2.1) Rb-1 challenge

Regarding immune and inflammation responses, IFN-γ and TNF-α are believed to be the most acute and crucial cytokines in the immune system (Locksley *et al.*, 2001; Bonjardim, 2005). For example, lymphocytes secreted IFN-γ and TNF-α upon activation instantly. Secretion of IFN-γ and TNF-α were considered as the markers indicating splenocytes activation. Since Rb-1 did not induce any proliferation of splenocytes. It was of interest to see whether Rb-1 triggered cytokines production or not. Before quantification of IFN-γ and TNF-α level, standard curves of each cytokine had been established (Appendix III and IV). Subsequently, secretions of IFN-γ and TNF-α induced by various dosages of

Rb-1 were investigated. Experimental design and set-up was described in Materials and Methods (Section 2.5.1). No detectable level of IFN-γ and TNF-α was found after either 24- or 48-hour incubation with Rb-1 alone. Results were summarized in Table 4.1 below.

Analysts	IFN-γ	TNF-α
Incubation period	24-hour & 48-hour	24-hour & 48-hour
Medium Control	Negligible amount (NA) (Sensitivity > 15.6 pg/ml)	Negligible amount (NA) (Sensitivity > 7.8 pg/ml)
Rb-1 1 μg/ml	NA	NA
Rb-1 5 μg/ml	NA	NA
Rb-1 10 μg/ml	NA	NA
Rb-1 50 μg/ml	NA	NA
Rb-1 100 μg/ml	NA	NA

Table 4.1 – ELISA result of Rb-1-induced IFN-γ and TNF-α secretion after 24-hour or 48 hour incubation.

4.2.2) Immunomodulatory effects of Rb-1 on mitogen-induced cytokines production

Given that Rb-1 did not directly induce the secretion of IFN-γ and TNF-α, it would be of interest to find out if action of Rb-1 is immunomodulatory in nature.

In order to find that an external stimulant was needed, LPS is a potent inflammation inducer in the immune system. Moreover, mechanisms of LPS binding and its activation pathway in lymphocyte cells had been documented (Triantafilou *et al.*, 2002). Therefore, in this section, LPS was applied as cytokines inducer. In addition, concanvalin-A (Con-A) was also added into splenocytes culture and act as a co-stimulatory factor of T-cells. 1.25 μg/ml of LPS and 1.25 μg/ml Con-A was used as sub-optimal concentration in inducing lymphocytes culture.

4.2.2.1) Pre-incubation of Rb-1 prior to LPS/ Con-A stimulation

In this preliminary experiment, it was hypothesized that Rb-1 exerted its modulatory effects before LPS-activation. Hence, splenocytes were pre-incubated with Rb-1 with different duration before LPS stimulation. For example, 0-hour (co-incubation with LPS), 24-hour and 48-hour pre-incubation was assayed in this experiment. Again, referring to the standard curves (Appendix III and IV), absolute amounts of IFN-γ and TNF-α secreted were calculated. Data were shown in mean $\pm$ S.E.M. (n = 4) and analyzed by student's t-test. Results were presented in the following figures.

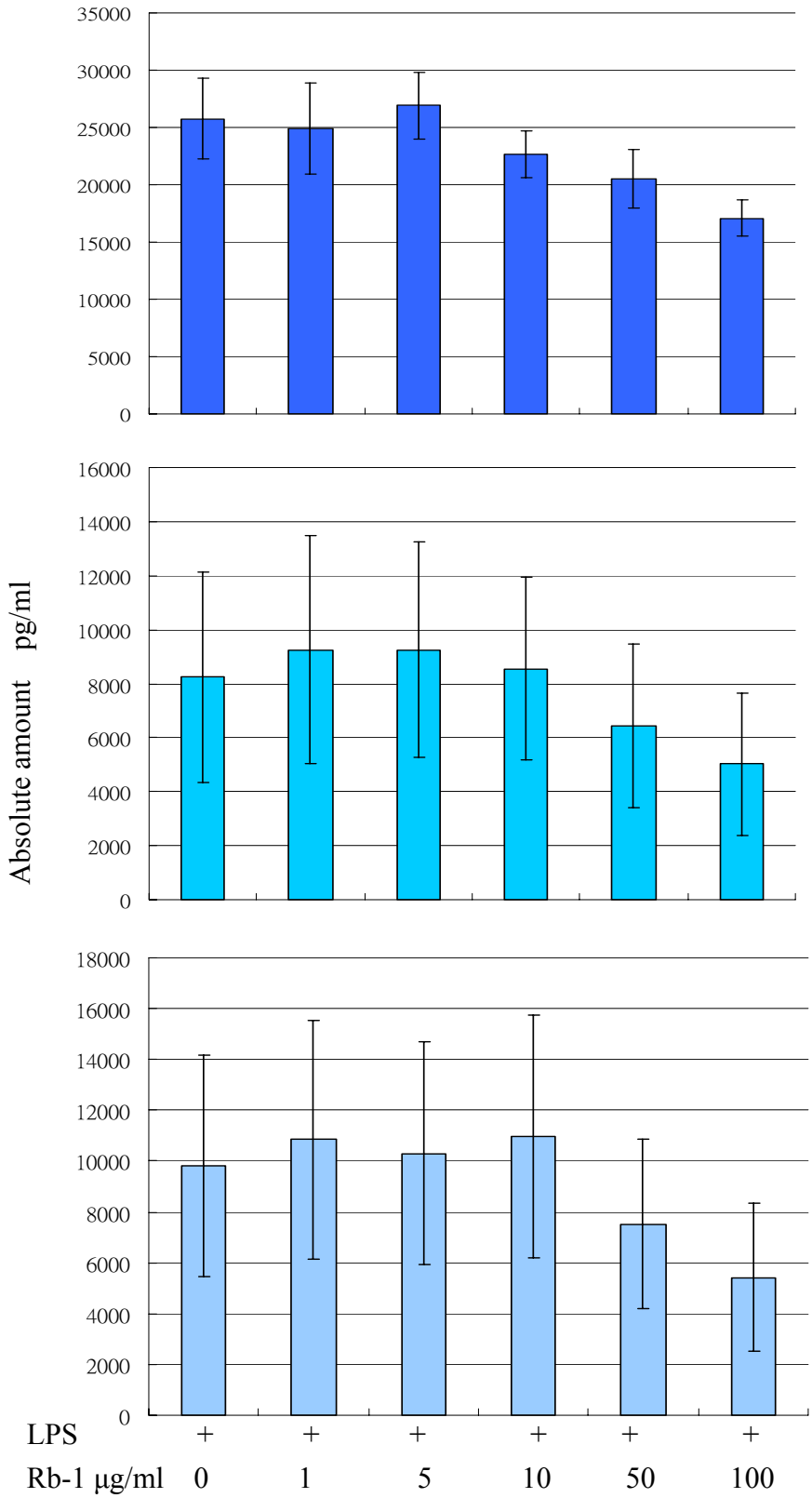


Figure 4.3a

0-hour

(Co-incubation)

Figure 4.3b

24-hour

Pre-incubation

Figure 4.3c

48-hour

Pre-incubation

Figure 4.3(a-c) – LPS-induced IFN-γ secretion by splenocytes with different pre-incubation periods (a-c) at various concentrations of Rb-1. Mean ± S.E.M. (n = 4)

From Figure 4.3a – 4.3c, it was found that secretion of IFN-γ from Rb-1 pre-incubated splenocytes was lowered than those without Rb-1 pre-incubation. For example, 26000 pg/ml of IFN-γ was found from culture without pre-incubation (Figure 4.3a). However, only 8000 – 10000 pg/ml of IFN-γ was found from pre-incubated splenocytes (Figure 4.3b & 4.3c). This indicated that the immune responses of LPS-stimulated IFN-γ production were inhibited after pre-incubation with Rb-1. Beside IFN-γ, amounts of TNF-α secreted were also measured. The following figures represented the absolute amount of LPS-induced TNF-α secreted against different concentrations of Rb-1. Again, various periods of Rb-1 pre-incubation such as 0-hour (co-incubation), 24-hour and 48-hour was set up.

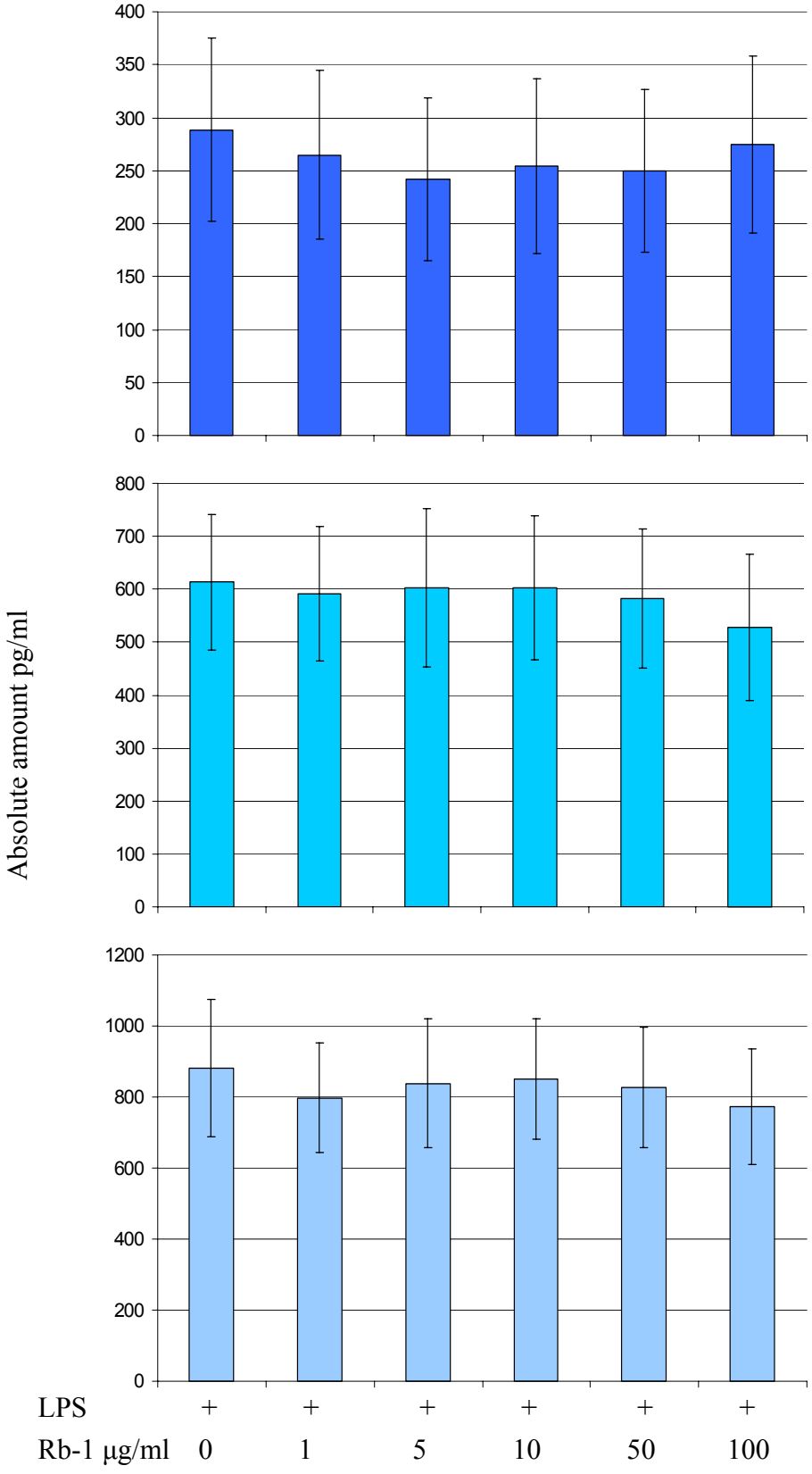


Figure 4.4a
0-hour
(Co-incubation)

Figure 4.4b
24-hour
Pre-incubation

Figure 4.4c
48-hour
Pre-incubation

Figure 4.4(a-c) – LPS-induced TNF-α secretion by splenocytes with different pre-incubation periods (a-c) at various concentrations of Rb-1. Mean ± S.E.M. (n = 4)

In Figure 4.4a – c, it was found that Rb-1 did not modulate the LPS-induced TNF-α production. Pre-incubation of Rb-1 did not contribute any effects to the LPS-induced TNF-α production. In this section, cytokines were measured after 16 hours LPS activation. It illustrated that pre-incubation of Rb-1 in splenocytes culture could not bring any modulatory effects on either IFN-γ or TNF-α secretion. Nevertheless, it was found that Rb-1 modulated the LPS-induced IFN-γ secretion.

4.2.2.2) Immunomodulatory effects of Rb-1 on LPS/ Con-A-induced cytokines production

In this section, immunomodulatory effects of Rb-1 on LPS/ Con-A-induced cytokines secretion were examined. With this preliminary experiment, LPS-induced and Rb-1-modulated cytokines secretion profiles were established. Different from previous experiment, a total 8 rats of were used in this experiment.

According to the results obtained previously, pre-incubation of Rb-1 on splenocytes was not necessary and hence excluded. Subsequently, concentration of 1 μg/ml, 10 μg/ml and 100 μg/ml Rb-1 was added to splenocytes culture.

Results were compared to the control group (with LPS as stimulant). In addition, in order to establish serial cytokines secretion profiles, supernatant samples were collected as time 0-hour, 8-hour, 16-hour and 24-hour after LPS stimulation.

Levels of IL-2 (a growth-regulatory cytokine); IFN-γ, TNF-α, IL-1α, IL-6 (pro-inflammatory cytokines); IL-10 (inhibitory cytokines) were measured. Absolute amount of cytokines was quantified from standard curve of each cytokines (Appendix III – VIII). Results (mean $\pm$ S.E.M) were presented in the following figures and analyzed by one-way ANOVA pair t-test (performed by Prism v4.02). It was because splenocytes from the same rat were used for different treatments (e.g. LPS-stimulation or Rb-1 addition or both). Responses of splenocytes against different treatments were compared with its control (medium) group. Therefore, each set of treatment and result could be pair-up and compared (n=8). A level of confidence of more than 95%, [$p<0.05$] was required before the results were considered as siginificant differences. First of all, background profiles with LPS acts as mitogen against splenocytes and growth factor of B cells and T cells were measured.

I) Interleukin-2 (IL-2)

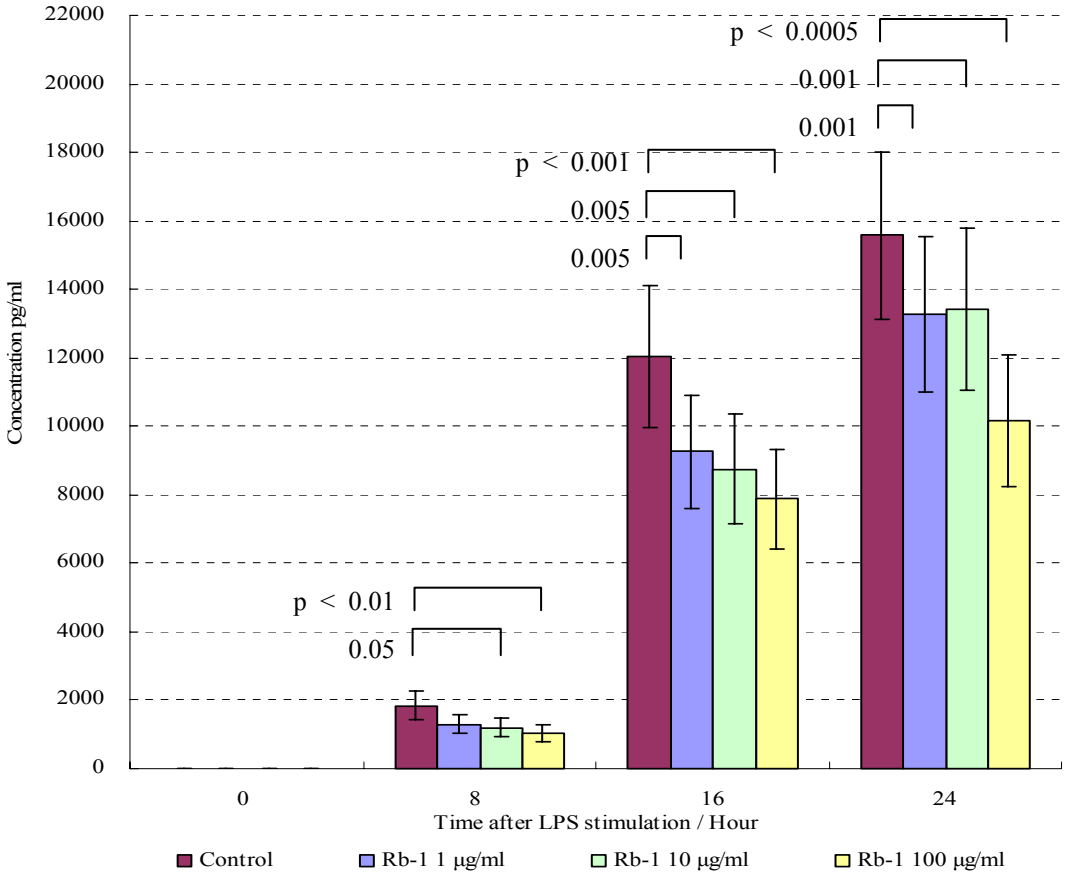


Figure 4.5 – Secretion profile of IL-2 upon LPS stimulation. Data was shown as mean ± S.E.M. (n = 8) (p < value ⌐)

Upon LPS stimulation, secretion of IL-2 was increased. Furthermore, for those splenocytes culture with Rb-1 addition, secretion of IL-2 was obviously lowered than that of control group. It was highlighted that IL-2 level reached a high level from 16-hour onwards after LPS stimulation. As evident from the p values, IL-2 secretion was suppressed by the addition of Rb-1.

II) Tumor necrosis factor-alpha (TNF-α)

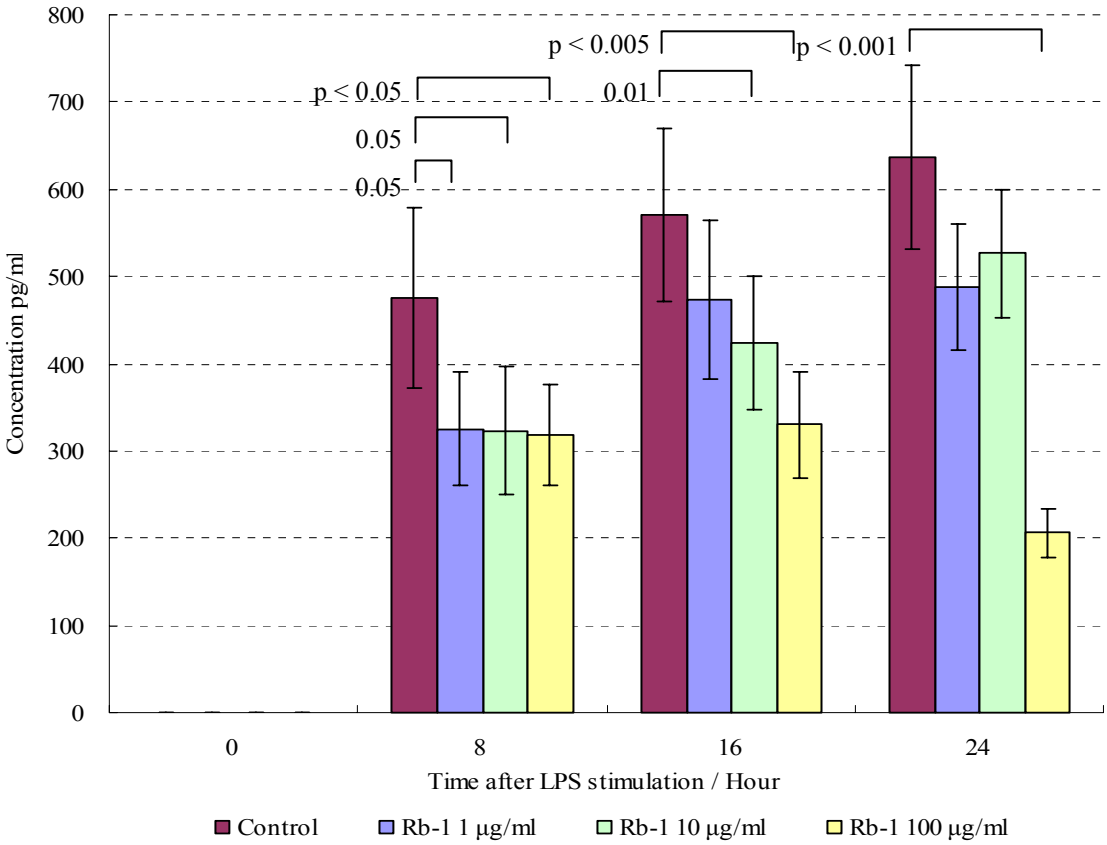


Figure 4.6 – Secretion profile of TNF-α upon LPS stimulation. Data was shown as mean ± S.E.M. (n = 8) (p < value ⌜ ⌝)

Result from the control group showed that level of TNF-α increased with time. However, secretion level of TNF-α decreased after 8-hour incubation with Rb-1. Rb-1 apparently significantly suppressed the secretion of TNF-α. When comparing control group and 100 μg/ml Rb-1 groups, TNF-α level was decreased by more than 60%.

III) Interferon-gamma (IFN-γ)

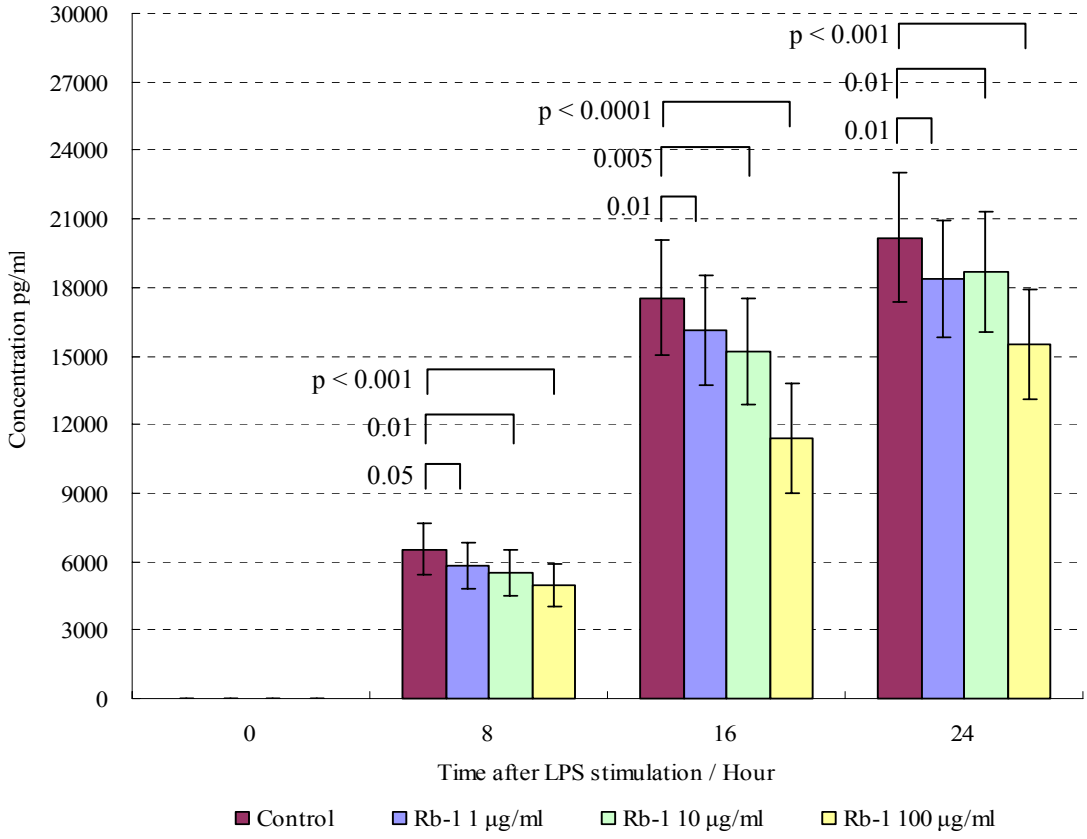


Figure 4.7 – Secretion profile of IFN-γ upon LPS stimulation. Data was shown as mean ± S.E.M. (n = 8) (p < value ⌜ ⌝)

From the figure, it was found that IFN-γ was secreted after LPS addition. During 8-hour to 16-hour of incubation, level of IFN-γ secreted was boosted to nearly 3-folds. Student's t-test analysis revealed that Rb-1 decreased IFN-γ secretion even at the first 8-hour. Similarly, secretion of IFN-γ was suppressed in the 16- and 24-hour of incubation of 10 µg/ml or more Rb-1.

IV) Interleukin-1 alpha (IL-1α)

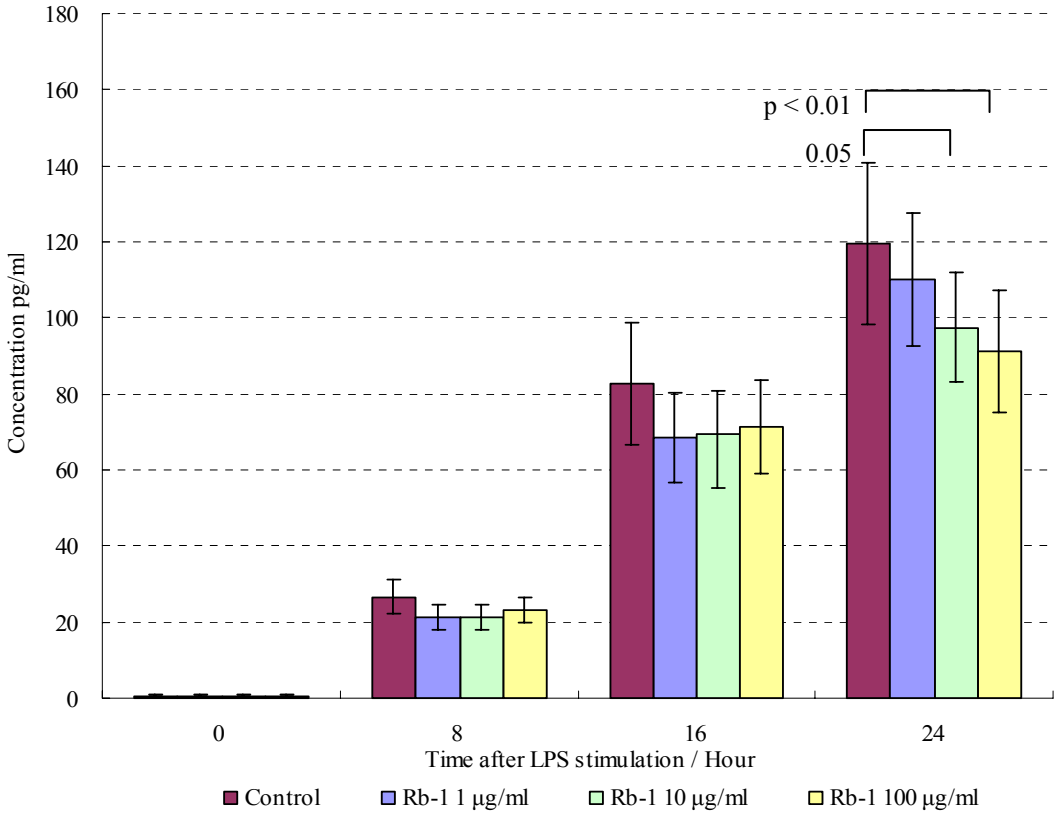


Figure 4.8 – Secretion profile of IL-1α after LPS stimulation. Data was shown as mean ± S.E.M. (n = 8) (p < value ⌐)

As seen above, upon LPS stimulation, secretion of IL-1α from splenocytes without the presence of Rb-1 was increased with time. However, for those with Rb-1 added, IL-1α secretion was lowered than that of control. After 24-hour of stimulation, significant difference between (a) 10 µg/ml and control, and (b) 100 µg/ml and control ($p<0.05$ and $p<0.01$ respectively) were found. Such readings indicated that secretion of IL-1α was suppressed.

V) Interleukin-6 (IL-6)

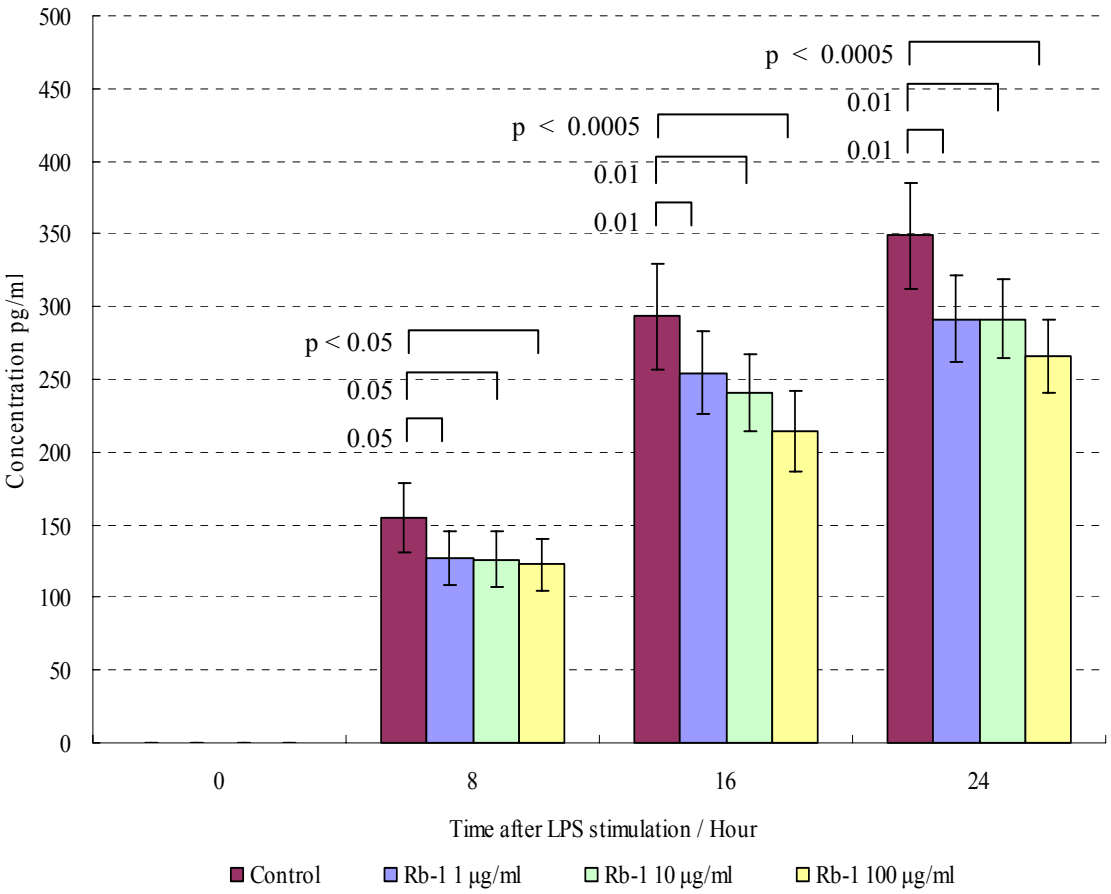


Figure 4.9 – Secretion profile of IL-6 upon LPS stimulation. Data was shown as mean ± S.E.M. (n = 8) ($p <$ value ⌐)

Being an inflammation inducer, pro-inflammatory cytokine IL-6 secretion level in splenocytes without Rb-1 was increased upon LPS stimulation. Secretion of IL-6 was suppressed by addition of Rb-1. For instances, highest dose of Rb-1 (100 µg/ml) showed an obvious significant differences ($p < 0.0005$) than other concentration (e.g. 10 µg/ml; $p<0.01$)

VI) Interleukin-10 (IL-10)

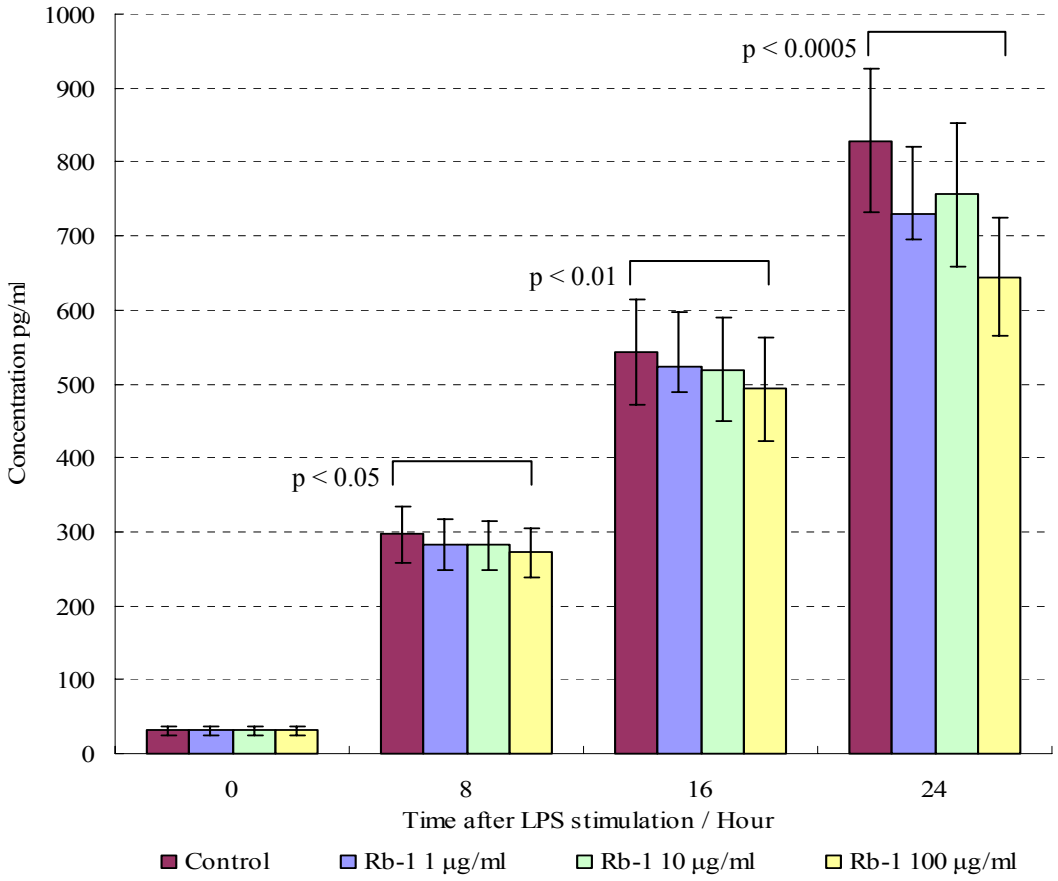


Figure 4.10 – Secretion profile of IL-10 upon LPS stimulation. Data was shown as mean ± S.E.M. (n = 8) (p < value ⌜)

Different from other cytokines, basal level of IL-10 was detectable in supernatant of splenocytes (~30 pg/ml), as shown at time-point 0-hour. Similar to other cytokines, secretion of IL-10 in splenocytes culture without Rb-1 was increased with time. However, immunomodulatory effect of Rb-1 on IL-10 secretion was not obvious, except with the highest dosage of Rb-1 dosage, i.e. 100 µg/ml.

As mentioned in literature, it was difficult to analyze the functions of individual cytokines. Instead, evaluation of cytokines network was more important in studying immune system. In this section of the results, immunomodulatory effects of Rb-1 were investigated. It was demonstrated that Rb-1 alone did not induce secretion of any cytokine. Hence, LPS added as an external stimulant or mitogen in our splenocytes culture amplified the secretion levels of cytokines and made them detectable.

First of all, LPS act as mitogen and induced lymphocytes to proliferate. Therefore, it was found that secretion factor of growth factor IL-2 was boosted at the first 8 to16-hour sample (from 2000 pg/ml to 12000 pg/ml) (Figure 4.5). This boosting action was considered as a result of LPS mitogenic effect. At the same time, LPS triggered signals that activated inflammation responses (Guha *et al.*, 2001). As a result, expression of pro-inflammation and inflammation cytokines were induced. This was illustrated by secretion profiles of pro-inflammation cytokines TNF-α (Figure 4.6) and IFN-γ (Figure 4.7) in samples without the addition of Rb-1. However, production of both TNF-α and IFN-γ was relieved after 16-hour of incubation of Rb-1. TNF-α amounts detected at 24-hour was even lower than that of the 16-hour sample. This might represent that, not only

no newly synthesized TNF-α was secreted but TNF-α was also utilized in the cellular mixture. Subsequently, pro-inflammation cytokines induced secretion of inflammation cytokines such as IL-1α and IL-6. In Figure 4.8 and 4.9, it was observed that production of IL-1α in splenocytes alone was induced after 8-hour while IL-6 level was increased with time. In order to maintain homeostasis of immune system and prevented hypersensitivity, inhibitory cytokine IL-10 was also secreted. Therefore, IL-10 level was increased proportional to time, as shown in Figure 4.10. On the other side, it was found that Rb-1 modulated the production of cytokines. Especially, immunosuppressive effect of Rb-1 was most obvious in cases of IL-2, TNF-α, IFN-γ and IL-6 secretion. Since these cytokines were first-response elements against LPS activation. Hence, it was speculated that Rb-1 exerted its effects on the signaling pathways which mediated acute inflammatory responses.

4.3) Modulatory effect of Rb-1 on LPS-induced nitrite production

In the previous sections, secretory levels of various cytokines regulating inflammation and anti-inflammation upon LPS activation were studied. Results obtained illustrated that Rb-1 suppressed the secretion of all cytokines to different degrees. Incidentally, it was known that NF-κβ was responsible not only

for cytokines gene transcription (Li *et al.*, 2002), NF-κβ also played an important role in expression of inducible nitric oxide synthase (iNOS) (Kleinert *et al.*, 2004). Because of utilization of the common transcription factor, it was of interest to find if iNOS activity was affected as well.

Subsequent to previous experiments which indicated that, cytokines productions were significantly suppressed at highest dosage of Rb-1 used. Hence, dosage of 100 μg/ml Rb-1 was applied in splenocytes culture and the level of NO_2 was measured. Experimental set-up was described in Materials and Methods (Section 2.6). A total of 12 rats were used in this experiment. By plotting standard curve of nitrite (Appendix IX), concentration of nitrite in samples supernatant of splenocytes after incubation with Rb-1 were calculated. Data was analyzed by student's t-test and presented in the following figure.

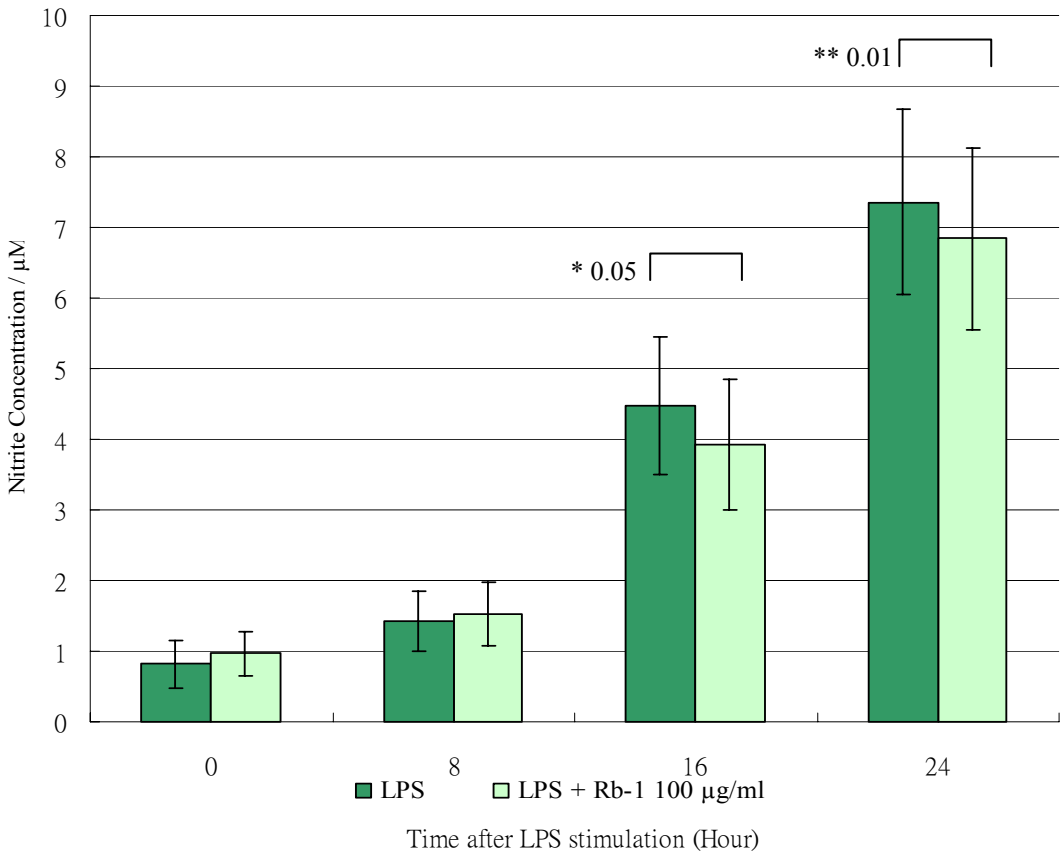


Figure 4.11 – Production of nitrite by LPS-stimulated splenocytes treated with medium (control) or with 100 μg/ ml Rb-1. Mean ± S.E.M. (n=12) (* $p < 0.05$, ** $p < 0.01$)

In Figure 4.11, two observations could be made. First, amount of nitrite produced was increased with time in cultures incubated with LPS alone. At time 0-hour, basal level (~ 1μM) of nitrite present in medium was detected. Once endotoxin LPS was added, splenocytes were stimulated and acute inflammation response was activated. Therefore, iNOS expression was induced (Wu-Hsieh *et al.*, 1998).

Afterwards, nitric oxide (NO) was synthesized as product. NO was extremely unstable and difficult to measure. However, NO was converted to nitrite which accumulated with time. Therefore, nitrite amount was measured as an illustrator of iNOS expression. Increase in iNOS expression would inevitably increase production of more NO while NO was converted and accumulated as nitrite. It was found that nitrite level was lowered in Rb-1-treated splenocytes culture. For example, nitrite amount in Rb-1 treated splenocytes culture (16-hour: 3.92 μM; 24-hour: 6.84 μM) was comparatively lowered than that of the LPS control group (16-hour: 4.47 μM; 24-hour: 7.35 μM). According to student's pair t-test analysis (n = 12), it showed significant differences (* $p<0.05$; ** $p<0.01$) between two groups.

To summarize results of the cytokines assays and nitrite measurement, it was found that Rb-1 modulated the immune system through a suppression mechanism. LPS-induced cytokines production was significantly suppressed by Rb-1. Although no Rb-1-mediated modulatory evidence on iNOS expression was obtained, nitrite (by-product of NOS) production was also inhibited by high dosage of Rb-1 (100 μg/ml). It was highlighted that, both cytokines and iNOS transcriptions were initiated by a universal transcription factor NF-κβ.

Differential expression of cytokines and activities of iNOS were both dependent on the extent of activation of this nuclear factor-κβ (NF-κβ). Hence, it was postulated that Rb-1 might interfere with the NF-κβ pathway, resulting in suppression of cytokines secretion and iNOS activity.

Chapter 5) Mode of action of Rb-1

5.1) Signaling Pathway Studies

From the previous result chapters, it was found that Rb-1 modulated the immune system. Furthermore, LPS-induced cytokines secretion and LPS-induced nitrite production were significantly suppressed in Rb-1 treated splenocytes. In this chapter, mechanism of how Rb-1 modulated the immunologic functions was examined. It was well known that LPS activated immune response through the TLR-4 signaling pathway (Takeda *et al.*, 2004). Moreover, TLR-4 signaling was connected by a step-wise process of phosphorylation between upstream signal transducers and downstream substrates. From literature review of this thesis, it had been discussed that transcription factor NF-κβ is the first responsive signaling element upon LPS stimulation, resulting in cytokines production and iNOS expression etc. Hence, it is hypothesized that Rb-1 modulates immune response via interfering of the NF-κβ signaling. Besides, activation of NF-κβ was critically dependent on the phosphorylation of its inhibitor I-κβ. Therefore, Rb-1 was believed to exert some sort of effects on either the activation of NF-κβ or its inhibitor I-κβ. In order to prove this hypothesis, phosphorylation activities of NF-κβ and its inhibitor I-κβ were investigated.

Including the NF-κβ signaling pathway, TLR-4 diverse its signals to several downstream signaling pathways. These included the MAP kinase pathway (Matsuguchi *et al.*, 2003) and protein kinase C (PKC) pathway (Asehnoune *et al.*, 2005). Therefore, either direct or indirect downstream signal transducers of TLR-4 pathway were studied. These candidates included JNK, p38 MAPK, ATF-2, p90RSK and Akt. Similarly, their phosphorylation activities were assayed.

In order to study phosphorylation activity of each signaling candidate, three figures of protein statuses were assayed with the aid of Bio-plex suspension array system. These were a) phosphorylated status, b) total protein expression and c) phosphorylated to total amount ratio. Detail protocol was described in Materials and Methods (Section 2.7.1.2). Briefly, 1.25 μg/ml LPS and 1.25 μg/ml Con-A were applied as external stimulants on the splenocytes culture. 100 μg/ml of Rb-1 was added into splenocytes in treatment groups. Results were plotted as mean fluorescent intensity (MFI) against time in minutes after LPS stimulation (mean $\pm$ S.E.M.) (n = 8).

5.1.1) p38MAPK – Analysis of phosphorylated form and total amount of p38MAPK expressed

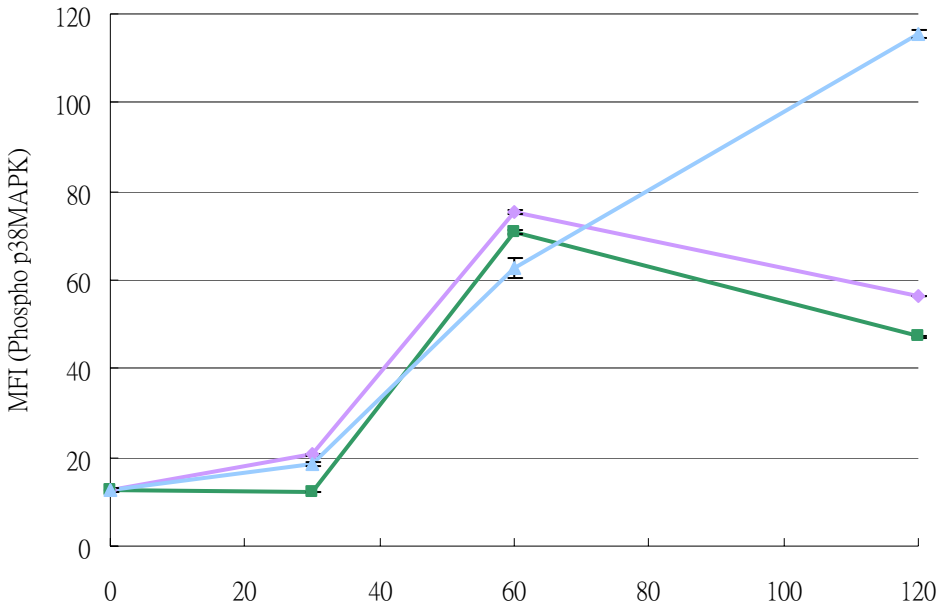


Figure 5.1a

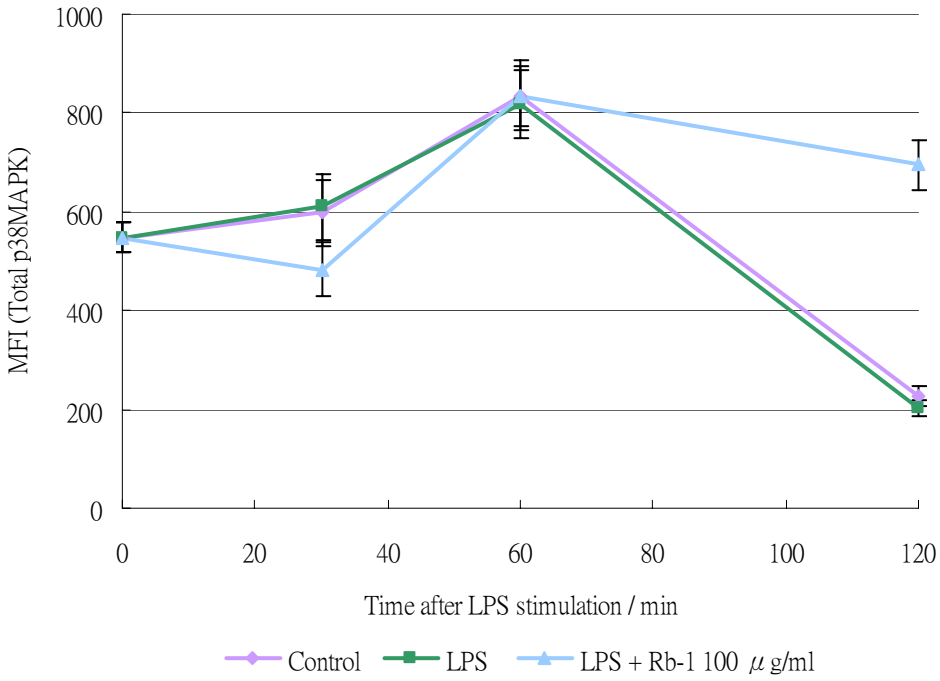


Figure 5.1b

Figure 5.1a & b – Change of mean fluorescent intensity (MFI) of (a) phosphorylated p38MAPK and (b) total p38MPAK of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

5.1.1) p38MAPK – Analysis of ratio (phosphorylated to total amount) of p38MAPK

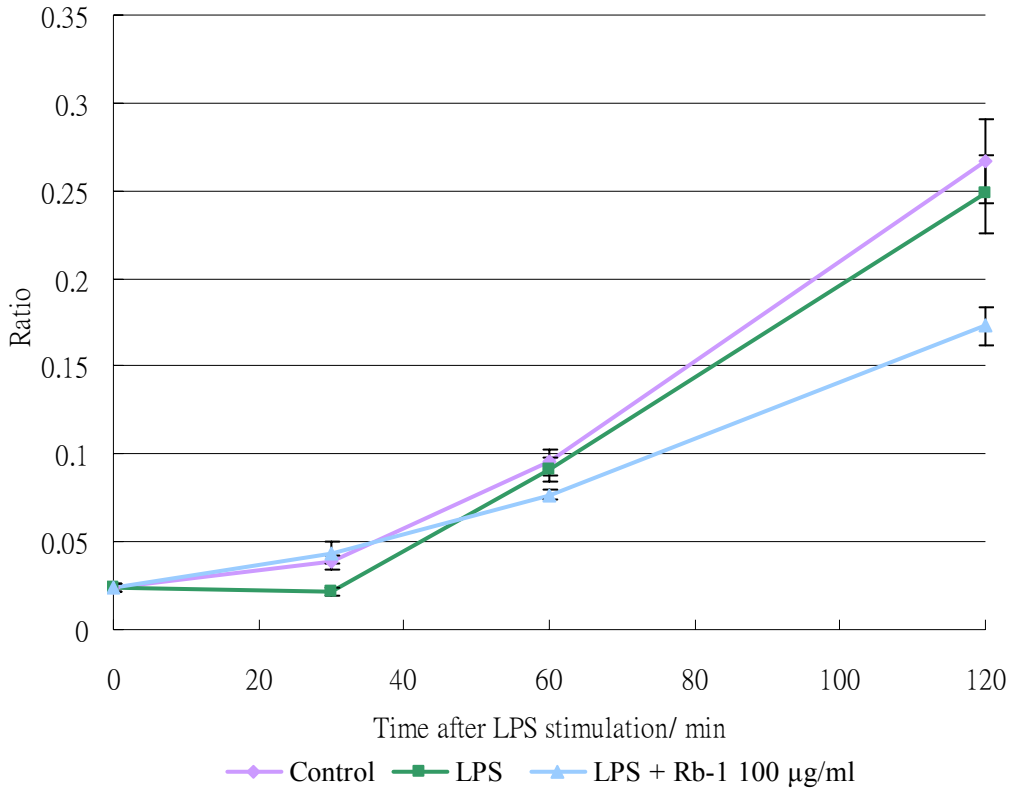


Figure 5.1c – Change of ratio of (a) phospho-p38MAPK / (b) total p38MAPK of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

From Figure 5.1(a-b), it was found that both phosphorylated form and total amount of p38MAPK in Rb-1 treated splenocytes was higher than that of those without treatment at 120 minutes. However, regarding to the ratios (Figure 5.1c), there was no significant differences in terms of phosphorylation activities.

5.1.2) JNK – Analysis of phosphorylated form and total amount of JNK

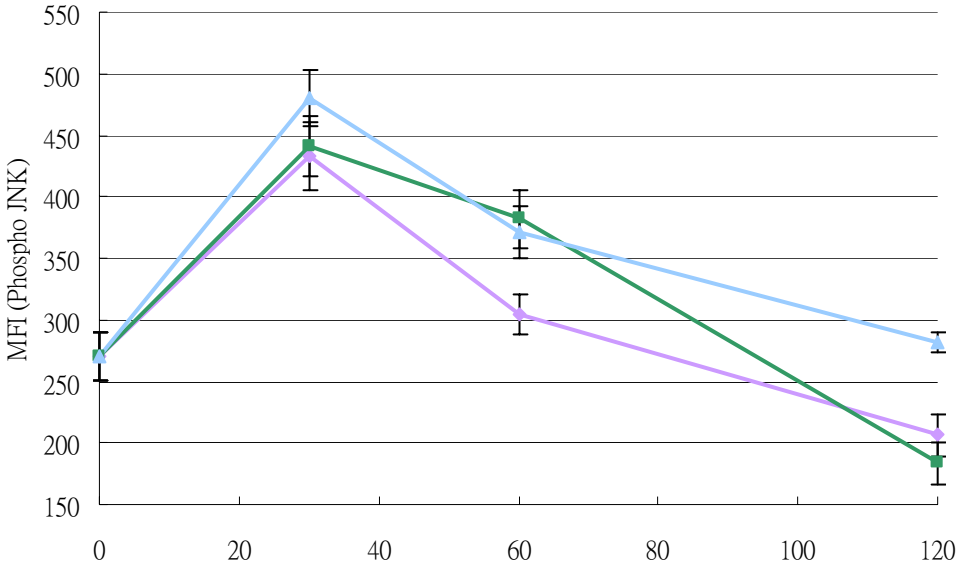


Figure 5.2a

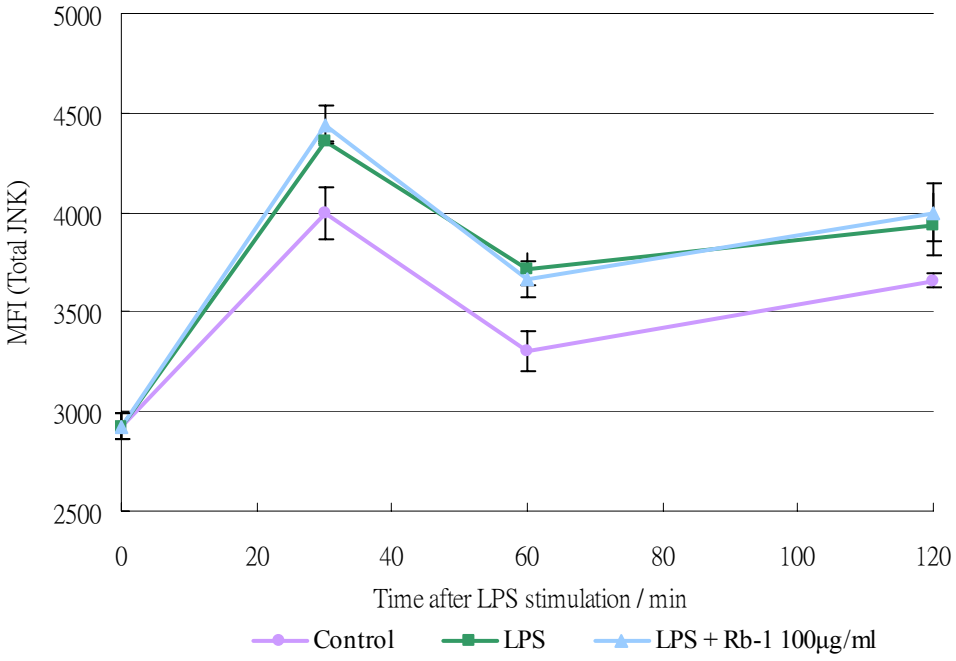


Figure 5.2b

Figure 5.2a & b – Change of mean fluorescent intensity (MFI) of (a) phosphorylated JNK and (b) total JNK of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

5.1.2) JNK – Analysis of ratio (phosphorylated to total amount) of JNK

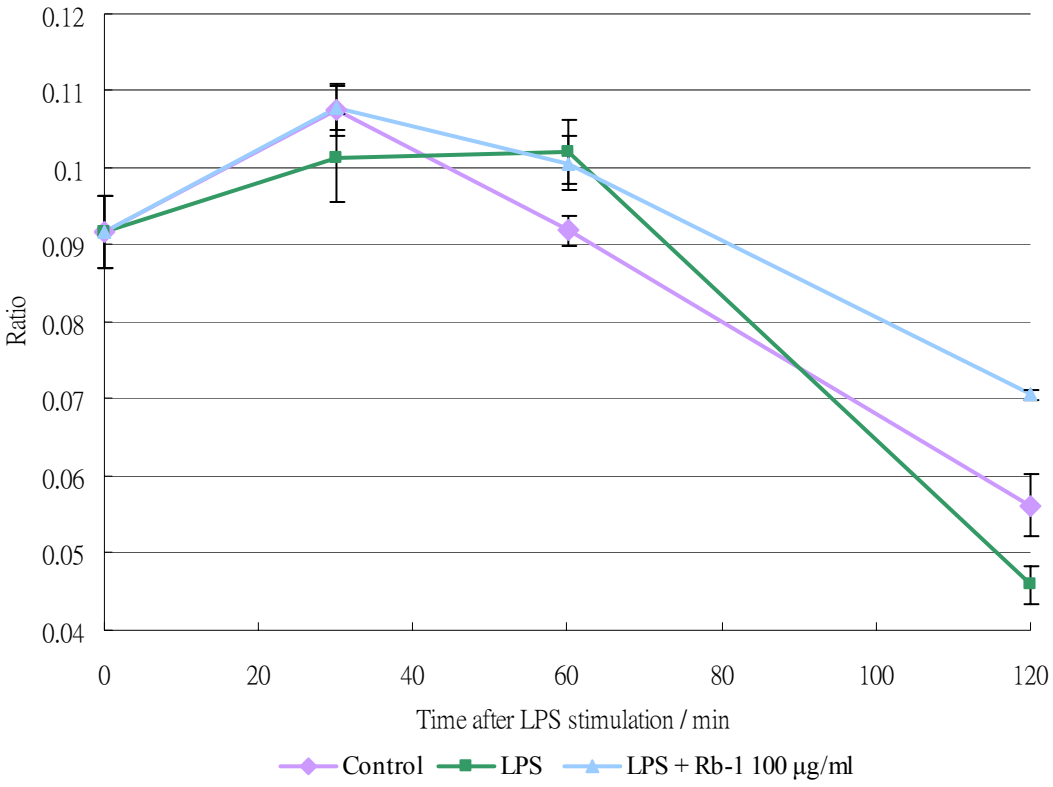


Figure 5.2c – Change of ratio of (a) phosphorylated JNK / (b) total JNK of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

In case of the JNK, in terms of phosphorylated form, total protein amount and ratio of phosphorylated to total protein, there was no differences between splenocytes with or without Rb-1 treatment. In addition, all three parameters demonstrated the same trend against time, as illustrated in Figure 5.2 (a-c).

5.1.3) ATF-2 – Analysis of phosphorylated form and total amount of ATF-2

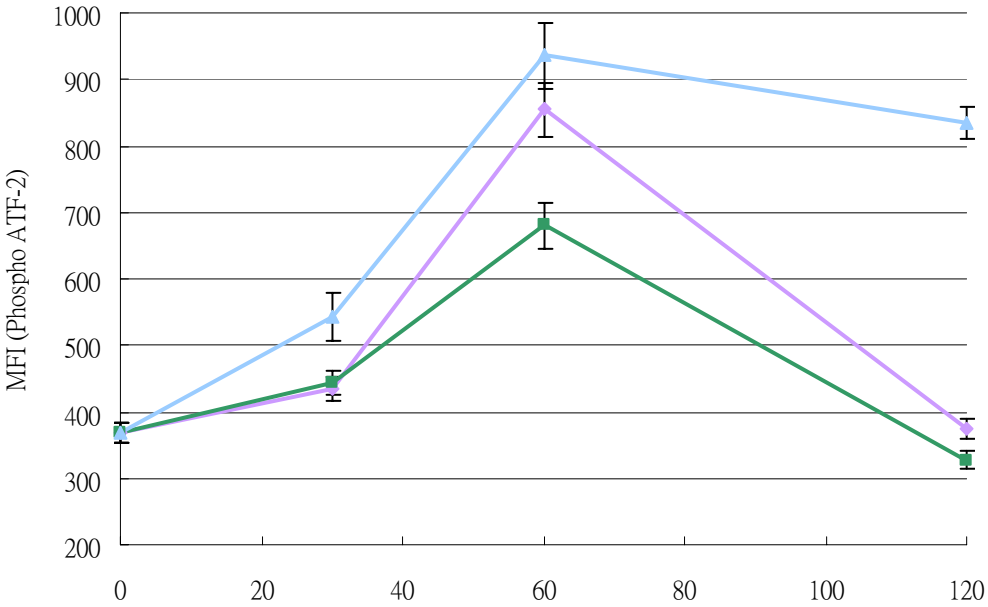


Figure 5.3a

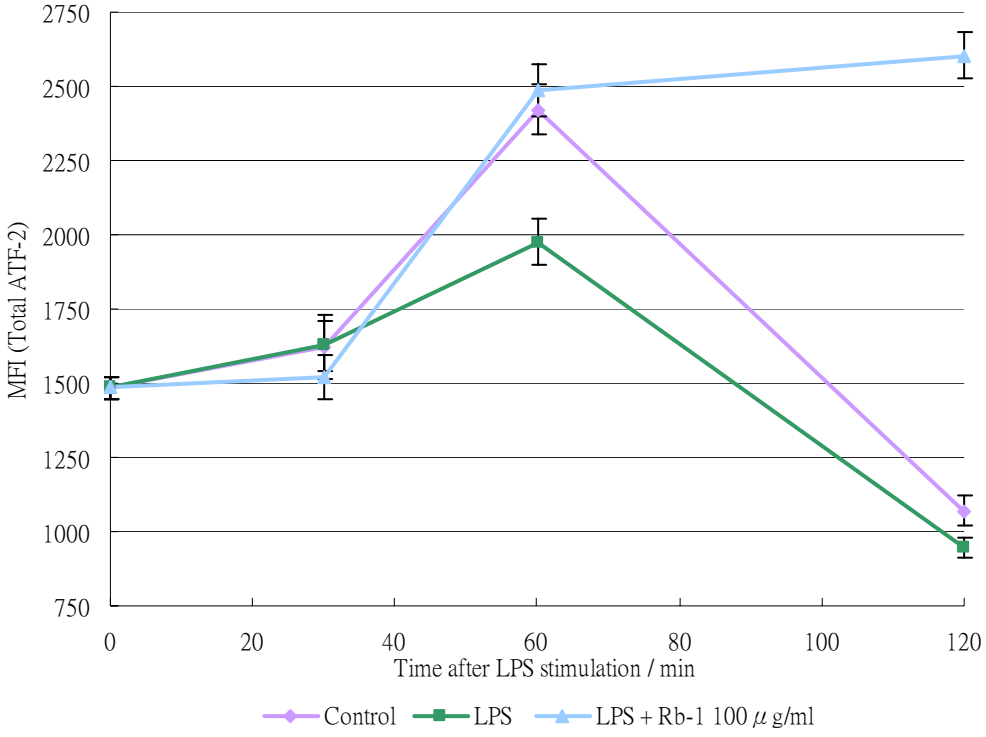


Figure 5.3b

Figure 5.3a & b – Change of mean fluorescent intensity (MFI) of (a) phosphorylated ATF-2 and (b) total ATF-2 of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

5.1.3) ATF-2 – Analysis of ratio of (phosphorylated to total amount) ATF-2

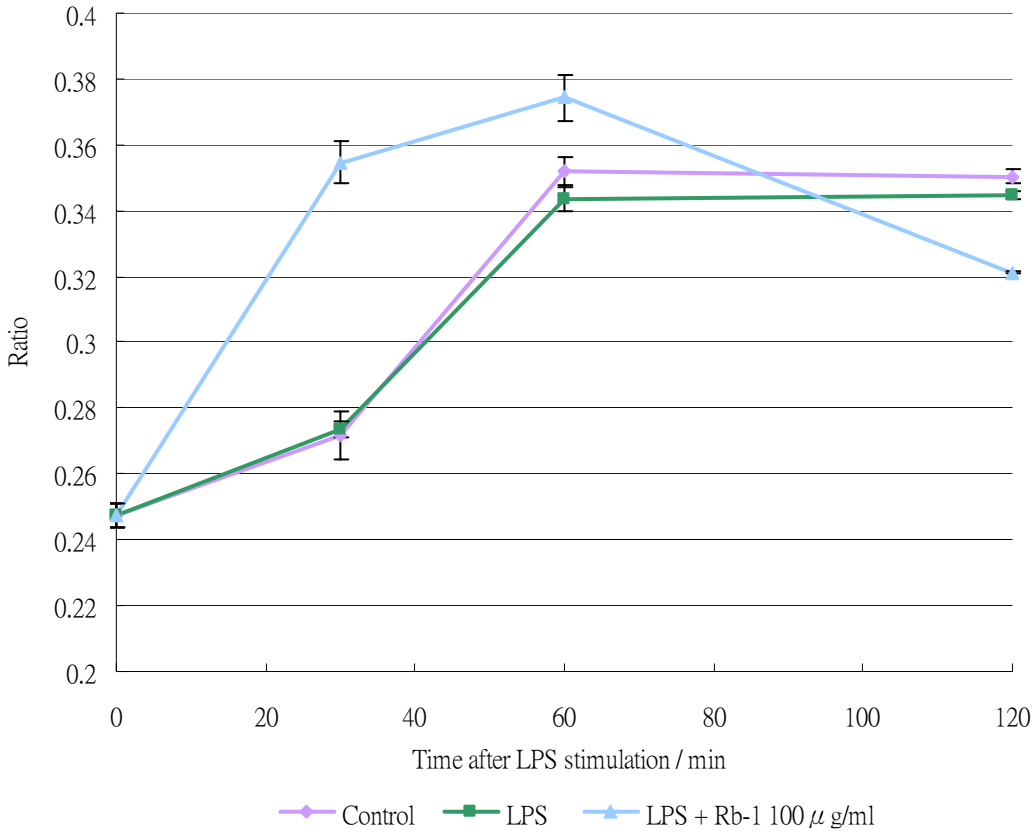


Figure 5.3c – Change of ratio of (a) phosphorylated ATF-2 / (b) total ATF-2 of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

In Figure 5.3a, phosphorylated form of ATF-2 in Rb-1 culture was slightly higher than that without Rb-1, especially from 60 – 120 minutes onwards. In case of the total protein expressed, Rb-1 treated cells also showed higher amount of total protein expressed at 120 minutes (Figure 5.3b). However, there was no difference in ratios at 120 minutes between the two groups (with or without Rb-1 and control). Instead, higher ratio of phosphorylated to total protein of ATF-2 was measured in Rb-1 treated cells at 30 minutes, as shown in Figure 5.3c.

5.1.4) p90RSK – Analysis of phosphorylated form and total amount of p90RSK

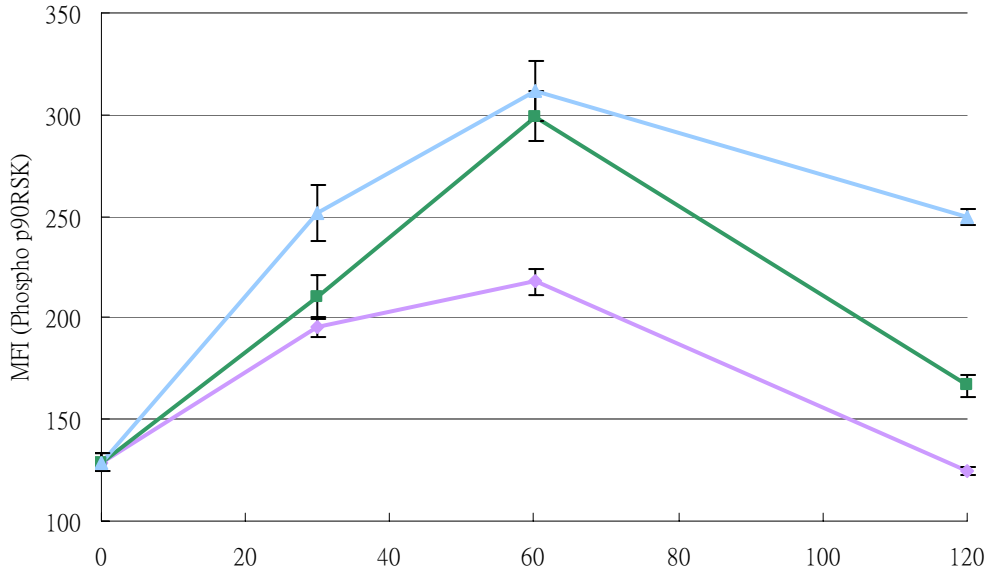


Figure 5.4a

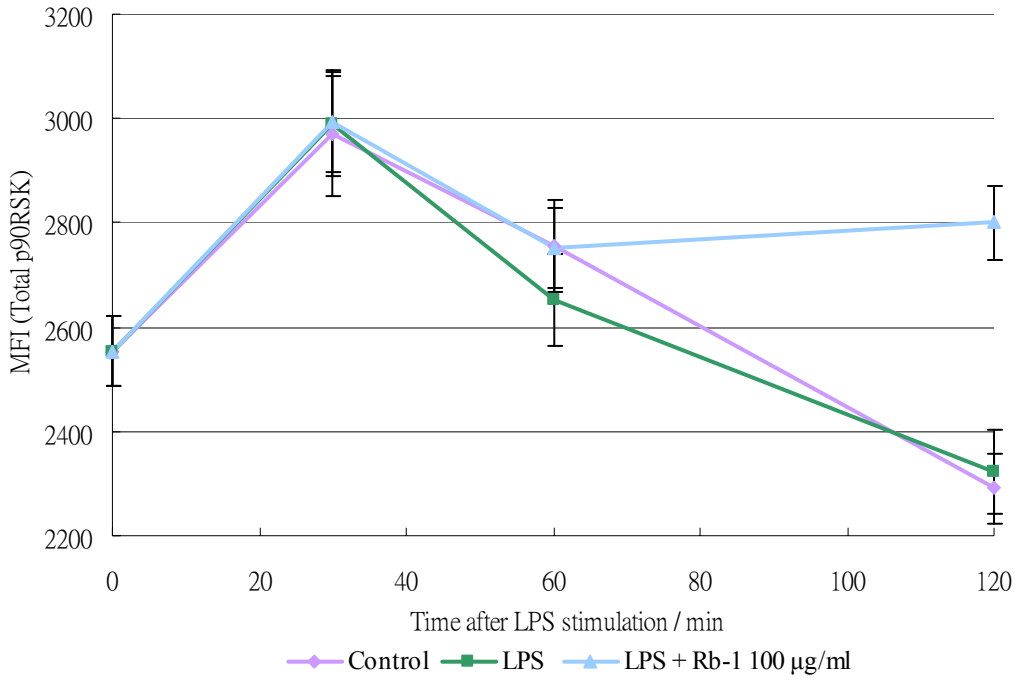


Figure 5.4b

Figure 5.4a & b – Change of mean fluorescent intensity (MFI) of (a) phosphorylated p90RSK and (b) total p90RSK of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

5.1.4) p90RSK – Analysis of ratio (phosphorylated to total amount) of p90RSK

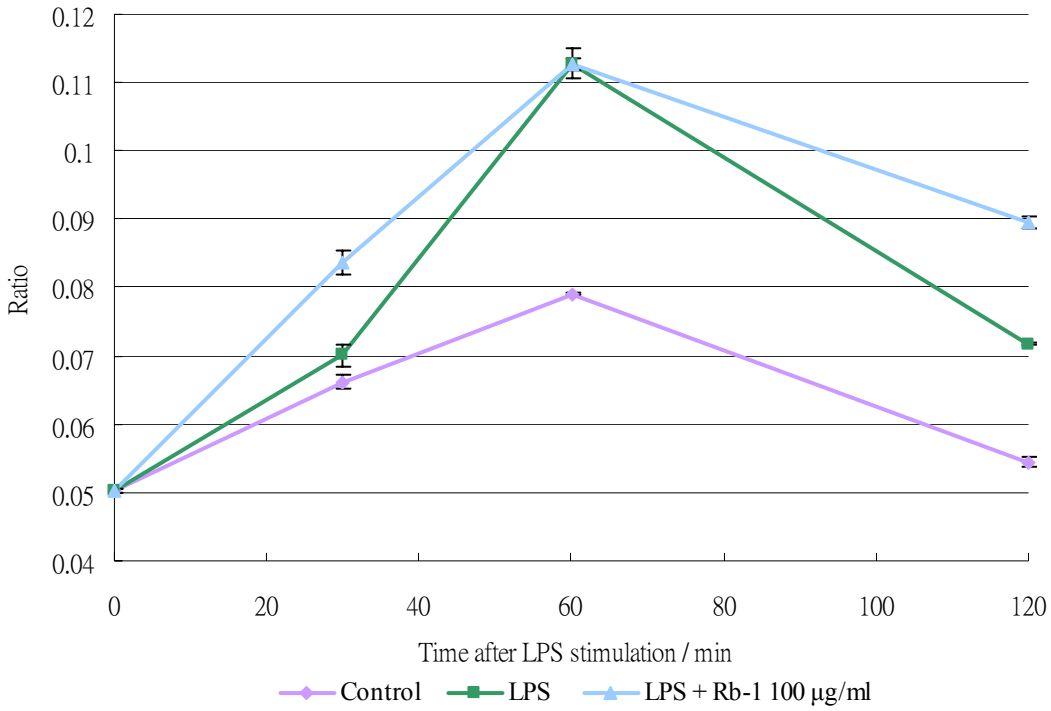


Figure 5.4c – Change of ratio of (a) phosphorylated p90RSK / (b) total p90RSK of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

In Figure 5.4a, more p90RSK was phosphorylated in Rb-1 treated cells than those without Rb-1. On the other hand, except the time-point at 120 minutes, levels of total p90RSK in both Rb-1 treated and without Rb-1 treated splenocytes were the same (Figure 5.4b). As regard to the phosphorylated/ total protein ratio, both groups (with Rb-1 and without Rb-1) showed similar trend. However, Rb-1 treated cells were slightly higher than that of control group, as illustrated in Figure 5.4c.

5.1.5) Akt – Analysis of phosphorylated form and total amount of Akt

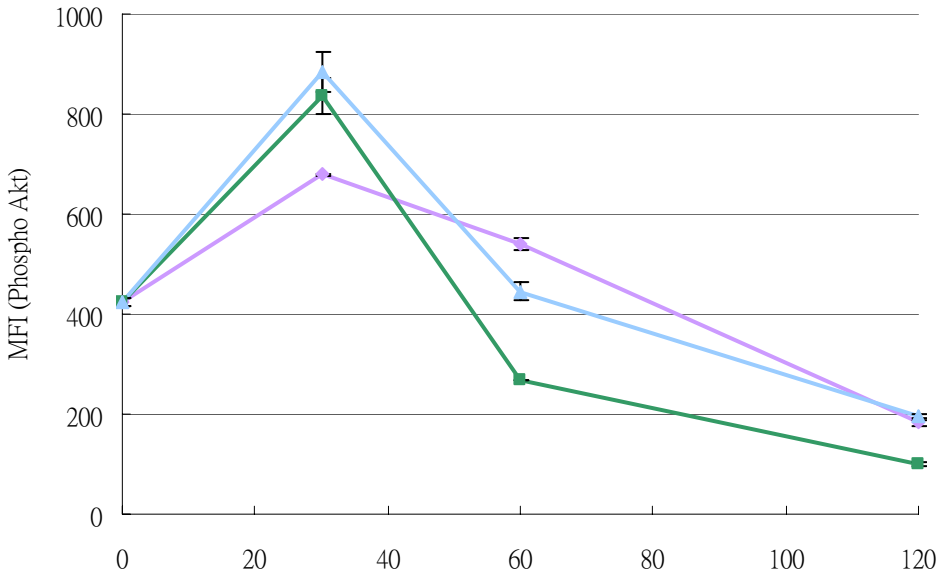


Figure 5.5a

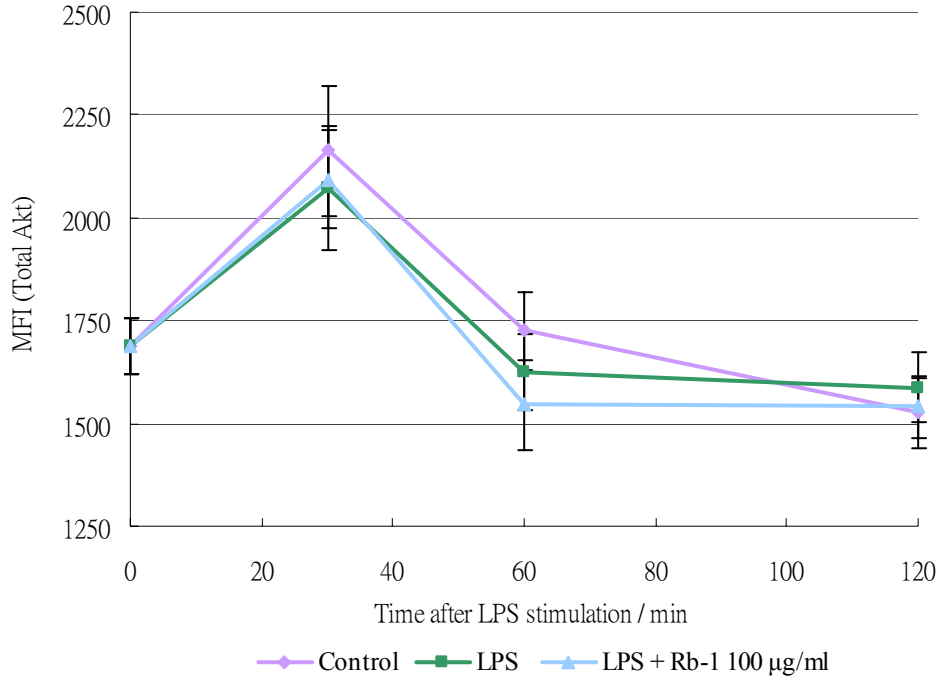


Figure 5.5b

Figure 5.5a & b – Change of mean fluorescent intensity (MFI) of (a) phosphorylated Atk and (b) total Atk of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

5.1.5) Akt – Analysis of ratio of (phosphorylated to total amount) of Akt

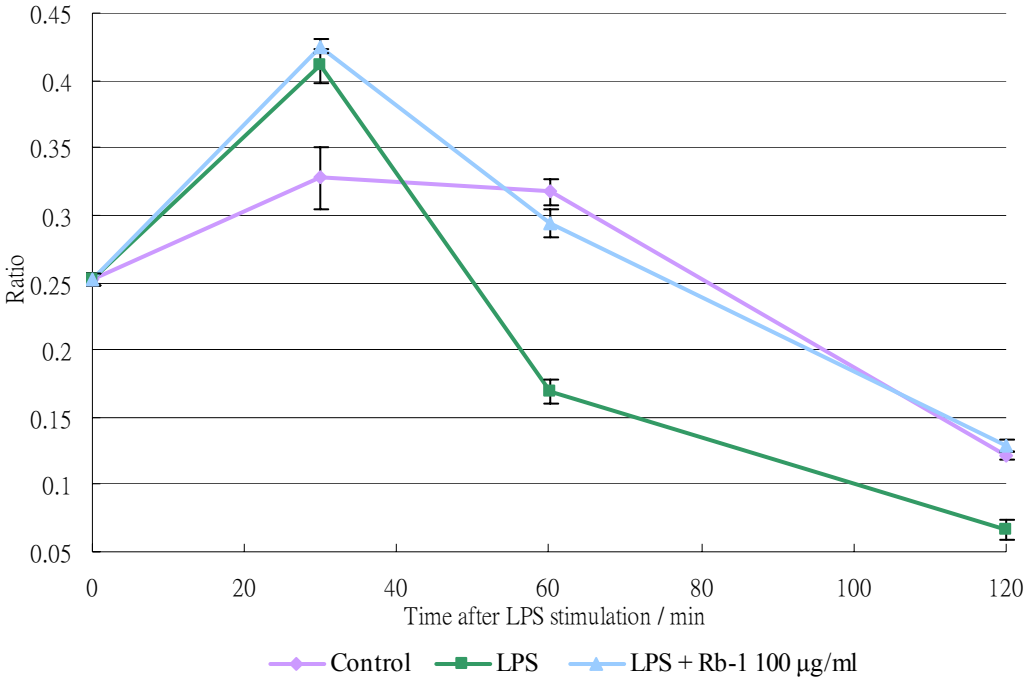


Figure 5.5c – Change of ratio of (a) phosphorylated Akt / (b) total Akt of splenocyte treated with or without Rb-1. Mean ± S.E.M (n = 8)

In the case of Akt, both splenocyte groups (with or without Rb-1) illustrated that there was similar trend in term of phosphorylated Akt. Except that the phosphorylation level in Rb-1 group was slightly higher than that of control (Figure 5.5a). However, Rb-1 group and without-Rb-1 group had the same total protein expression level of Akt (as illustrated in figure 5.5b). In term of phosphorylated Akt to total Akt, both groups demonstrated the same trend. Except at time 60 minutes, ratio of Rb-1 group was lower than control group (Figure 5.5c).

5.1.6) Analysis of phosphorylated form and total amount of inhibitor of κβ (I-κβ)

In this section, the amounts of total I-κβ, phosphorylated I-κβ were measured by the Bio-plex suspension array system. Again, 1.25 μg/ml LPS and 1.25 μg/ml Con-A would be applied as external stimulant against splenocyte. Detail protocol was described in materials and methods. However, experiments were conducted twice with different incubation period. They are 0 -120 minutes and 0 – 240 minutes respectively. The following graphs showed the results of the first section (0 – 120 minutes).

5.1.6.1) I-κβ – Analysis of phosphorylated form and total amount of I-κβ

(0 – 120 minutes)

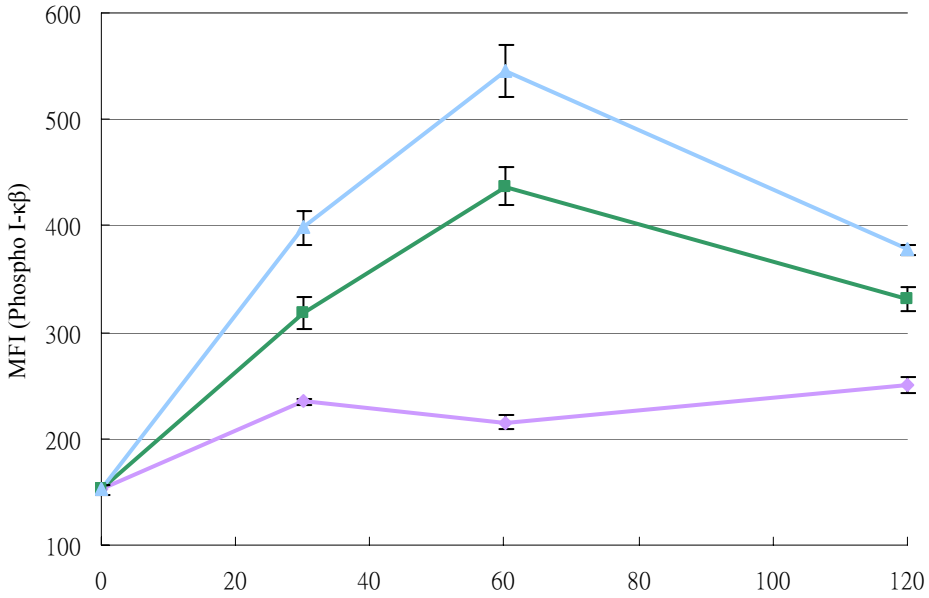


Figure 5.6a

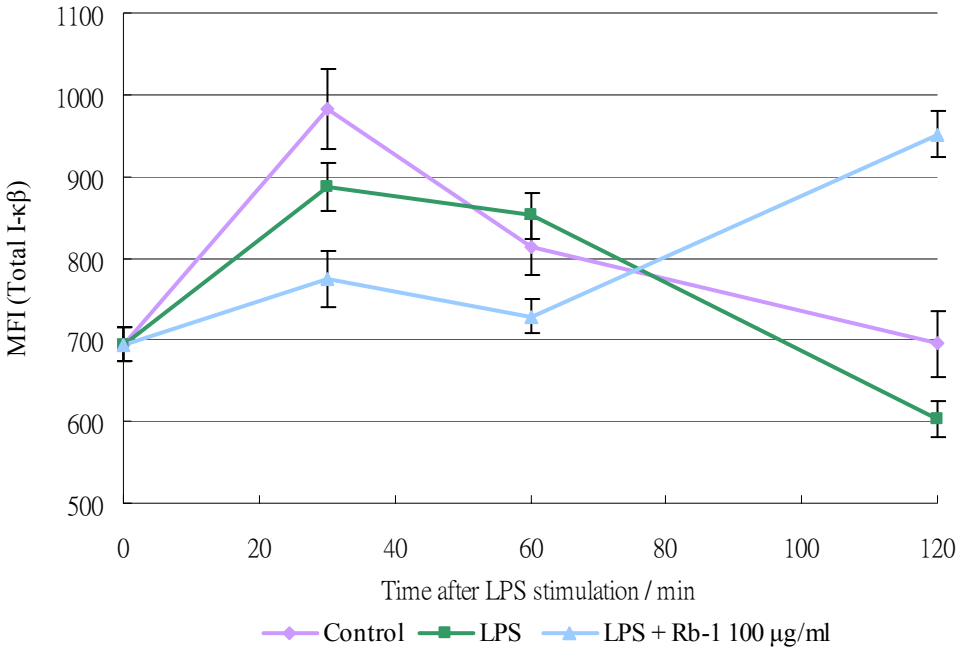


Figure 5.6b

Figure 5.6a & b – Change of mean fluorescent intensity (MFI) of (a) phosphorylated I-κβ and (b) total I-κβ of splenocyte treated with or without Rb-1 at 0 – 120 minutes. Mean ± S.E.M (n = 8)

5.1.6.1) I-κβ – Analysis of ratio of (phosphorylated to total amount) of I-κβ (0 – 120 minutes)

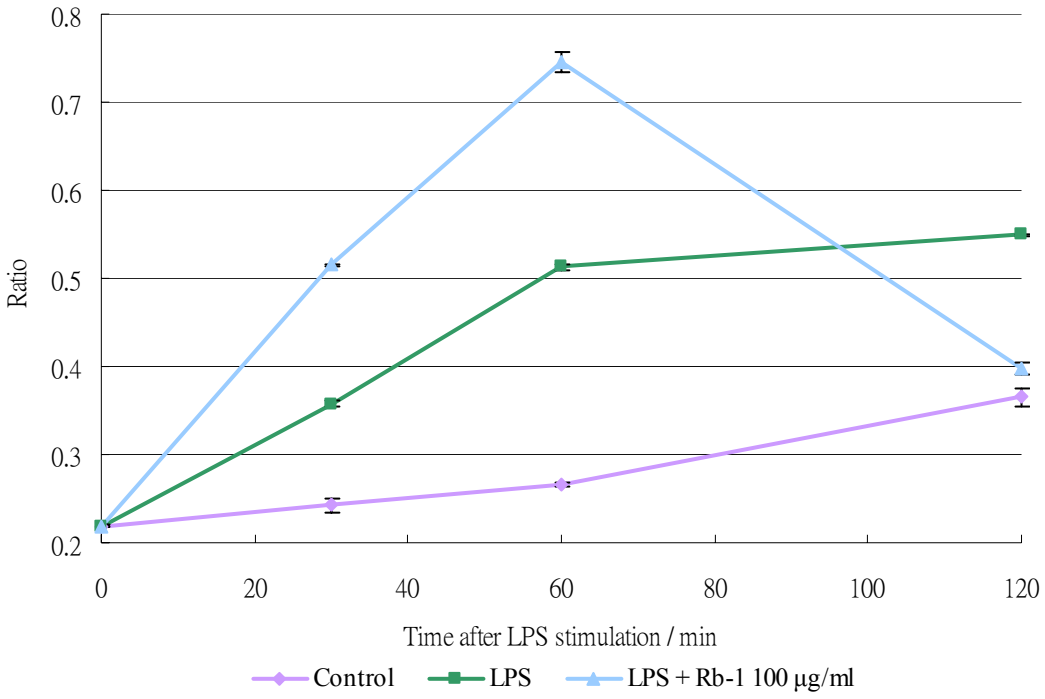


Figure 5.6c – Change of ratio of (a) phosphorylated I-κβ / (b) total I-κβ of splenocyte treated with or without Rb-1 from 0 -120 minutes. Mean ± S.E.M (n = 8)

In Figure 5.6a, it was observed that phosphorylated form of I-κβ in both Rb-1 treated splenocytes and without Rb-1 group was higher than that of control group. In addition, Rb-1 group was further higher than that of those without Rb-1 group. However, readings of total protein expressed in all groups seemed to be fluctuated widely, as illustrated in Figure 5.6b. In Figure 5.6c, however, there were differences among the three groups.

First of all, ratios of phosphorylated I-κβ to total I-κβ in the LPS group and the LPS + Rb-1 group were higher than that of control. Secondly, at 60 minutes ratio of LPS + Rb-1 group splenocytes was higher than that of the LPS group. Nevertheless, this situation was reversed at time 120 minutes and was mainly because of the sudden drop of readings in the LPS + Rb-1 group (Figure 5.6c).

5.1.6.2) I-κβ – Analysis of phosphorylated form and total amount of I-κβ (0 – 240 minutes)

In order to verify the phosphorylation activities of I-κβ, a longer incubation time period such as 240 minutes was used in this experiment. Results were shown as follows.

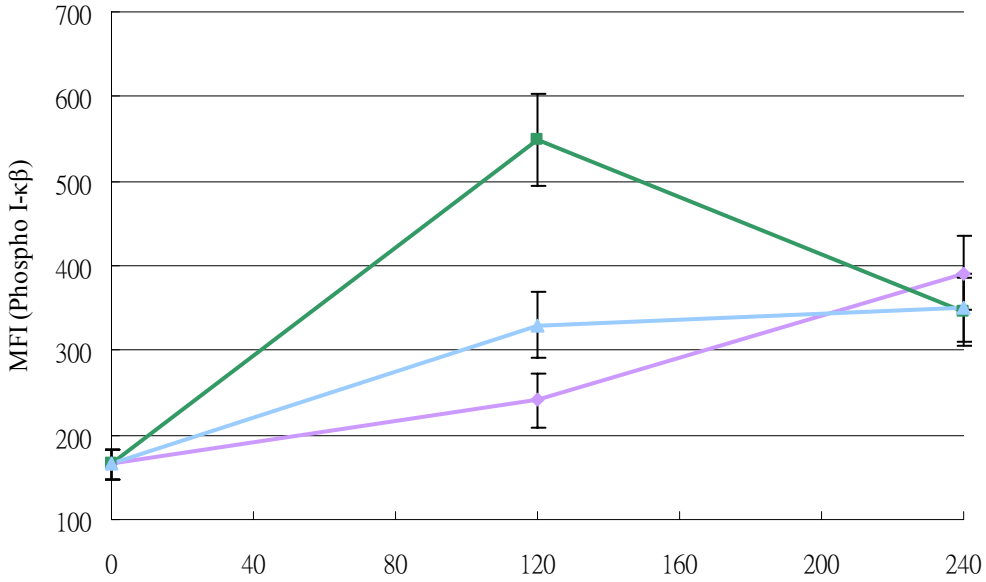


Figure 5.7a

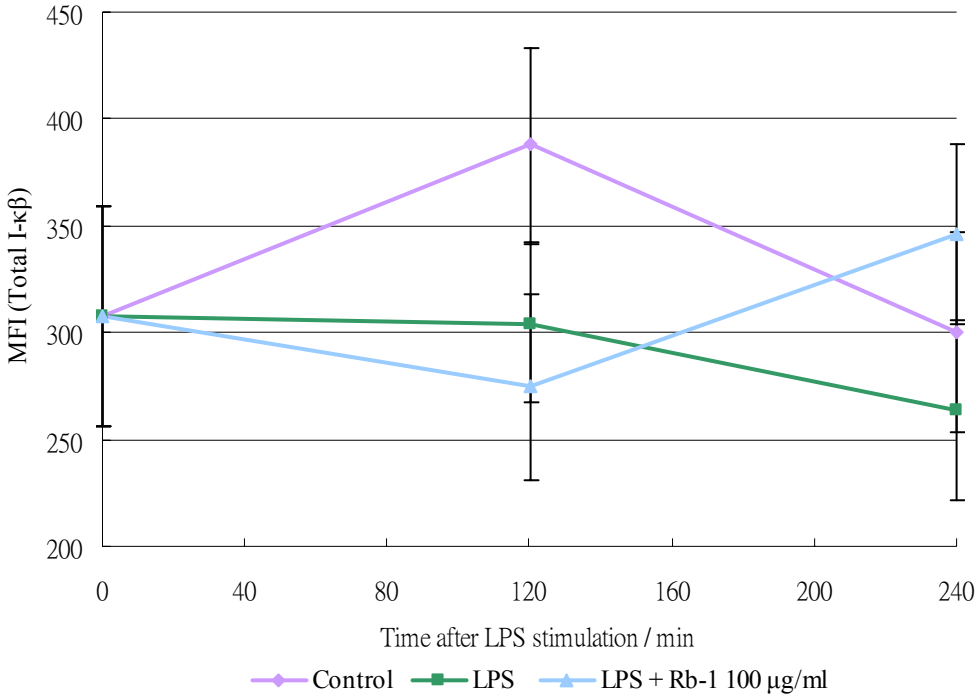


Figure 5.7b

Figure 5.7a & b – Change of mean fluorescent intensity (MFI) of (a) phosphorylated I-κβ and (b) total I-κβ of splenocyte treated with or without Rb-1 at 0 – 240 minutes. Mean ± S.E.M (n = 8)

5.1.6.2) I-κβ – Analysis of ratio of (phosphorylated to total amount) of I-κβ (0 – 240 minutes)

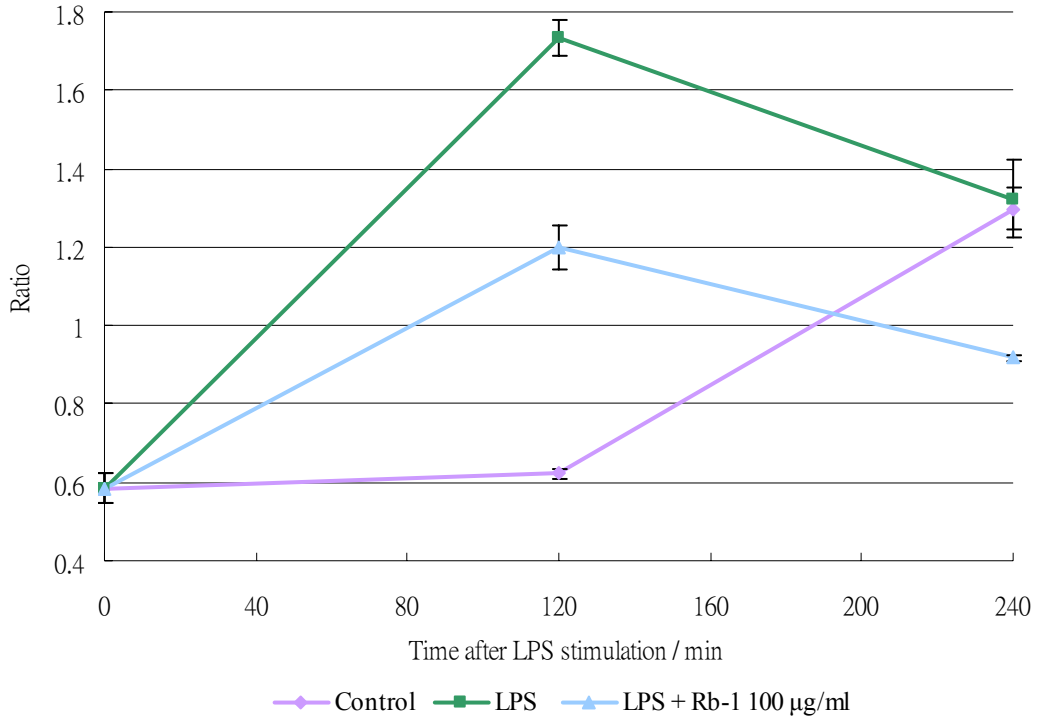


Figure 5.7c – Change of ratio of (a) phosphorylated I- κβ / (b) total I-κβ of splenocyte treated with or without Rb-1 from 0 -240 minutes. Mean ± S.E.M (n = 8)

From Figure 5.7a, similar to the last experiment with 120 minutes incubation time, phosphorylated I-κβ in LPS group and LPS + Rb-1 group was higher than that of the normal control group. Furthermore, it was found that phosphorylated I-κβ in LPS group at 120 minutes was higher than Rb-1 group. Again, measurement of total I-κβ expression in both Rb-1 splenocytes and without Rb-1 splenocytes was not consistent (Figure 5.7b). However, in term of

phosphorylated/ total ratio, same result was obtained when compared with last experiment (Figure 5.6c). That is, ratio of phosphorylated I-κβ to total I-κβ in LPS group was obviously higher than that of Rb-1 group at time 120 minutes and 240 minutes.

5.1.6.3) Summary of phosphorylation and total amount of I-κβ (0 & 120 minutes)

Since same results were obtained from the two experiments (5.6c & 5.7c). Readings of these Bio-plex experiments were grouped together appropriately and calculated again. The results were summarized in following figures.

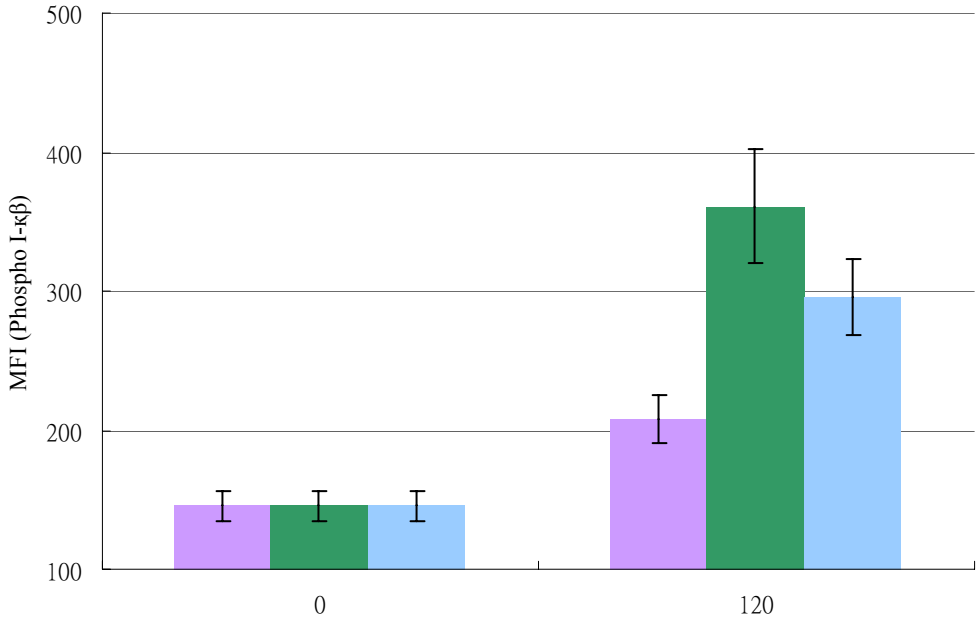


Figure 5.8a

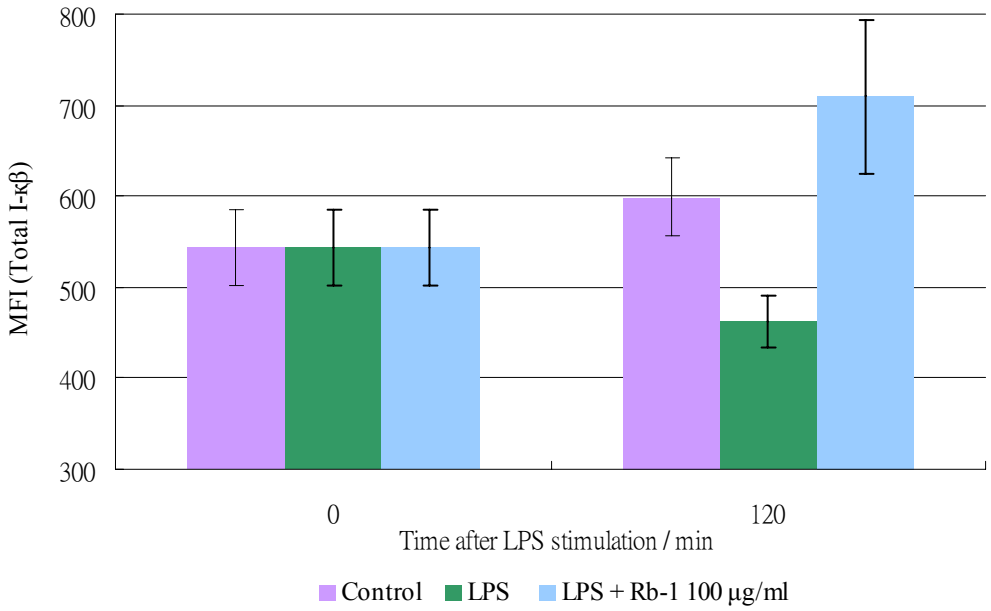


Figure 5.8b

Figure 5.8a & b – Summary of changes of mean fluorescence intensity (MFI) of (a) phosphorylated I-κβ and (b) total protein of I-κβ in splenocytes culture treated with and without Rb-1 from 0 – 120 minutes. Data showed as mean ± S.E.M (n = 8)

5.1.6.3) Summary of ratio (phosphorylation to total amount) of I-κβ (0 & 120 minutes)

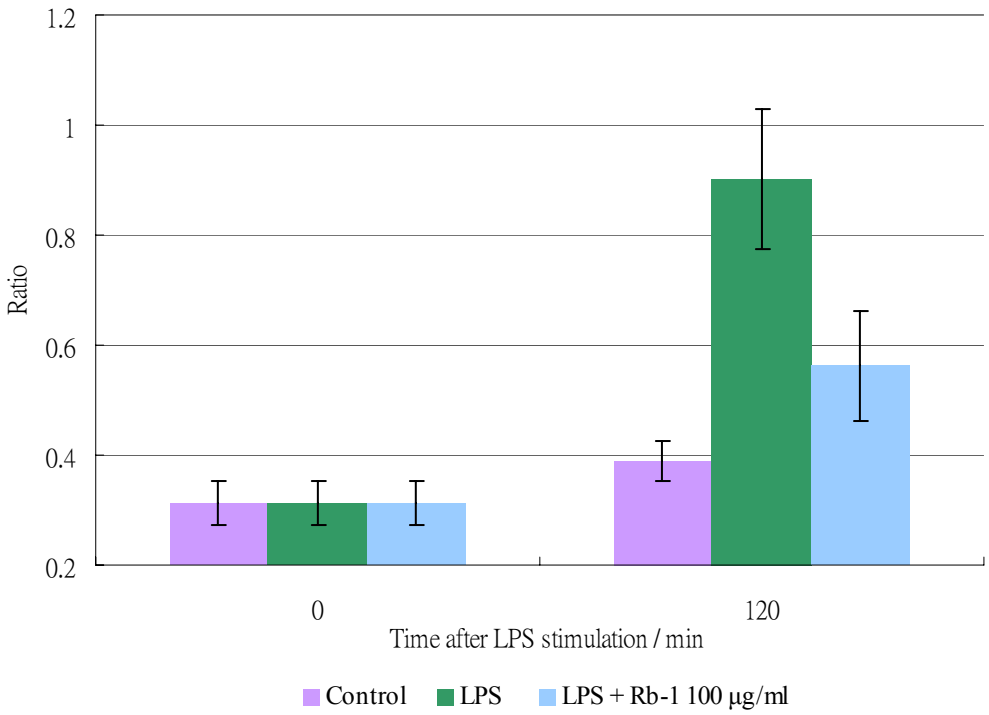


Figure 5.8c – Summary of changes of ratio of phosphorylated/ total I-κβ protein in splenocytes culture with or without Rb-1from 0 – 120 minutes. Data showed as mean ± S.E.M (n = 8)

In Figure 5.8a, it was found that phosphorylated I-κβ of LPS group and Rb-1-added/ LPS-stimulated group was higher upon LPS stimulation (0 min vs. 120 min). In addition, levels of phosphorylated I-κβ in both groups were also higher than that of the control group at time 120 minutes. However, I-κβ in LPS group was higher than that of Rb-1 group. Regarding to the total I-κβ expression,

total I-κβ expressed in LPS treated splenocytes was lower than that of Rb-1-added/ LPS-stimulated splenocytes. In Figure 5.8c, differences of phosphorylated I-κβ to total I-κβ ratio in LPS group and Rb-1-added/ LPS-stimulated group was found. In this figure, ratio of LPS group was obviously higher than that of the Rb-1-added/ LPS-stimulated group. It was probably mediated by having more I-κβ phosphorylated in the LPS group than that of the Rb-1 treated group, assuming total I-κβ amount was not different in the 3 groups.

5.2) Studies on expression of NF-κβ

In the last section, phosphorylation activities of I-κβ were investigated. Bio-plex result showed that phosphorylation I-κβ was suppressed by Rb-1 in the first few hours of incubation. It was then speculated that activity of NF-κβ was also influenced because of I-κβ inactivation. In other words, NF-κβ dimer was not dissociated from the I-κβ-complex and was not free for translocation. In order to validate this hypothesis, cytoplasm as well as nuclear extracts of splenocytes incubated with Rb-1, were probed with anti-NF-κβ antibodies in a Western blotting set-up. The following figure showed the results obtained.

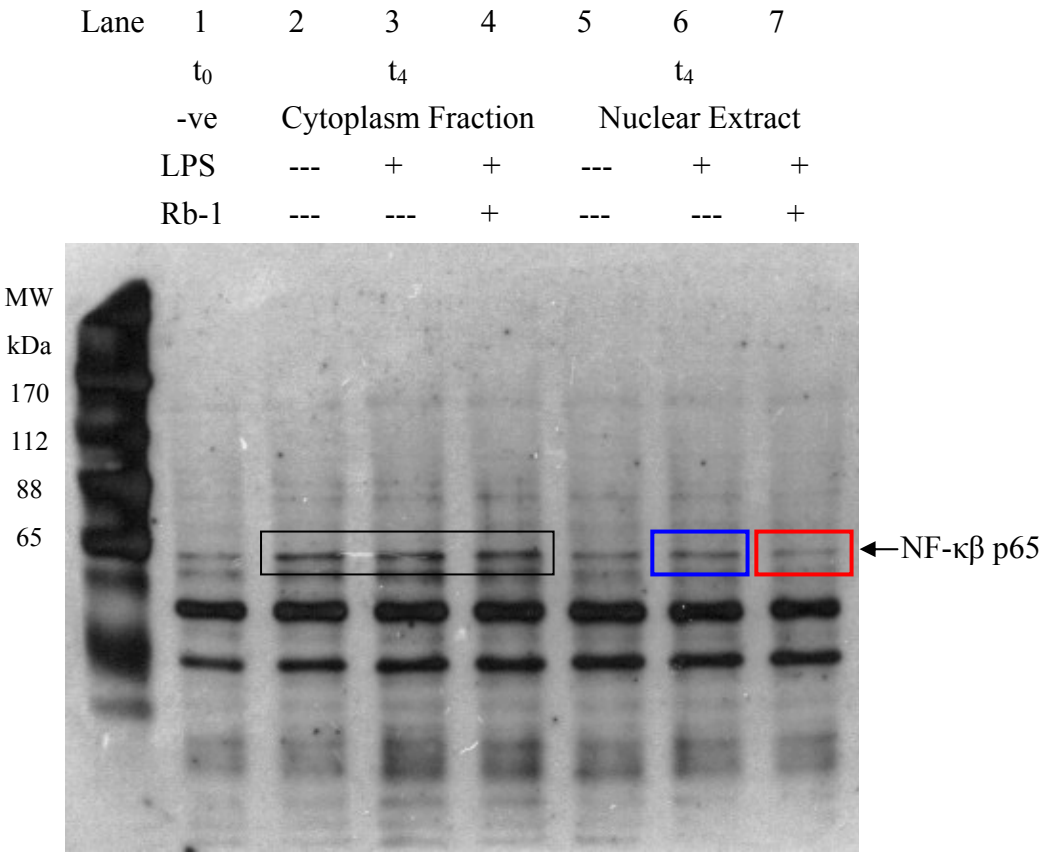


Figure 5.9 – Representative Western Blot result of NF-κβ p65 in cytoplasm and nucleus extracts after LPS activation. (t_0 = zero time-point; t_4 = samples taken at the fourth hour after incubation)

In Figure 5.9, it was found that amount of the NF-κβ in cytoplasm was elevated after LPS stimulation (comparing line 2 with other lines in the black box). It was because LPS triggered several signaling pathways that induced NF-κβ activation. As a result, a portion of NF-κβ was released from its inhibitor and present in cytosolic compartment. Due to instant translocation, amount of NF-κβ was increased in nuclear extract (Blue box) in splenocytes. In Rb-1-supplemented

splenocytes, however, level of NF-κβ was lowered than that of without Rb-1 culture (Red box). This result was in line with those obtained on the levels of I-κβ and its phosphorylated form using the Bio-plex. Hence, it was evident that Rb-1 inhibited NF-κβ translocation by suppression of phosphorylation of I-κβ.

Chapter 6) Two dimensional-polyacrylamide gel electrophoresis (2D-PAGE) analysis of protein expression in splenocytes with or without the presence of Rb-1

As evident from results presented in the previous chapters, it was found that ginsenoside Rb-1 suppressed LPS-induced cytokines secretion. Moreover, Rb-1 mediated its suppression through the inhibition of the phosphorylation of the inhibitor I-κβ, resulting in decrease of NF-κβ translocation from cytoplasm into the nucleus (Figure 5.9). Although LPS activation in immune cells had been well documented (Triantafilou *et al.*, 2002; Asehnoune *et al.*, 2005), not much on collaterally stimulated pathways triggered concurrently by LPS were defined. It was of interest to study if Rb-1 alters other cellular functions. Therefore, the differential proteome expressions of splenocytes responding to LPS and Rb-1 were studied. Due to limited splenocytes and other resources, only a specific dosage of Rb-1 (100 μg/ml) was applied in splenocytes culture in this study.

Before attempting any comparison, we had made several assumptions: (a) LPS induced splenocytes activation will result in altered protein expression; (b) Rb-1 affected splenocytes activation and hence its protein expression; (c) Rb-1 would modulate LPS-induced protein expression in splenocytes. To validate these

assumptions, different sets of 2D proteome maps were established first. They were (i) normal splenocyte; (ii) LPS-stimulated splenocytes and (iii) Rb-1-added splenocytes and (iv) Rb-1-added / LPS-stimulated splenocytes. Make up of these groups were summarized in the following table:

Group	Splenocytes	LPS	Rb-1 (100 μg/ml)
(i) Normal (control)	√	X	X
(ii) LPS-responsive	√	√	X
(iii) Rb-1-responsive	√	X	√
(iv) Rb-1-modulated/ LPS-added	√	√	√

Table 6.1 – Composition of varies treatments of different groups

(√ = presence; X = not added)

In order to investigate the activities of Rb-1, these 2D-maps were compared. For instances, by comparing group (i) and (ii), protein-expression altered by LPS was labeled first (e.g. blue dot-line region) (Figure 6.1). Secondly, Rb-1-induced protein expression was also obtained by comparison between groups (i) and (iii) (since there were no differences in protein expression, hence results were not shown). Lastly, those differentially expressed protein spots shown between groups (ii) and (iv) were assigned as Rb-1-modulated proteins (red line region)

(Figure 6.1). Applying this strategy, immunomodulatory effects of Rb-1 in term of differential protein expression, other from known LPS pathways (e.g. NF-κβ and MAPK etc), were investigated. The strategy of 2D-map proteome study was outlined schematically as follows:

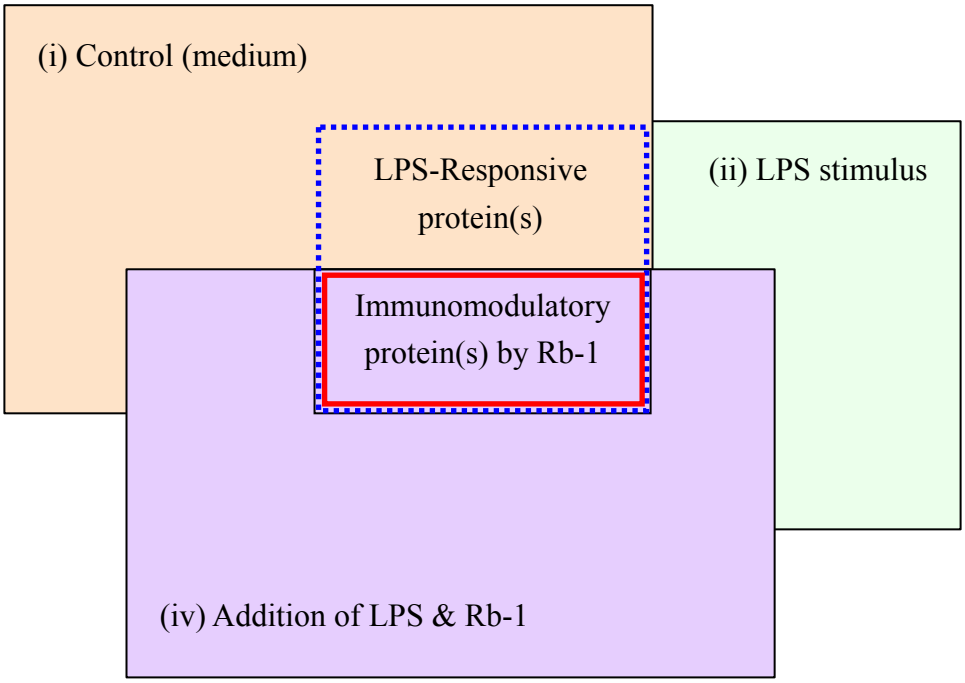


Figure 6.1 – Strategy of 2D-PAGE analysis (in studying immunomodulatory effect of Rb-1). By this strategy, those differential expressed proteins under LPS-influence was compared and labeled (Blue area ⋯⋯). Those Rb-1-modulated LPS-induced protein(s) were marked within red region (——)

Prior to image analysis, 2D-maps were established according to the protocol as described in Materials and Methods (Section 2.8). However, in order to have a better picture on the effects induced in the subcellular compartments, two cellular fractions were prepared. One was the cytosolic compartment and the other was the nuclear extract (NE) (this NE was the same sample used in western blot analysis before). Protein samples were prepared (with the addition of 0.1% NP-40) as previously described.

Briefly, 2D-maps of cytosolic fractions of each group were performed by using pH gradient 5 – 8 and 50 μg of protein sample was loaded in each 2D-map. 2D-maps of cytosolic fraction were performed in triplicates. On the other hand, nuclear extracts were prepared in group (ii) (LPS-added splenocytes) and group (vi) (LPS-/ Rb-1-added splenocytes) were performed by using pH gradient 6 – 11 with 50 μg load of proteins. 2D-maps were then visualized by silver staining (Shevchenko *et al.*, 1996).

6.1) Comparison of 2D-map of cytosolic fraction between (i) control and (ii) LPS-stimulated splenocytes

In this section, it was aimed to find LPS-responsive proteins. Therefore, 2D-maps of control (normal splenocytes) and LPS-stimulated splenocytes were compared. In the experiment, proteins samples were performed in triplicates. The following figures showed a representative 2D-map of normal splenocytes (Figure 6.2) and that of LPS-stimulated splenocytes (Figure 6.3).

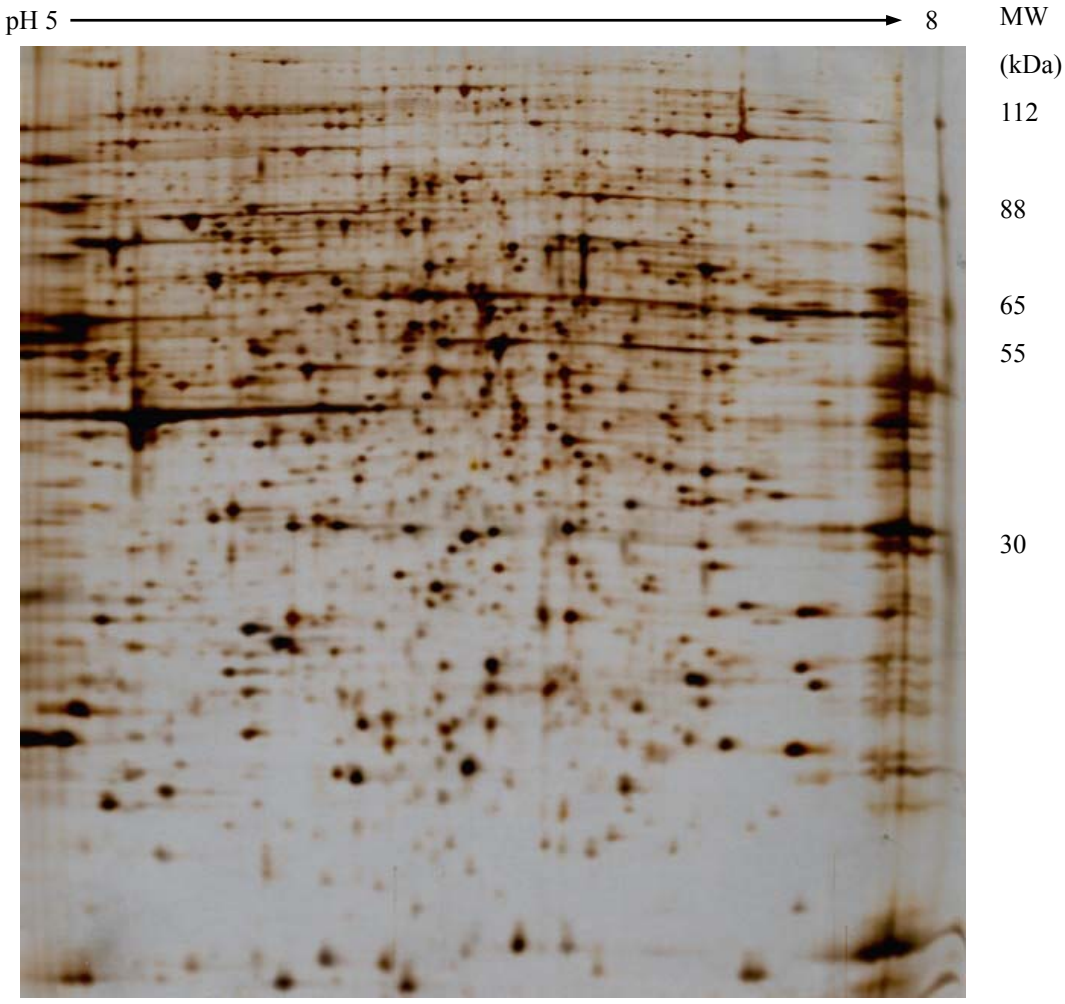


Figure 6.2 – 2D-map of cytosolic fraction of normal splenocytes [Group (i)] (50 μg protein, pH 5 – 8, 10% gel)

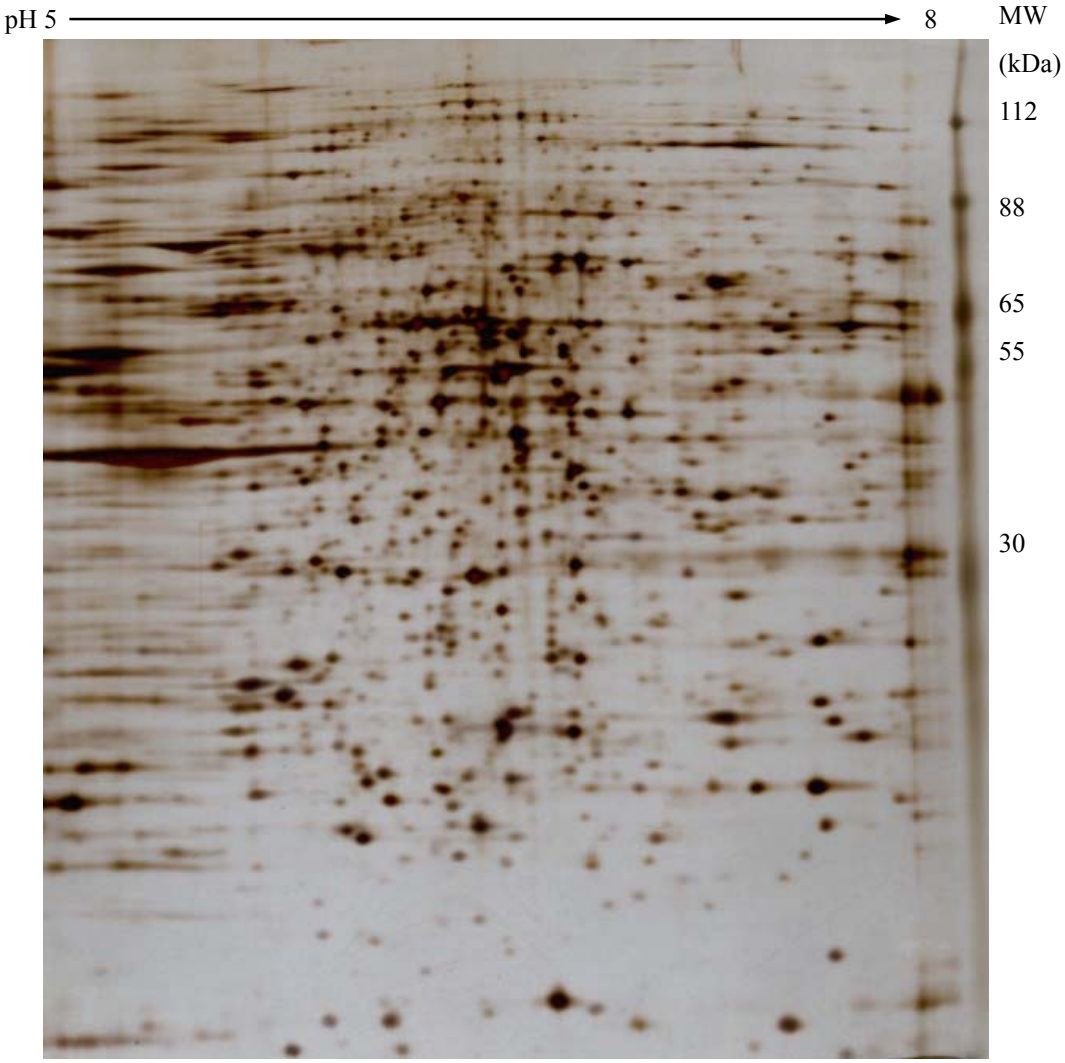


Figure 6.3 – 2D-map of cytosolic fraction of LPS-stimulated splenocytes [Group (ii)] (50 μg protein, pH 5 – 8, 10% gel)

2D-map images analysis were processed by gel alignment, calibration and normalization with the aid of software (Melanie v4.0, Gene-Bio). Firstly, during the staining process, dimensions of each 2D-map would vary and interfere with the spot detection process later. Therefore, images of each-pair gels were aligned into same dimension before spot detection, calibration and normalization. This

was achieved by labeling different landmarks onto the images manually, neglecting the expression level but exact spot position. By matching and pairing these landmarks, two gels with different dimension scales were aligned and fitted. As a result, different protein spots were matched and paired between two 2D-maps (Figure 6.4).

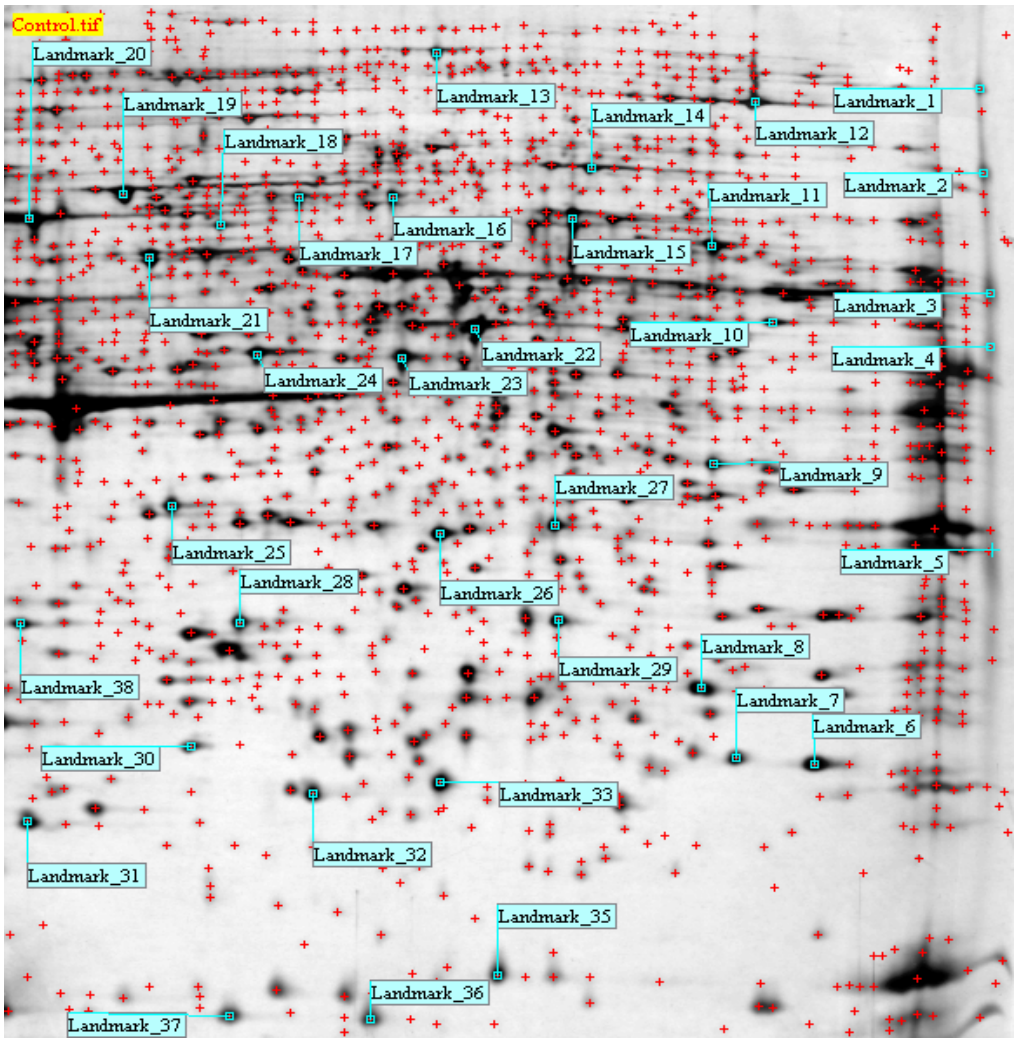


Figure 6.4 – Arbitrary landmarks of 2D-map [2D-map of control was used as reference (Figure 6.2)]

In case of Figure 7.4, a total of 38 landmarks were evenly assigned on the 2D-map of control splenocytes arbitrarily. Assignment of evenly distribution landmarks would facilitate successful image comparison of the 2D-maps. Subsequently, two different 2D-maps of two different samples could be paired-up for comparison. Results of pairing of (i) control and (ii) LPS-stimulated splenocytes were shown in Figure 6.5 below.

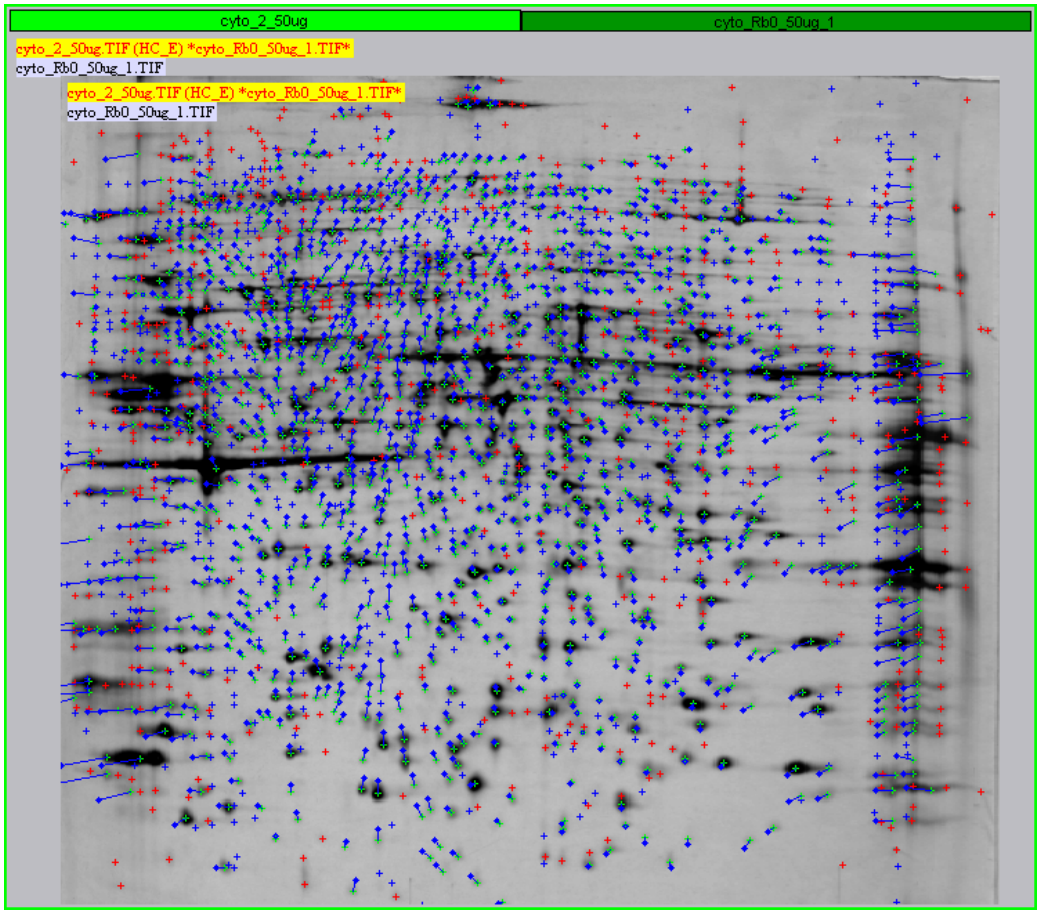


Figure 6.5 – Pairing of 2D-map of (i) control group and (ii) LPS-stimulated splenocytes

In this Figure 6.5, spots were shown in green or red that was linked by a blue arrow. Green spots represented protein spots on 2D-map of control while red spots represented protein spots on 2D-map of LPS-stimulated group. Blue arrow meant those matched and paired protein spots between two groups. After pairing, protein spots were then analyzed and summarized as follows tables.

	Control (Normal)	LPS-stimulated		
2D-map	No. of spots detected	No. of spots detected	Matched Pair of spots	Percentage matched
1	1307	1232	937	73.8 %
2	1075	1127	685	61.9%
3	1289	1506	959	68.6%

Table 6.2 – Summary of 2D-maps analysis between control and LPS-stimulated splenocytes.

After spots detection and pairing, differential protein expression comparison, i.e. difference in level of expression, was performed. Since the image was visualized by silver staining, it was necessary to calibrate the staining or sensitivity variation. In case of paired 2D-maps (e.g. Figure 6.2 & 6.3), spot volume (Vol) reading of each protein (on each map) was measured by the software Melanie. Subsequently, these reading was scattered in terms of [Spot] against [Vol] for one

2D-map (e.g. Figure 6.2) while another 2D-map (e.g. Figure 6.3) was plotted [Vol] against [Spot] (Appendix XI). By combining two scatter plots, the slope value (*m*) provided the calibration constant. By changing spot detection parameters, each 2D-map was calibrated according to this calibration constant. In other words, two distinct 2D-maps were then calibrated to each other as a result. Moreover, higher calibration constant value also indicated higher reproducibility between the two proteome maps. Furthermore, in this study, spot expression level was measured by the volume [Vol] reading instead of optical density (OD). Protein expression was normalized by spot Vol/ total Vol (% Vol). Subsequently, the percentage of Vol (% Vol) of each spot was referred as expression level. Expression level of protein with 3-fold differences was then classified arbitrarily as differentially expressed proteins.

As mentioned previously, each group of samples were performed in triplicates. Among these three sets of proteome maps (control vs. LPS-stimulated), a total of 32 protein spots were found to have either up-regulated or down-regulated in LPS-stimulated splenocytes. These proteins were illustrated as the following figures.

(i) Control (normal)

(ii) LPS-stimulated splenocyte

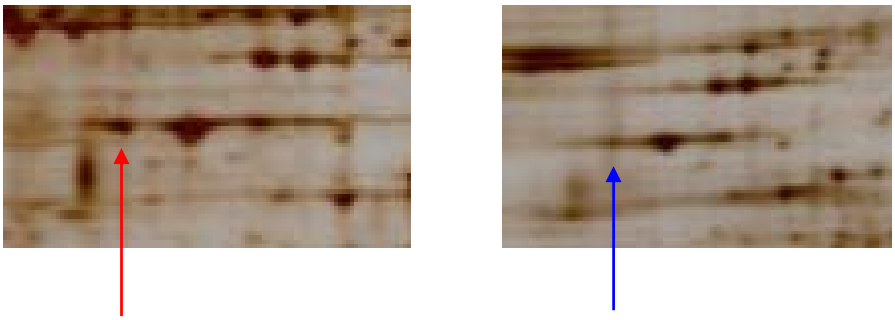


Figure 6.6a

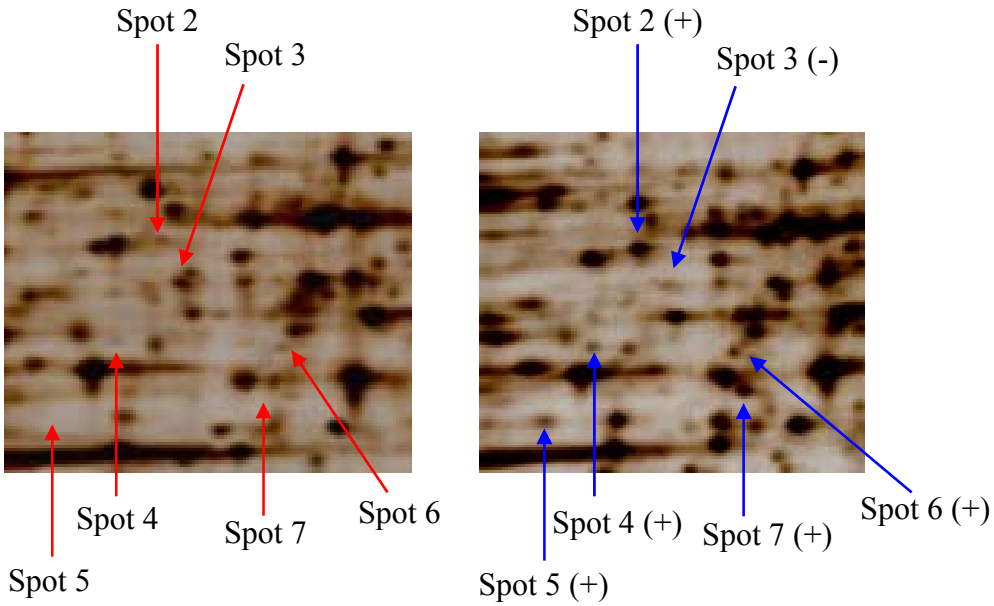


Figure 6.6b

Figure 6.6a-b –Zoomed regions of 2D-maps of differential proteins (a: spot 1; b: spot 2 – 7) between (i) control group and (ii) LPS-stimulated splenocytes (Only representative 2D-maps were presented) [(-): down-regulated protein; (+): up-regulated protein]

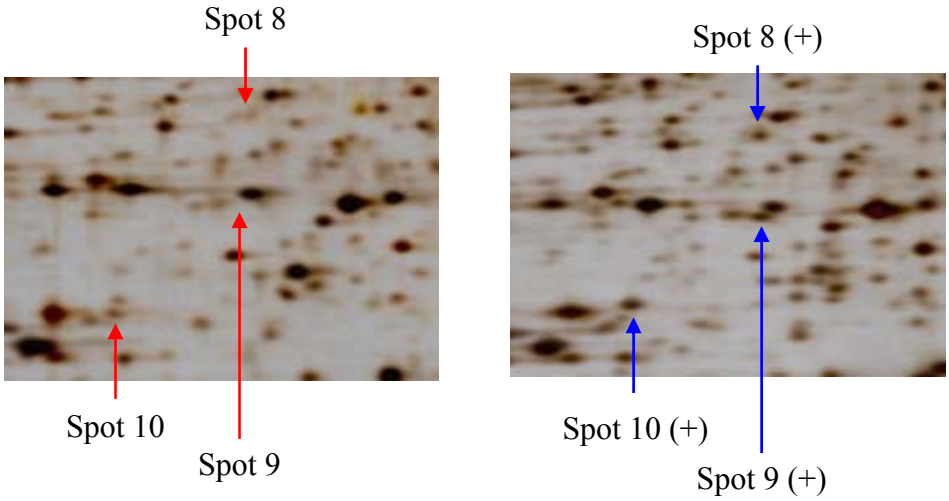


Figure 6.6c

Figure 6.6d

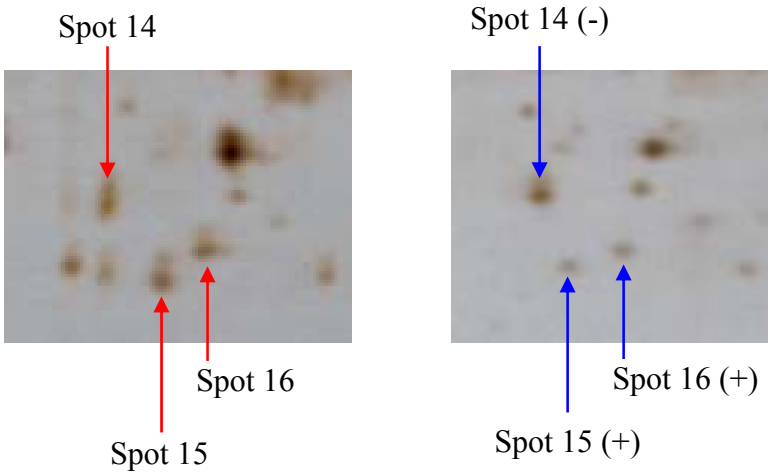


Figure 6.6e

Figure 6.6c-e – Zoomed regions of 2D-maps of differential proteins (c: spot 8 – 10; d: spot 11 – 13; e: spot 14 – 16) between (i) control group and (ii) LPS-stimulated splenocytes (Only representative 2D-maps were presented) [(-): down-regulated protein; (+): up-regulated protein]

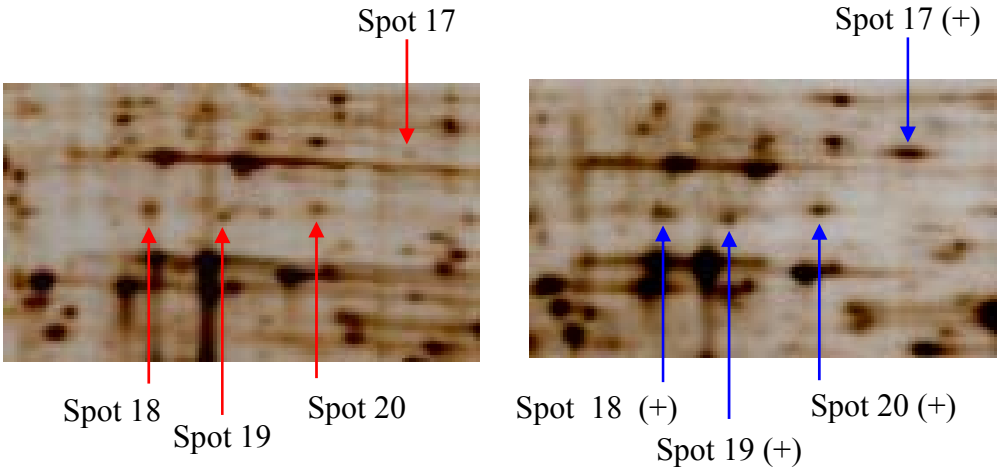


Figure 6.6f

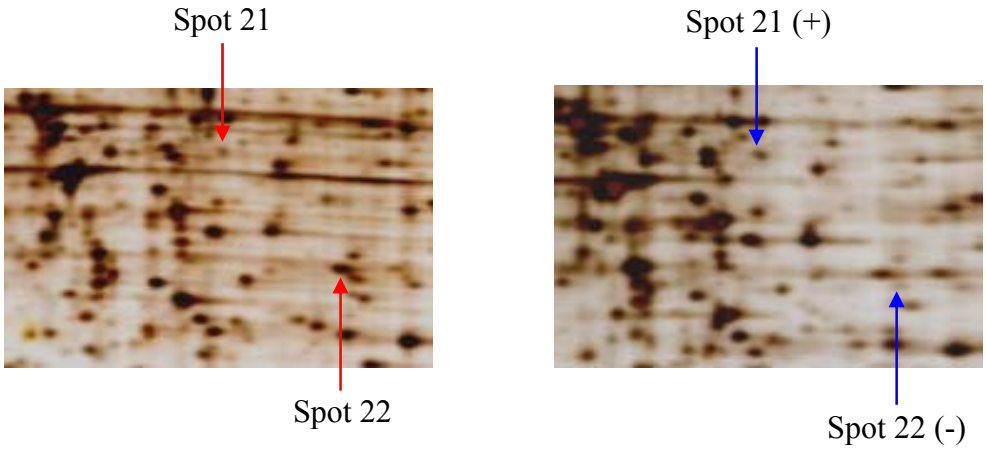


Figure 6.6g

Figure 6.6f-g – Zoomed regions of 2D-maps of differential proteins (f: spot 17 – 20; g: spot 21 – 22) between (i) control group and (ii) LPS-stimulated splenocytes (Only representative 2D-maps were presented) [(-): down-regulated protein; (+): up-regulated protein]

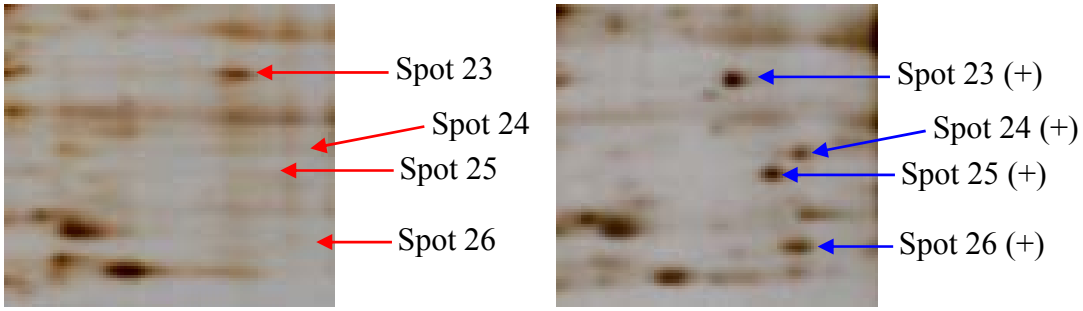


Figure 6.6h

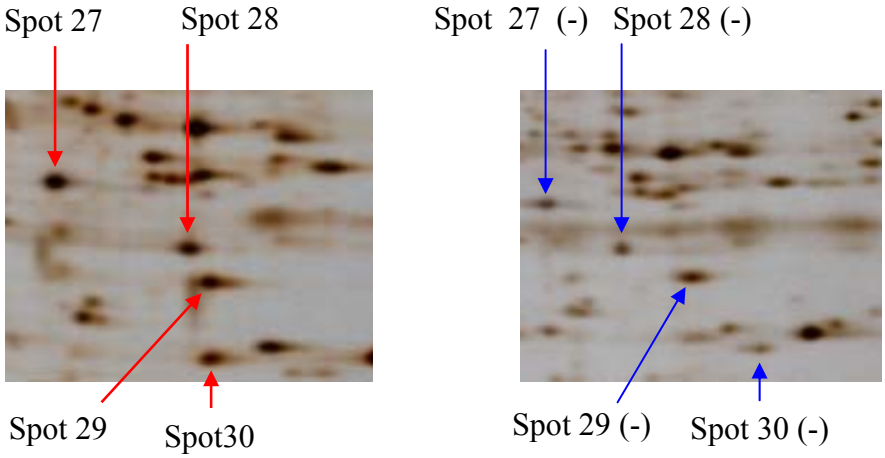


Figure 6.6i

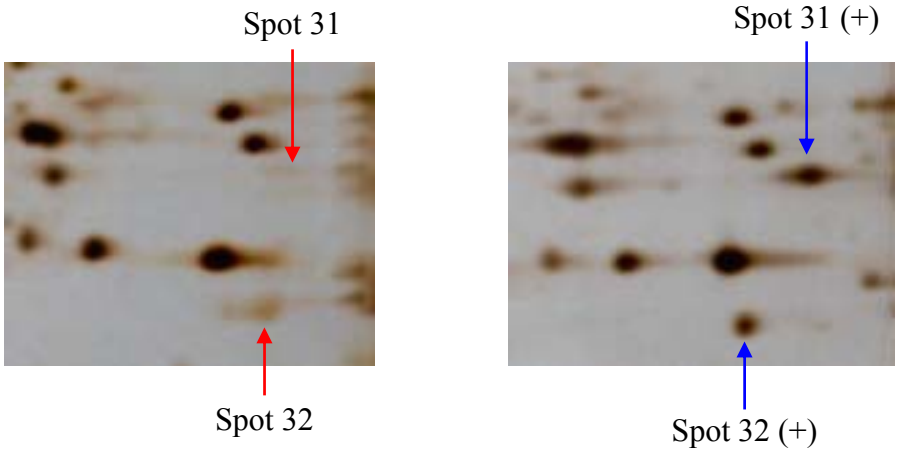


Figure 6.6j

Figure 6.6h-j – Zoomed regions of 2D-maps of differential proteins (h: spot 23 – 26; i: spot 27 – 30; j: spot 31 – 32) between (i) control group and (ii) LPS-stimulated splenocytes (Only representative 2D-maps were presented) [(-): down-regulated protein; (+): up-regulated protein]

6.2) Comparison of 2D-map of cytosolic fraction between (i) control and (iii) Rb-1-added splenocytes

In this section, those differential expressed proteins by Rb-1 addition were studied. 2D-maps of cytosolic fraction of normal control group and Rb-1-added splenocytes culture were compared. A representative 2D-map of Rb-1-treated splenocytes was shown in Figure 6.7. However, no significant differences in term of differential proteins were found these two groups (hence data not shown).

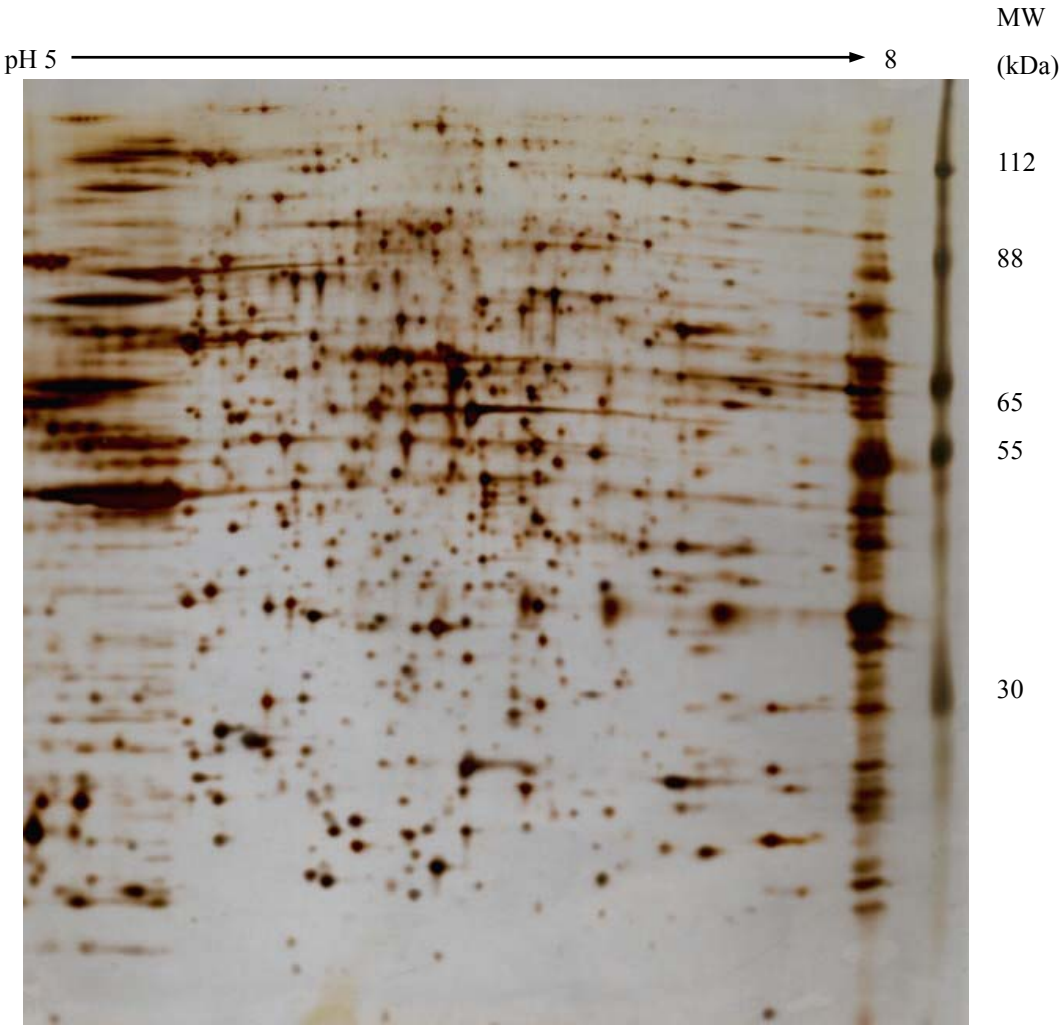


Figure 6.7 – 2D-map of cytosolic fraction of Rb-1-added splenocytes [Group (iii)] (50 μg protein, pH 5 – 8, 10% gel)

6.3) Comparison of 2D-map of cytosolic fraction between (ii) LSP-stimulated splenocytes and (iv) Rb-1-added/ LPS-stimulated splenocytes culture

In order to find out the modulatory effects of Rb-1, comparison of 2D-maps of (ii) LPS-stimulated splenocytes and (iv) Rb-1/ LPS-stimulated splenocytes culture was performed. According previous procedures, 2D-maps of Group (ii) and (iv) were matched and analyzed. The following figures demonstrated representative 2D-maps of cytosolic fraction of (ii) LPS-stimulated and (iv) Rb-1/ LPS-stimulated splenocytes culture.

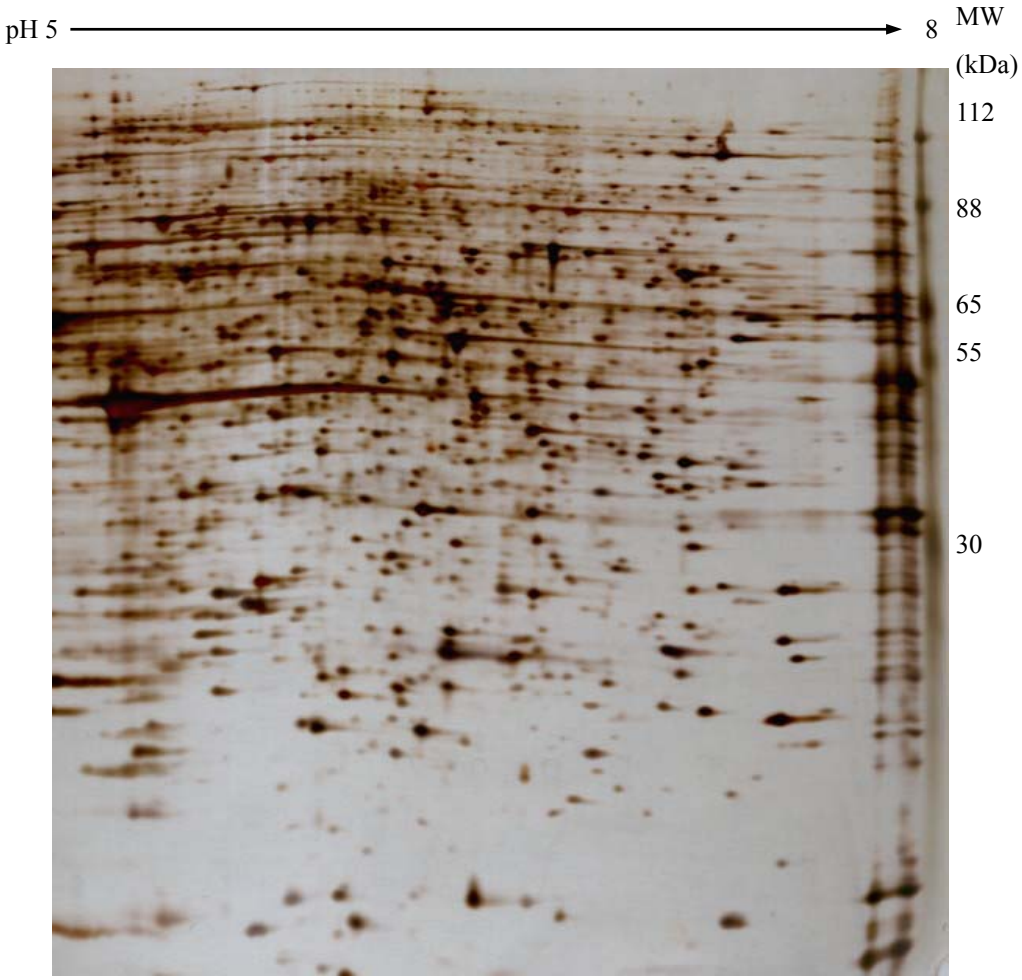


Figure 6.8 – Representative 2D-map of cytosolic fraction of LPS-stimulated splenocytes [Group (ii)] (50 μg protein, pH 5 – 8, 10% gel)

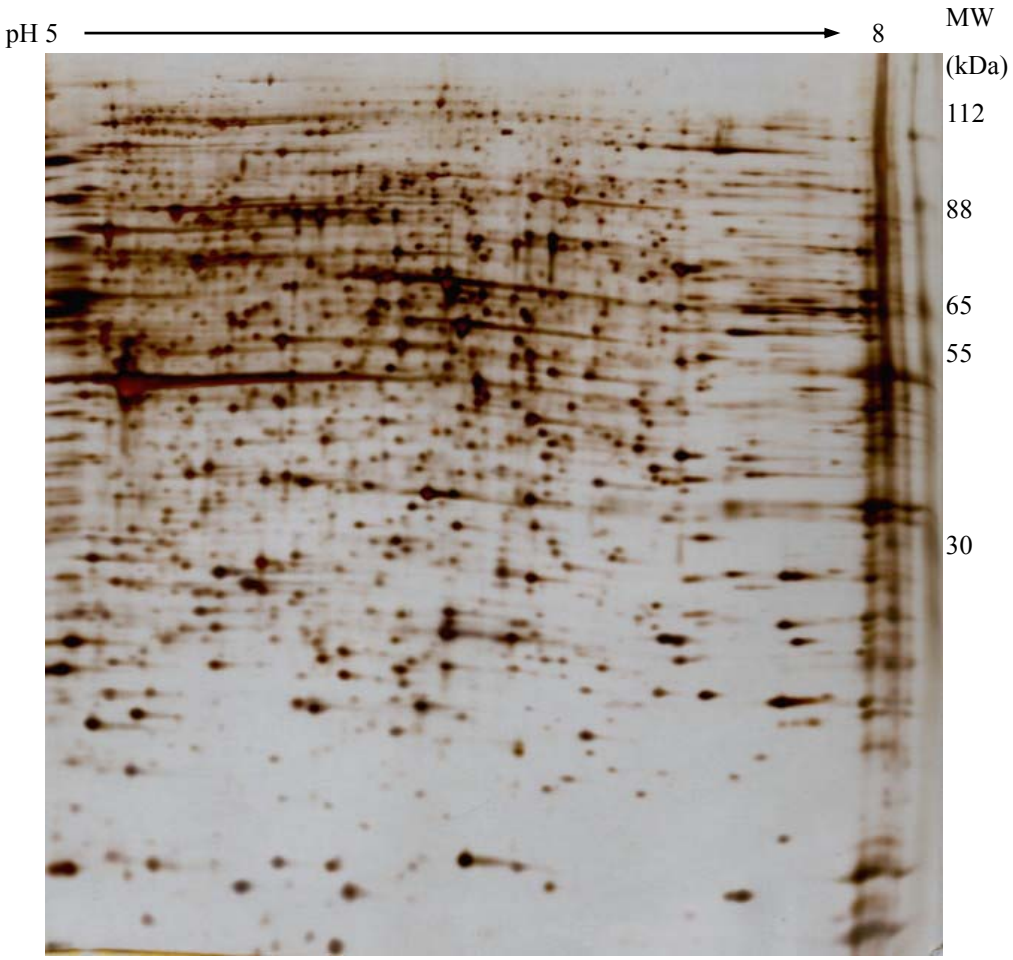


Figure 6.9 – Representative 2D-map of cytosolic fraction of Rb-1-added/ LPS-stimulated splenocytes [Group (iv)] (50 μg protein, pH 5 – 8, 10% gel)

After landmark labeling and spot detection, number of protein spots and matching pair found were summarized as follow:

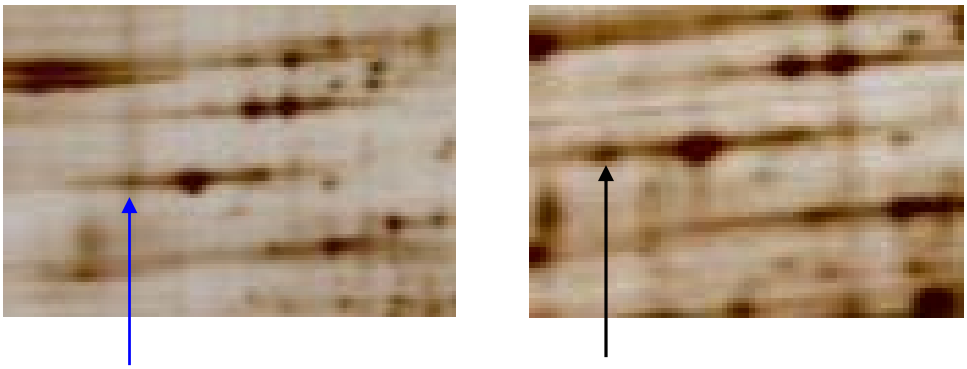
	LPS stimulated	Rb-1-added/ LPS-stimulated		
2D-map	No. of spots detected	No. of spots detected	Matched pairs of spots	Percentage matched
1	1232	1135	813	68.6 %
2	1127	1089	687	62.0 %
3	1506	1231	1057	77.2 %

Table 6.3 – Summary of 2D-maps analysis between LPS-stimulated and Rb-1/ LPS-stimulated splenocytes.

In the last section, a total of 32 proteins were found to be LPS-responsive when compared with control group (Figure 7.6a – j). On the other hand, by comparing the 2D-maps of LPS-stimulated and RB-1-added/ LPS-stimulated splenocytes, only two spots were found to be differentially expressed among these 32 spots. These spot images were illustrated as follows.

(ii) LPS-stimulated splenocyte

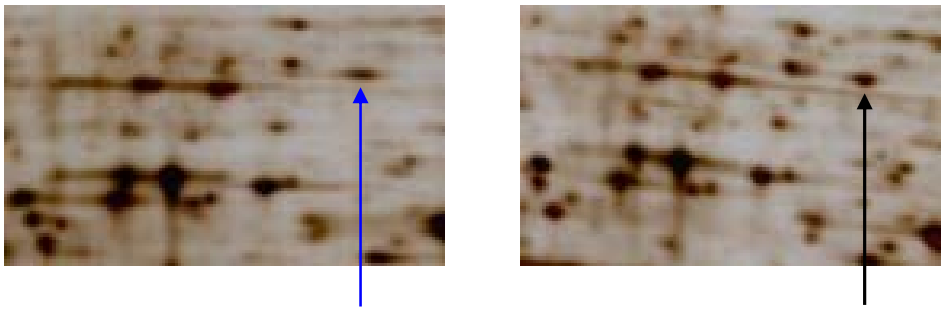
(i) Rb-1-added/ LPS-stimulated splenocytes



Spot 1

Spot 1 (+)

Figure 6.10a



Spot 17

Spot 17 (+)

Figure 6.10b

Figure 6.10a & b) – Zoomed regions of 2D-maps of differential proteins (a: spot 1; b: spot 10) between (ii) LPS-stimulated and (iv) Rb-1-added/ LPS-stimulated splenocytes [(-): down-regulated protein; (+): up-regulated protein].

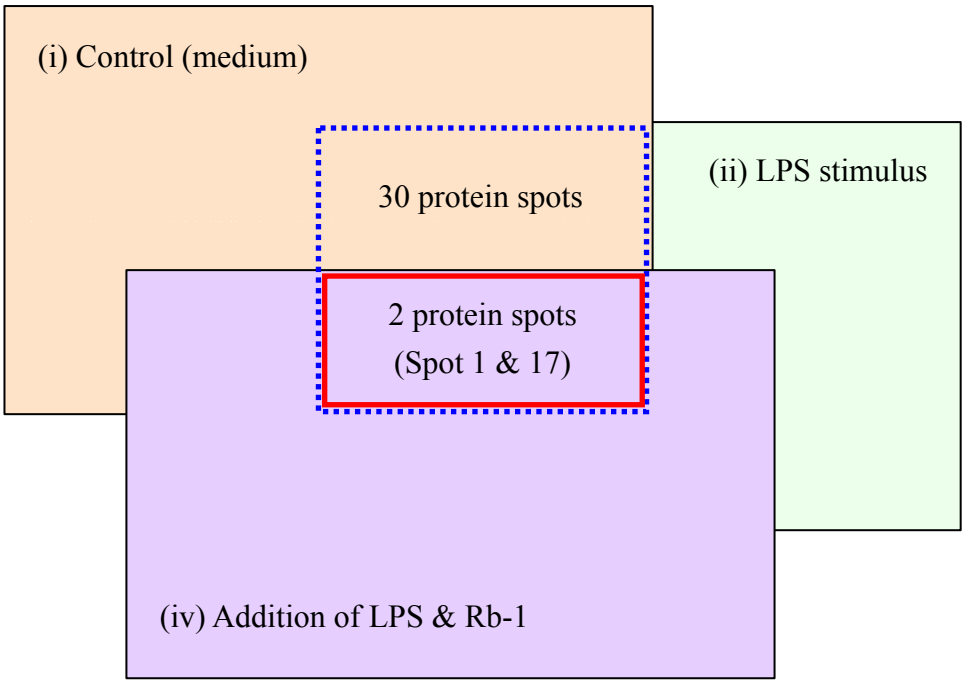


Figure 6.11 – Summary of studies of differential expressed protein spots, among (i) normal; (ii) LPS-stimulated and (iv) Rb-1-added/ LPS-stimulated splenocytes.

As a summary, as shown in Figure 6.11, it was found that a total of 32 spots were differentially expressed against LPS-stimulation. In addition, by comparing the cytosolic fraction of LPS-stimulated and Rb-1-added/ LPS-stimulated splenocytes culture, only 2 protein spots were considered as Rb-1-mediated proteins (Figure 6.10a & b).

In order to identify these protein spots and gain an insight into their possible functions, these 32 spots were subjected to protein identification through in-gel

trypsin digestion and matrix-assisted laser desorption/ ionization – time of flight mass spectrometry (MALDI-TOF MS). Preparation and procedures were described previously (materials and methods). Out of these 32 spots, only 12 proteins were successfully identified and details of their identification were summarized in the following table (Table 6.4)

Spot Number	Protein Name	NCBI accession number	Experimental pI	Theoretical pI	Experimental MW/ kDa	Theoretical MW/ kDa	Mascot Score	Expect value	Sequence matched	Sequence recovery
1	Gelsolin	gi|51260019	6.0	5.7	111	86.4	82	1.4e-006	11	18.6 %
2	Tudor domain containing 3 protein	gi|55947	6.2	5.5	69	58	127	7e-009	14	35.6%
3	Mitochondria aldehyde dehydrogenase	gi|25990263	6.3	5.6	65	63	63	0.018	5	12.5%
9	Acidic ribosomal phosphoprotein PO	gi|11693176	6.4	5.9	34	34.4	65	0.01	9	43.2%
10	Proteosome activator subunit 3	gi|30410794	6.2	5.6	28	29.6	134	1.4e-009	14	45.7%
17	Myxovirus resistance 1 protein	gi|71534287	7.4	6.5	95	75	240	3.5e-20	25	40.3%
19	Reticulo endothelial oncogene	gi|62660921	7.0	6.1	90	68.9	60	0.033	8	14.2%
25	Aspartate transaminase (cytosolic)	gi|91997	7.9	6.9	46	46.6	75	0.0011	9	26.6%
27	LIM & SH3 protein	gi|4249130	7.2	6.7	33	30.4	146	3.5e-010	14	47.9%
29	LIM	gi|47940150	7.4	6.8	31	36	139	4.4e-010	10	32.0%
30	Phosphoribosyl pyrophosphate synthetase	gi|57277447	7.5	6.6	30	35.3	105	1.1e-006	8	36.2%
31	Tpi protein	gi|38512111	7.9	7.8	26	27.2	148	5.5e-011	12	58.1%

4	Un-identified		6.2		59					
5	Un-identified		6.0		52					
6	Un-identified		6.4		59					
7	UN-identified		6.4		56					
8	Un-identified		6.5		42					
11	Un-identified		6.9		33					
12	Un-identified		6.9		32					
13	Un-identified		6.9		21					
14	Un-identified		7.0		21					
15	Un-identified		7.1		19					
16	Un-identified		7.1		20					
18	Un-identified		7.0		91					
20	Un0identified		7.1		90					
21	Un-identified		7.0		64					
22	Un-identified		7.3		49					
23	Un-identified		7.8		51					
24	Un-identified		7.9		48					
26	Un-identified		7.9		43					
28	Un-identified		7.3		32					
32	Un-identified		7.8		22					

Table 6.4 – Identification of differentially expressed protein spots in cytosolic fractions by peptide mass fingerprint (PMF) searching.

6.4) Comparison of 2D-maps of nuclear extracts of LPS-stimulated and Rb-1-added/ LPS-stimulated splenocytes

In the previous sections, the cytosolic proteomes of LPS-stimulated splenocytes with or without Rb-1 treatment were studied. Several proteins were found to be differentially expressed. Two of them (Gelsolin and myxovirus resistance-1) were identified as Rb-1-related proteins. In addition, in order to acquire more information on possible nuclear protein expression, the nuclear extracts of these splenocytes were also studied by 2D-PAGE. However, only nuclear extracts of LPS-stimulated splenocytes and Rb-1-added/ LPS-stimulated splenocytes were compared. The 2D-maps were performed in duplicates. The following figures showed representative 2D-maps of nuclear extract of different samples.

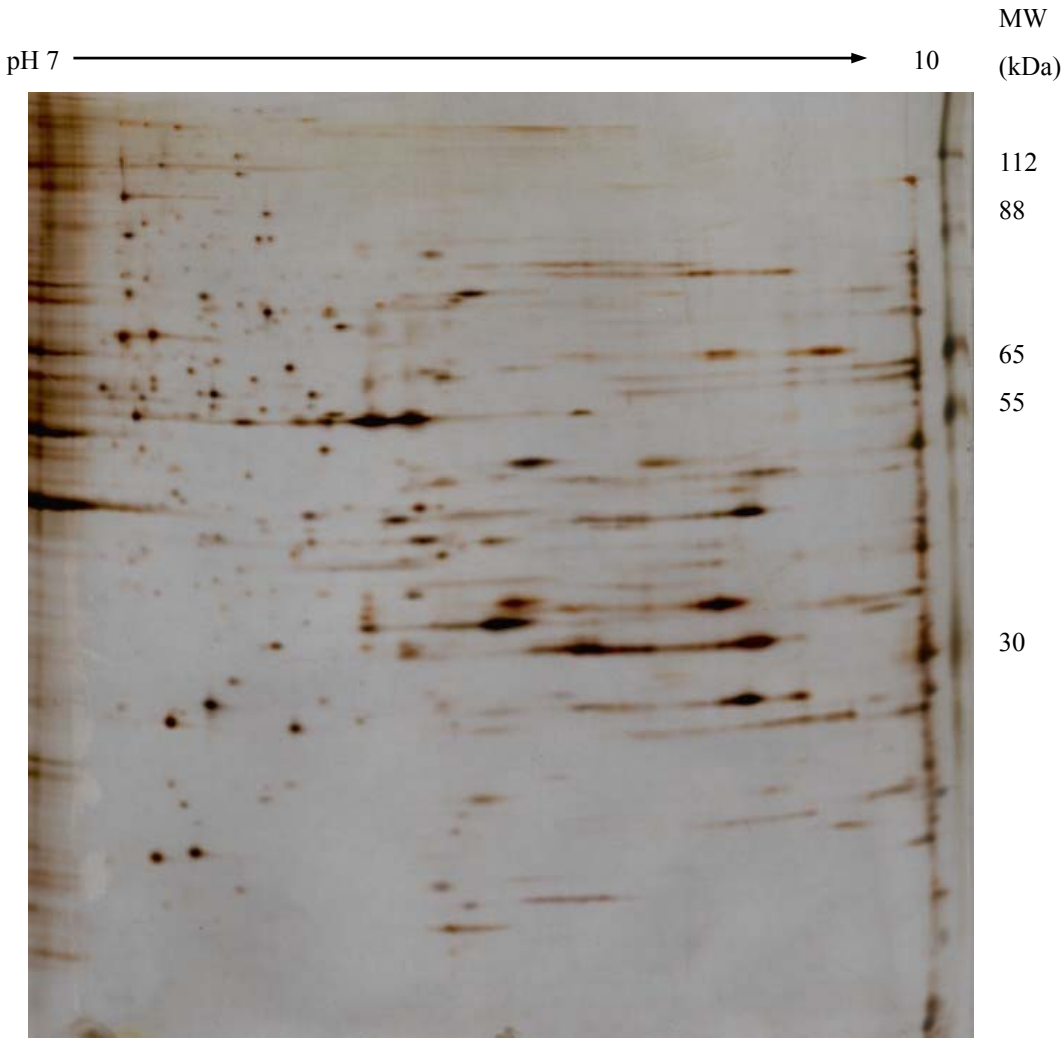


Figure 6.12 – 2D-map of nuclear extract of (ii) LPS-stimulated splenocytes (50 µg, pH 7 – 10, 10% gel).

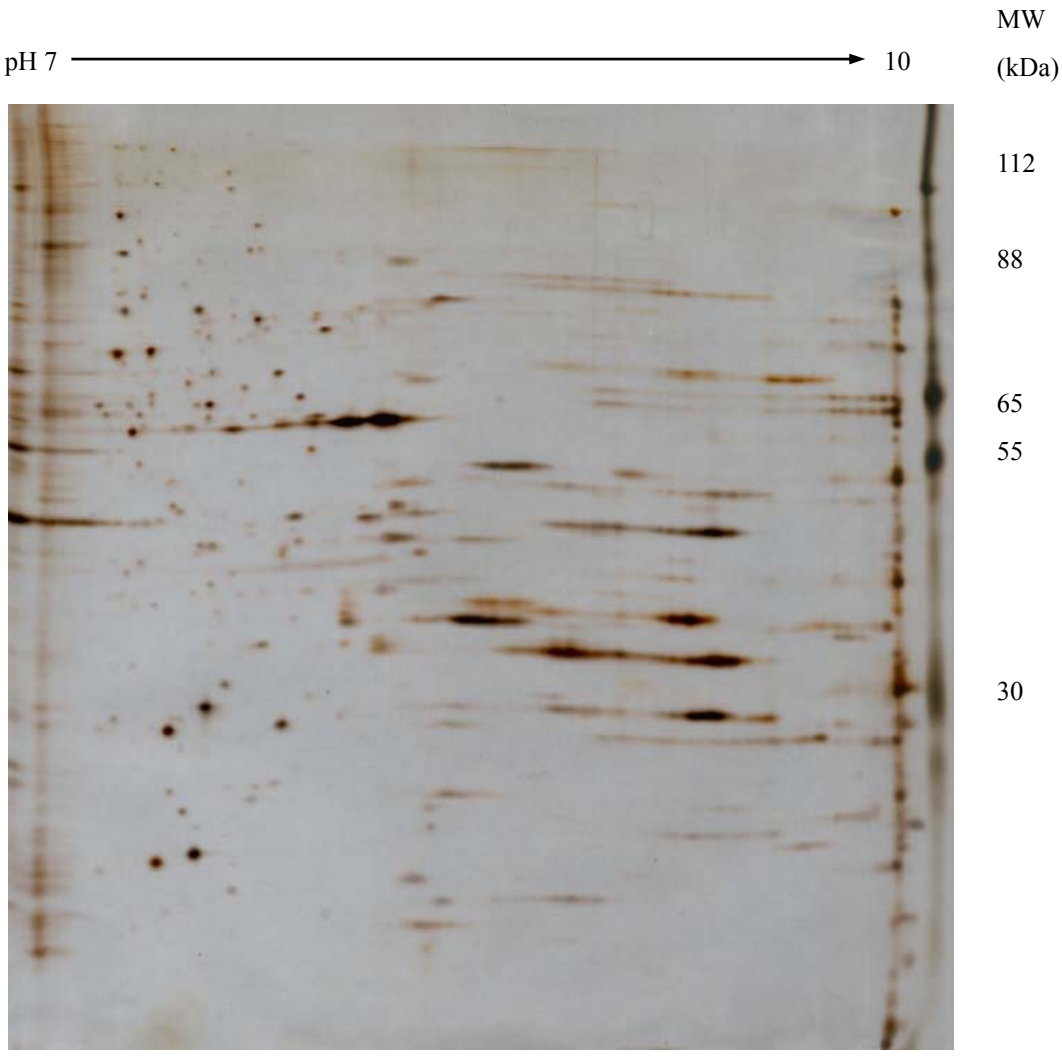


Figure 6.13 – 2D-map of nuclear extract of (iv) Rb-1-added/ LPS-stimulated splenocytes (50 μg, pH 7 – 10, 10% gel).

In Figure 6.12 and 6.13, it was found that number of protein spots in nuclear extract was obviously lower than that of cytosolic fraction. Similar to the cytosolic fraction studies, 2D-maps were labeled with designated landmarks and then paired with these landmarks. Labeled spots of 2D-maps and matched spot-pairs were shown in Figure 6.14. Furthermore, Table 6.4 had summarized the proteins and pairing reports.

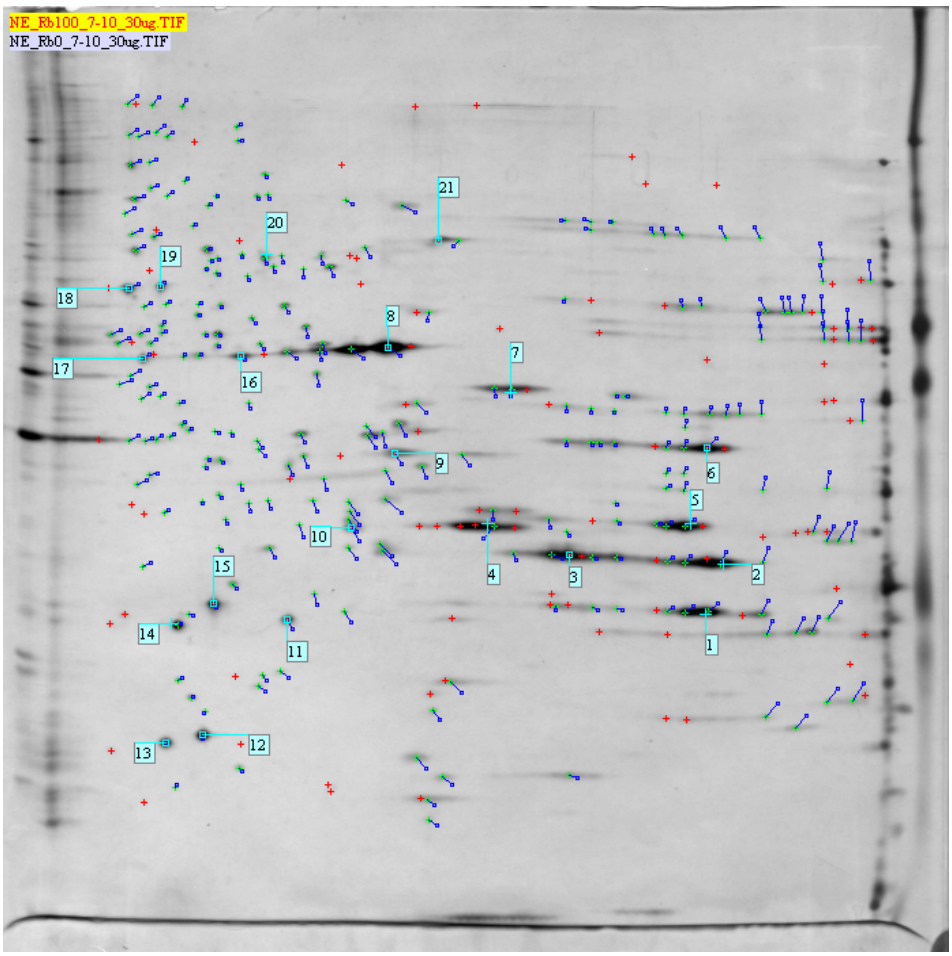


Figure 6.14 – Pairing of 2D-maps of (ii) LPS-stimulated and (iv) Rb-1/ LPS-stimulated splenocytes. Blue spots represented the paired spots from one gel and green arrow represented the pairing pathway against the other.

	LPS-stimulated	Rb-1-added/ LPS-stimulated		
2D-map	No. of spots detected	No. of spots detected	Matched pairs of spots	Percentage matched
1	362	334	243	69.8 %
2	419	416	277	65.3 %

Table 6.5 – Summary of 2D-maps analysis of nuclear extract between (ii) LPS-stimulated and (iv) Rb-1/ LPS-stimulated splenocytes.

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After spots matching and pairing, their expression levels were measured in term of relative volume (% Vol) as described before. However, none of the spot was found to be differentially expressed.

To summarize, in this chapter, it was intended to study if Rb-1 alter any cellular functions. Differential protein expression of splenocytes exposing to LPS with or without Rb-1 incubation were analyzed by 2DE and subsequent MALDI-TOF MS protein analysis. Of the 12 proteins eventually identified, only 2 were found to be differentially expressed due to the addition of Rb-1. Spot 1 (gelsolin) was down-regulated due to the addition of RB-1. It is speculated that Rb-1 inhibited the NF-κβ pathway which in turn decreased the production of gelsolin (a protein found in the cytoskeleton) in our environment where synthesis of the

cytoskeleton of splenocytes are induced to increase dramatically in the presence of LPS. However, its mechanism and exact mode of action is unknown. Another explanation of the phenomena is that, due to the presence of LPS, splenocytes grow immensely. Synthesis of cytoskeleton protein will also be increased drastically. However, in the presence of Rb-1, the synthesis of gelsolin was somehow decreased. Obviously, this postulation had to be validated by further experiments which are outside the scope of this thesis.

As for the second identified protein spot, myxovirus resistances 1 protein, it was found to be up-regulated. It is known that this protein is an IFN-γ-responsive protein. From the results of my earlier experiments, it is known that Rb-1 suppressed the production of IFN-γ in LPS-stimulated splenocytes. Therefore, it is unknown how Rb-1 induced the up-regulation of an IFN-γ-responsive protein while decrease of the production of IFN-γ is found. Again, the mechanism and physiological significance of this finding is yet to be confirmed. Nevertheless, the findings of two differentially expressed proteins under the influence of Rb-1 in LPS-stimulated splenocytes had never been reported previously. This opens up more opportunities for research in the effects of Rb-1 in the proteome expression of splenocytes being activated by mitogen such as LPS.

Chapter 7) DISCUSSION AND CONCLUSION

At any given time in our daily life, immune system of our body faces challenges from millions of foreign pathogens. Numerous components make up the immune system while lymphocytes play the major and leading role. Lymphocytes not only regulate innate immunity but also direct the development of adaptive immunity (Applequist *et al.*, 2002). On the other hand, thousands of substances are believed to have immunomodulatory effects. Chinese traditional medicine (TCM) is one set of them (Wilasrusmee *et al.*, 2002b). Among them, ginseng is a well-known TCM and has been in safe use for thousands of year. Therefore, immunomodulatory effects of one of the major components Rb-1 (Shibata *et al.*, 1963) was investigated in this study.

Firstly, preparation of unstimulated lymphocytes was preformed (Nussenzweig *et al.*, 1997). Ficoll-Paque density gradient solution provided a simple, rapid and reliable method for isolating lymphocytes from a mixture of cells (Romeu *et al.*, 1992). After isolation and washing, lymphocytes or splenocytes were examined by Wright-Giemsa morphology staining assay. Wright-Giemsa dye is a pH-sensitive dye and changes its colors according to the surrounding pH values (Woronzoff-Dashkoff, 2002). Hence, it is adopted for intracellular morphology

study and cell type identification (Alleman *et al.*, 1999). For example, eosinophil is identified by its red-orange granules while basophil's granule is in dark bluish-purple color. Polymorphonuclear neutrophil appears a pale pink color in cytoplasm, lilac granules and reddish-purple nuclei. However, a reddish-purple nucleus is also observed in lymphocytes and with pale blue in cytoplasm. In our study (Figure 3.3a), splenocytes that were prepared consisted of lymphocytes in majority. Further, high nucleus to cytoplasm ratio was seen (Figure 3.3b) (van *et al.*, 1981). This finding conformed to the expected results from the instruction manual of Ficoll-Paque. 95 $\pm$ 5 % of cells present in the interphase fraction were mono-nucleocytes.

Secondly, although lymphocytes were prepared, it is necessary to enumerate the relative populations of B cells and T cells in the preparation. CD molecules are well accepted surface markers of these immune cells. These molecules can be recognized by antibodies and applied in cell type analysis. CD 20 was the adherent component of BCR (Mathas *et al.*, 2000) while CD3 was associated with TCR (Timon *et al.*, 1993). Hence, antibodies against CD20 and CD3 were used for B cells and T cells sorting. With FACS analysis, 45 $\pm$ 3 % of B cells and 57 $\pm$ 3 % of T cells were found. Gravely *et al* reported that 50 % of B cells and

27 – 38 % of T cells respectively were found in spleen lymphocyte population (Gravely *et al.*, 1976). In Erbach's studies, only 34 % and 37 % of B cells and T cells were found in preparation of spleen cells (Erbach *et al.*, 1993). However, it should be noted that classical approach of light microscopy was used in these studies to enumerate the cells and the whole spleen cell populations were not sorted by FACS. Most recent research on relative populations of various cell types of the spleen by FACS was done on murine species. For example, Connoy and co-workers reported that murine spleen consists of 52 – 60 % of B cells and about 22 % of T cells. It should be stressed that sometimes different CD markers were taken as indication of B cells (Connoy *et al.*, 2006). Connoy and co-workers used CD19 as an indication of B cells while CD20 was used as the B cell biomarker in this study. Nevertheless, although there are percentage discrepancies of the exact figures, it was noted that preparation and measurement method of these studies were not exactly the same.

On the other hand, before starting the immunological studies of Rb-1, it is essential to obtain some background pharmacological properties of ginsenoside Rb-1. Ginsenoside Rb-1 was purchased from Sigma-Aldrich (Cat # - G-0777) and the purity of Rb-1 was certified to be more than 99% by high pressure liquid

chromatography analysis. In addition, Rb-1 was declared to be endotoxin-free. Therefore, proliferative activities and immunotoxicity of Rb-1 was assayed. In the past, several different assays were used for this type of work. MTT assay (Ferrari *et al.*, 1990) and H^3-thymidine incorporation (Osborne *et al.*, 1983) were the most common examples. Bounous *et al* had compared both methods and found these methods were comparable to each other (Bounous *et al.*, 1992). In addition, MTT and H^3 incorporation were also feasible in cell number determination. However, MTT assay bears several advantages than H^3-thymidine incorporation (Bounous *et al.*, 1992). Obviously, although H^3 has a limited radiation diameter of a few centimeters, it is a radioactive isotope. Moreover, MTT is an easy-handling method and simple apparatus is required. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is a water soluble yellowish tetrazolium salt. Soluble MTT was converted to an insoluble MTT formazan by enzyme dehydrogenase which was abundant in active mitochondria. Hence, viable cells converted soluble MTT into insoluble purple MTT formazan salt. Insoluble formazan salts was re-solubilized by iso-propanol and yield a purple color which was measured by absorbance 570 nm. This reading was considered as a function of population of viable cells (Niks *et al.*, 1990). It should be emphasized that activities of mitochondrial dehydrogenase

were the critical parameter in the MTT assay. However, activity of dehydrogenase in lymphocytes was comparatively lower than that in other cell types, such as epithelial cells (Mosmann, 1983). Therefore, in order to solve this constringency, Chen *et al* suggested a larger population of splenocytes to be cultured (for example 5 x 10^6 cells/ml) for the production of a more accurate MTT absorbance (Chen *et al.*, 1990). Therefore, a standard curve of splenocytes number, which ranged from 6.25 x 10^4 – 10^7 cells, against absorbance had been constructed and used in this study (Appendix II, the standard curve showed that MTT assay was compatible in determining a wide range of splenocytes population).

Thirdly, with the aid of MTT assay, the question of whether ginsenoside Rb-1 induced proliferation of splenocytes was examined. From the results of several groups, they had reported that ginseng/ ginseng extract exerted proliferative activities or growth effects on immune cells (Kenarova *et al.*, 1990; Lee *et al.*, 1996; Wilasrusmee *et al.*, 2002a; Lee *et al.*, 2004). However, ginseng extract rather than purified Rb-1 were used in these studies. In 2004, Popovich and colleagues reported that Rb-1 alone had no proliferative effects (Popovich *et al.*, 2004). My study also found that Rb-1 did not have any direct proliferative

activities against splenocytes culture, even at a relatively high dosage, such as 100 μg/ml. As various types of preparation of ginseng and different extracts (with or without endotoxin contamination) were used in various studies on the proliferation effects on lymphocytes/ splenocytes, there were a lot of different factors at play. Subsequently, contradictory results were reported. Nevertheless, in this study, the Rb-1 used had neither endotoxin activity (as guaranteed by the manufacturer, Sigma Alrich) nor proliferative activity on the splenocytes.

Fourthly, regarding to the cytotoxic test of Rb-1, it was found that our Rb-1 had no cytotoxicity even at concentration of 100 μg/ml. This result is similar to that reported previously (Kang *et al.*, 2000). These authors examined the maximum concentration tolerance of ginsenosides. They found that the maximum dosage of Rg-1 and Rb-2 was 1000 μg/ml and 277 μg/ml respectively. Although ginsenoside Rb-1 was not assayed in their report, maximum concentration of Rb-2 was considered as a reference as they have very similar structure. Furthermore, several studies also utilized 10 – 200 μg/ml Rb-1 as a normal working concentration (Kim *et al.*, 1996; Chang *et al.*, 1999; Liu *et al.*, 2005). Hence, a dosage of Rb-1 such as 100 μg/ml was well accepted and commonly used. Therefore, a highest dose of 100 μg/ml Rb-1 was used in this study.

Fifthly, the immunomodulatory effects of Rb-1 were studied. This was attempted at different levels. Initially, possible direct effects of Rb-1 on cytokine production were examined. In section 4.2.1, it was found that Rb-1 did not induce secretion of pro-inflammatory cytokines. However, Song *et al* had reported that ginseng extract induced TNF-α production dose-dependently in macrophages (Song *et al.*, 2002). Oppositely, another report indicated that neither Asian ginseng nor Siberian ginseng alone could induce expression of TNF-α and IL-1β (Wang *et al.*, 2003). Smolinski *et al* reported that there were no direct relationship between induction of IL-6, TNF-α, with treatment of Rb-1 RAW 264.7 cells (Smolinski *et al.*, 2003). Despite their results are conflicting, it is stressed that ginseng extract was used these studies rather than purified ginsenoside. In the literature review section, it had been mentioned that more than twenty ginsenoside species was identified (Fujita *et al.*, 1962). Up to date, it was still uncertain about the exact functions of each individual ginsenoside. Moreover, it was not surprising that some ginsenoside species was either an agonist or antagonist to another (Attele *et al.*, 1999). Given the fact that some ginseng extract or ginsenoside preparation was contaminated with endotoxin, interpretation of some results became very difficult. Hence, investigation of single highly purified ginsenoside is most beneficial to the scientific community.

Subsequent to knowing that the endotoxin-free Rb-1 that was used did not show any direct effects on the splenocytes, it is important to know if the addition of an external stimulus, LPS, would make any difference. Therefore, LPS was used added with Rb-1 in order to visualize the costimulatory effects of Rb-1 (Guha *et al.*, 2001). As shown in Figure 4.5 – 4.10, it was clearly demonstrated that Rb-1 suppressed LPS-mediated cytokines secretion.

It should be stressed that Smolinski and colleagues indicated that data from single cell-line culture did not accurately predict the result *in vivo* (Smolinski *et al.*, 2003). However, such discrepancy is actively compensated in the splenocytes culture setup in this study. It is because the splenocyte culture was composed of several cell types, such as B cells and T cells (a very small amount of macrophages could be seen attached to bottom of the culture dish). Such environment was essential in studying immune system, as immune cells worked in network and not separately as single individuals.

After the secretion profiles of each cytokines were established, it is evident that Rb-1 demonstrated an inhibitory chain-reaction in splenocytes culture. Besides, Rb-1 exerted its inhibitory action on each individual cell. For example, being

pro-inflammatory cytokines, TNF-α and IFN-γ were one of the first-responsive agents upon LPS activation (Bonjardim, 2005; Ware, 2005). In addition, amount of TNF-α and IFN-γ regulate the production of pro-inflammatory cytokines such as IL-1α and IL-6 (Locksley *et al.*, 2001). Hence, decreased level of TNF-α resulted in decreased amount of other inflammatory cytokines. In Figure 4.6 and 4.7, amount of TNF-α and IFN-γ were greatly increased after incubating with LPS for 8-hour. On the other hand, inhibitory actions on TNF-α and IFN-γ secretion occurred after addition of Rb-1. In addition, levels of IL-1α and IL-6 produced were also decreased after 16-hour of incubation with Rb-1 (Figure 4.8 & 4.9). In other words, Rb-1-supressed-TNF-α level in turn relieved production of IL-1α and IL-6. In case of IL-10, its amount in splenocytes culture was also suppressed. As predicted by Pestka *et al*, less extent of inflammation status required less inhibitory cytokine like IL-10 to maintain homeostasis (Pestka *et al.*, 2004). However, it is not known if Rb-1 would alternate cellular activities of individual cells. This postulation is only hypothetical and more experimental proof is required.

Nevertheless, there are several points to note from the cytokines profiles. Individual cytokine secretory profiles further illustrated the role of distinct cell

types. Moreover, it is possible to manifest how Rb-1 exerted its inhibitory chain-effect in splenocyte network. As discussed before, T cells consists of two sub-classes according to their cytokines production profiles (Van *et al.*, 1992; Siebenlist *et al.*, 2005). For instances, T_H1 cells produced IL-2, TNF-α and IFN-γ while T_H2 secreted IL-6 and IL-10 (Locksley *et al.*, 2001; Ware, 2005). Being activated, T_H2 produced appropriate cytokines that in turn affected T_H1. Take IL-10 as an example, T_H2 produced IL-10 that inhibited the cytokines secretion of T_H1(Pestka *et al.*, 2004). Therefore, it was observed that cytokines from T_H1 was suppressed 16-hour after LPS activation (including TNF-α and IFN-γ etc). Nevertheless, cytokines secretion such as IL-6 and IL-10 from T_H2 was continuous throughout the whole time. Furthermore, results in my experiments demonstrated that T_H1 and T_H2 were mutually exclusive to each other in my splenocytes culture setup (Eyles *et al.*, 2003). It was because IL-10 inhibited the development of T_H1 that eventually affected T_H2 as well. Further, in regarding B cells, no appropriate and specific parameter was adopted in measuring B cells activities. Despite B cells produced cytokines, T cells were the main sources of secretory cytokines. Hence, cytokines activities were not applicable in studying B cells responses completely.

It was reminded that activity of macrophage was not measured in this study while macrophages play an important role in immune system (Taylor *et al.*, 2005). However, as illustrated in Chapter 3, negligible amount of macrophages were found in splenocytes preparation obtained by using Ficoll-Paque solution. In addition, macrophages were the main source of nitric oxide (NO) in response to inflammation (MacMicking *et al.*, 1997). Hence, amount of NO represented some sort of extent of inflammatory status. Nevertheless, B cells and T cells was able to produce NO during activation (Tiscornia *et al.*, 2004; Vig *et al.*, 2004).

In my study, nitrite level in supernatant of splenocyte culture was measured as an indicator of the presence of nitric oxide (Wu-Hsieh *et al.*, 1998). However, if a negative control was added in the experimental set, interpretation of the results will be more convincing. This negative control is L-NMMA (N(G)-monomethyl-L-arginine), it is a NOS inhibitor and routinely added into culture or sample to provide a negative result (Scheller *et al.*, 1998; Perner *et al.*, 2001).By application of L-NMMA, conversion of arginine to NO and citrulline by iNOS was terminated. As a result, production of NO was terminated and nitrite will not be found. If this inhibitor had been added and nitrite level was indeed lowered, it would be more certain that it is NO which produces the nitrite.

From my data, nitrite amount in Rb-1-treated splenocytes culture was lower than that of un-treated splenocytes (Figure 4.11). Consistently, Oh *et al* demonstrated that protopanaxatriol of ginseng (e.g. Rg-1) suppressed the iNOS mRNA expression (Oh *et al.*, 2004). Furthermore, Park *et al* provided supporting data on results of Oh's group (Park *et al.*, 2005). They reported that purified Rb-1 significantly reduced the production of NO in RAW 264.7 cells. However, Song *et al* reported that ginseng extract had stimulatory effects on iNOS mRNA expression and NO production (Song *et al.*, 2002). It should be stressed again that whole ginseng extract was used rather than single component Rb-1. Similar to results of the cytokines studies, Rb-1 is believed to exert its inhibitory effects on iNOS though its common transcription factor – NF-κβ (Li *et al.*, 2002).

For the sixth sets of experiments, in-depth studies on the model of inhibitor of Rb-1 on cytokines production were performed using a newly developed technology. Bio-plex™ suspension array system (Bio-Rad, US) (Torrence, 2004) provided a platform to study several signaling molecules and/ or protein concurrently with the same sample. Comparing to traditional biochemical methods (western blot and ELISA), protein is often analyzed and quantified individually. Furthermore, these techniques are rather time-consuming and

labor-intensive. In contrast, the Bio-plex suspension array system offers a rapid, accurate and reliable method for measuring multiple analysts simultaneously.

In this study, many pathways are involved but, due to time constraint, only NF-κβ signaling transduction pathway was examined. Moreover, it was found that LPS greatly induced phosphorylation of I-κβ within 120 minutes. However, phosphorylation activity of I-κβ was suppressed by Rb-1 (Figure 5.7a). Furthermore, it was found that total amount of I-κβ in LPS group was significantly lower than that of the Rb-1-added/ LPS-stimulated splenocytes (Figure 5.8b). In Figure 5.8c, the suppression of I-κβ phosphorylation by Rb-1 was more obvious. As a result, it is postulated that the Rb-1 suppression of I-κβ phosphorylation in turn inhibited NF-κβ functions. Subsequently, production of proinflammatory cytokines was greatly reduced (Figure 4.6 – 4.7). Therefore, it was found that Rb-1, either directly or indirectly, regulated the activities of upstream signaling factors of I-κβ such as IKK.

Subsequent to results of cytokines studies and I-κβ phosphorylation activities obtained, two postulations were formulated. Firstly, as mentioned before (Section 1.7.3), ginsenosides share a high degree of homology in their structural skeletons

with steroid-like hormones. As glucocorticoid (GC) is a commonly known transcription regulatory element, GC could bind with other transcription factors (such as AP-1) within the glucocorticoid response element (GRE) (Herrlich, 2001). Moreover, GC is one of the anti-inflammatory agents that its mode of action was reported by several research groups. Auphan *et al* (1995) had reported that GC-analog dexamethasone (DEX) mediated immunosuppression through induction of I-κβ transcription. In addition, Scheinman *et al* (1995) had also reported similar results. In other words, newly synthesized I-κβ in turn inhibits NF-κβ activities and immune responses. Hence, it was not surprising that ginsenoside carried similar effects activities as GC (Lee *et al.*, 1997; Chan *et al.*, 2002; Ling *et al.*, 2005).

Another speculation was that, Rb-1 exerted their effects on upstream signal mediator(s) of the LPS-mediated pathway. LPS being a the potent inducer of various receptor pathways, including BCR, TCR and TLR-4 (Akira *et al.*, 2004; Weil *et al.*, 2004). Each of these receptors in turn distributes their surface signals into differential intracellular pathways. Signals from these receptor pathways not only stimulates NF-κβ pathway, but also regulates I-κβ activities. In addition, NF-κβ activation was not the sole consequence of these receptors activation.

In the Bio-plex experiment analysis, two assumptions were made. Firstly, it is assumed that the total repertoire pool of analyst of interest was not changed although the turn-over rate could be relatively fast (Lavner *et al.*, 2005). In other words, protein synthesis turn-over rate after sample preparation was not considered in the total protein expression. Otherwise, the ratio could have been changed by every second. Secondly, phosphorylation status of target analyst was either phosphorylated or un-phosphorylated. Therefore, phosphorylated and un-phosphorylated analyst then contributed to the total amount of repertoire pool. As a consequence, ratio of phosphoprotein to total protein directly represented the phosphorylation profile of analyst.

Although complicated network is involved, further work is possible to define which pathway does Rb-1 got involved. On behalf of specific signaling pathway, specific receptor blocker can be applied in order to define the relevant pathway. A newly synthesized molecule (A-420983) which inhibited Lyn and Src kinase activities in T cells could be used. This molecule also suppressed TCR-mediated ZAP-70 phosphorylation and proinflammatory cytokines production (McRae *et al.*, 2005). In case of B cells, AMN 107 is another commonly used and strong blocker in BCR pathway (Weisberg *et al.*, 2005). In addition, use of signal

transducer blockers is another strategy that could be applied in order to find out the signaling key in Rb-1-mediated immunomodulation. For example, TRAF-6 plays an important role in TLR4 signaling that transmit membrane signal to cytosol. However, TRAF-6-binding peptide is shown to be specific inhibitor to TRAF-6 that abrogated interaction with other signaling factor such as receptor activator of NF-κβ ligand (RANKL) (Bharti *et al.*, 2004). Hence, by applying distinct pathway blockers or mediator inhibitors, possible involvement of Rb-1 in particular pathway could be defined.

In the Western blot results set, nuclear translocation of NF-κβ was investigated. However, there were some practical difficulties during sample preparation. First of all, due to limited cells number, only a single time point samples (e.g. 4-hour after LPS stimulation) could be prepared instead of a 24-hour profile (which may consists of three or more time-points). This affected accurate interpretation of the translocate event. For example, the translocation profile of the NF-κβ was not fully investigated and that could lead to false positive result. Second, several groups had studied the biology of primary cell lines and found that their membranes (both plasma and nuclear membrane) were especially fragile (Osborne *et al.*, 1983; Bladon *et al.*, 1988; Antalis *et al.*, 1991). In these studies,

researchers discovered that inappropriate lysis solution greatly enhanced cross contamination of cytosol contents. Hence, usage of strong detergent must be avoided and appropriate solubilization buffer should be adapted (Dyer *et al.*, 1995). Dyer *et al* had suggested varies concentrations (0.1 – 0.5%) of detergent NP-40 to be applied for different cells. For instances, these authors reported that as lower as 0.1% NP-40 was used in fragile cells such as primary culture. In addition, Neamita *et al* had further improved the usage of 0.1% NP-40 with subsequent application of glycerol cushion (Neamati *et al.*, 1995). On the other hand, both LPS and Con-A were well-known stimulant against immune cells. Different experiments were conducted in order to investigate the molecular changes within nucleus of lymphocytes (Feuerstein *et al.*, 1987; Bladon *et al.*, 1988; Neamati *et al.*, 1995). However, no perfect and appropriate internal control could be found and applicable in these studies. Alternatively, electrophoretic mobility shift assay (EMSA) may be useful for the study of NF-κβ expression (Singh *et al.*, 1995; Cheng *et al.*, 2003).

In proteome analysis, it was found that addition of Rb-1 into LPS-stimulated splenocytes increased the expression of gelsolin (a component of cytoskeleton) and myxovirus resistance 1 protein (an IFN-γ-responsive protein). Gelsolin is an

actin regulatory family protein (Sun *et al*, 1999). It is speculated that Rb-1 increased the production of gelsolin via inhibition of the NF-κβ activation in an environment in which cytoskeleton of the splenocytes are induced to increase dramatically in the presence of LPS. However, its mechanism and exact mode of action is unknown. As for why Rb-1 induce the production of an IFN-γ-responsive protein – myxovirus resistance 1 protein (Al-Masri *et al*, 2006), it probably means that Rb-1 could mediate similar receptor to IFN-γ. Nevertheless, there is neither direct nor related evidence known currently, more experiments are required to verify these postulations.

Nevertheless, one of my last experiments in this project revealed the relationship between Rb-1 and phosphorylation of I-κβ. As it is commonly known that LPS stimulation signal was transmitted to NF-κβ pathway in which IKK was activated. Activated IKK then induced I-κβ phosphorylation and activation. Subsequently, phosphorylated I-κβ dissociated from NF-κβ complex. NF-κβ was free and will then be translocated into nucleus for LPS-responsive genes expression. However, if the phosphorylation of I-κβ was suppressed, dissociation of NF-κβ from its inhibitor I-κβ will also be terminated. My results showed that Rb-1 suppressed the phosphorylation of I-κβ. When I-κβ was not activated, it kept locking with

NF-κβ and translocation of NF-κβ will be abrogated. Inside the nucleus, hence, amount of NF-κβ was decreased. This was illustrated by the results of the Western blot. Furthermore, it is well known that cytokine expression is an NF-κβ-dependent process (Yasukawa *et al.*, 2000; O'Shea *et al.*, 2002). Results of cytokines secretory profiles in this study illustrated that Rb-1 relieved LPS-induced cytokines production and secretion. These results strongly support the conclusion that, during Rb-1 supplement, phosphorylation level of I-κβ was decreased which translated into a decrease of NF-κβ within nucleus which resulted in decrease of cytokines expression.

Appendix I – Solution Preparation

Agarose sealing gel

	Final Concentration	Amount
Agarose	0.5 % (w/v)	0.5 g
1X SDS electrophoresis buffer	/	100 ml
	Total	100 ml

Store at 4℃

30 % Acrylamide gel solution (29:1)

	Final Concentration	Amount
40 % acrylamide	29 % (v/v)	1000 ml
N', N'-methylenebisacrylamide	1% (w/v)	3.79 g
Distilled water	/	379 ml
	Total	1379 ml

Solution was filtered with 0.45 μm filter membrane
Store at 4℃

50 mM Ammonium carbonate solution

	Final Concentration	Amount
Ammonium carbonate	50 mM	1.95 g
Distilled water	/	500 ml
	Total	500 ml

Filter with 0.45 μm filter membrane

Antibody buffer

	Final Concentration	Amount
Bovine serum albumin (BSA)	1 % (w/v)	1 g
20 mM TBST, pH 7.6	/	100 ml
	Total	100 ml

Store at 4℃

10 % Ammonium persulafte (APS)

	Final Concentration	Amount
Ammonium persulfate *	10 % (w/v)	0.1 g
Distilled water	/	1000 µl
	Total	1000 µl

(*) Freshly prepared

Complete RPMI 1640 medium

	Final Concentration	Amount
RPMI 1640 medium	/	440 ml
Heat-inactivated fetal bovine serum (FBS)	10 % (v/v)	50 ml
10000 units/ml penicillin	100 units/ ml	5 ml
10000 µg/ml streptomycin	100 µg/ml	5 ml
Supplement with L-glutamine	/	/
Supplement with 25 mM HEPES	/	/
	Total	500 ml

Store at 4℃

Equilibration buffer

	Final Concentration	Amount
1.5 M Tris-HCl, pH 8.8	50 mM	33.5 ml
Urea	6 M	360.1 g
Glycerol	30 % (v/v)	333 ml
SDS	2 % (w/v)	20 g
Bromophenol blue	0.002 % (v/v)	20 µl
	Total	1000 ml

Prior to use 100 mg of DTT and 250 mg iodoacetamide are added

Erythrocyte lysing solution

	Final Concentration	Amount
Ammonium Chloride	155 mM	4.15 g
Sterile phosphate buffer saline (PBS), pH 7.4	/	500 ml
	Total	500 ml

Solution was filtered with 0.2 µm membrane

Store at 4℃

FACS cell blocking buffer

	Final Concentration	Amount
Bovine serum albumin (BSA)	1 %	1 g
Sodium azide	0.1 %	0.1 g
Phosphate buffer saline (PBS), pH 7.4	/	100 ml
	Total	100 ml

Store at 4℃

Gel washing solution

	Final Concentration	Amount
50 mM ammonium carbonate	25 % (v/v)	5 ml
100 %Acetonitrile (ACN)	50 % (v/v)	5 ml
	Total	10 ml

Giemsa stain solution

	Final Concentration	Amount
Giemsa stain powder	0.75 % (w/v)	3.8 g
Glycerol	50 % (v/v)	250 ml
100 % methanol	50 % (v/v)	250 ml
	Total	500 ml

Solution was heated at 60℃ for 2 hours with vortex

Solution was filtered with 0.45 μm membrane

Giemsa working solution

	Final Concentration	Amount
Giemsa stain solution	0.08 %	10 ml
Disilled water	/	80 ml
	Total	90 ml

Nuclear hypotonic buffer

	Total	
	Final Concentration	Amount
HEPES (100 mM)	10 mM	100 µl
KCl (1M)	10 mM	10 µl
$MgCl_2$ (1M)	1.5 mM	1.5 µl
EDTA (10 mM)	0.1 mM	10 µl
EGTA (10 mM)	0.1 mM	10 µl
DTT (100 mM)	1 mM	10 µl
PMSF (100 mM)	0.5 mM	5 µl
Leutinin (500 µg/ml)	2 µg/µl	0.4 µl
Aprotinin (200 µg/ml)	2 µg/µl	0.1 µl
Benzamidine (100 mg/ml)	0.5 mg/ml	5 µl
	Total	1000 µl

Freshly prepared before use
Store at 4℃

Lysis buffer, pH 8.5

	Final Concentration	Amount
Urea	7 M	12.6 g
Thiourea	2 M	4.6 g
CHAPS	4 % (w/v)	2.0 g
Tris	40 mM	0.24 g
	Total	50 ml

Aliquot in 1 ml and store at -20℃

Membrane blocking buffer

	Final Concentration	Amount
Bovine serum albumin (BSA)	3 % (w/v)	3 g
20 mM Tris-buffer saline (TBS), pH 7.6	/	100 ml
	Total	100 ml

Store at 4℃

Membrane stripping buffer, pH 6.8

	Final Concentration	Amount
Distilled water	/	87.5 ml
0.5 M Tris, pH 6.8	62.5 mM	12.5 ml
Sodium dodecyl sulfate (SDS)	2 % (w/v)	2 g
14.3 M β-mercaptoethanol (β-ME)*	100 μM	
	Total	100 ml

(*) Prior to use 70 μl of 14.3 M β-ME was freshly added to 10 ml buffer

Modified rehydration buffer

	Final Concentration	Amount
Urea	7 M	5 g
Thiourea	2M	1.82 g
Glycerol	5 % (v/v)	0.6 ml
Iso-propanol	10 % (v/v)	1.2 ml
CHAPS	4 % (w/v)	0.48 g
Bromophenol blue	0.002 % (v/v)	0.24 μl
DTT (*)	/	
IPG buffer (*)	/	
	Total	12 ml

Aliquot in 500 μl and store in -20 ℃

(*) Prior to use 2 mg of DTT and 1 % (i.e. 5 μl) of corresponding IPG buffer was freshly added to 500 μl rehydration

Nucleus isotonic buffer

	Total	
	Final Concentration	Amount
HEPES (100 mM)	20 mM	200 µl
NaCl	0.4 M	0.0234 g
$MgCl_2$ (1M)	1.5 mM	1.5 µl
EDTA (10 mM)	1 mM	100 µl
EGTA (10 mM)	1 mM	100 µl
DTT (100 mM)	1 mM	10 µl
PMSF (100 mM)	1 mM	10 µl
Leutinin (500 µg/ml)	2 µg/µl	0.4 µl
Aprotinin (200 µg/ml)	2 µg/µl	0.1 µl
Benzamidine (100 mg/ml)	0.5 mg/ml	5 µl
Glycerol	25 % v/v	250 µl
	Total	1000 µl

Freshly prepared before use

Store at 4℃

Peptide extraction solution

	Final Concentration	Amount
100 % Acetonitrile (ACN)	50 %	500 µl
10 % Trifluoracetic acid (TFA)	1 %	100 µl
Distilled water	/	400 µl
	Total	1000 µl

Phosphate buffer saline (PBS), pH 7.4

	Final Concentration	Amount
Sodium chloride	137 mM	8.0 g
Potassium chloride	2.7 mM	0.2 g
di-Sodium monophosphate	4.3 mM	1.54 g
Potassium di-phosphate	1.4 mM	0.19 g
	Total	1000 ml

Adjust pH with 0.1 N HCl to pH 7.4

Solution was autoclaved and filtered with 0.2 µm membrane

Store at 4℃

7.5 % SDS polyacrylamide separating gel

	Final Concentration	Amount
Distilled water	/	4.73 ml
30 % acrylamide	7.5 % (v/v)	2.5 ml
1.5 M Tris-HCl, pH 8.8	375 mM	2.5 ml
10 % SDS	1 % (v/v)	100 µl
10 % ammonium persulfate *	1 % (v/v)	100 µl
TEMED *	0.1 % (v/v)	10µl
	Total	10 ml

(*) Freshly added before use

10 % SDS polyacrylamide separating gel

	Final Concentration	Amount
Distilled water	/	39 ml
30 % acrylamide	10 % (v/v)	33.35 ml
1.5 M Tris-HCl, pH 8.8	375 mM	25 ml
10 % SDS	1 % (v/v)	1000 µl
10 % ammonium persulfate *	1 % (v/v)	1000 µl
TEMED *	0.1 % (v/v)	100 µl
	Total	100 ml

(*) Freshly added before use

5 % SDS polyacrylamide stacking gel

	Final Concentration	Amount
Distilled water	/	5.51 ml
30 % acrylamide	5 % (v/v)	1.67 ml
0.5 M Tris-HCl, pH 6.8	375 mM	2.5 ml
10 % SDS	1 % (v/v)	100 µl
10 % ammonium persulfate *	1 % (v/v)	100 µl
TEMED *	0.1 % (v/v)	10 µl
	Total	10 ml

(*) Freshly added before use

10 % Sodium dodecyl sulfate (SDS)

	Final Concentration	Amount
Sodium dodecyl sulfate (SDS)	10 % (w/v)	5 g
Distilled water	/	50 ml
	Total	50 ml

1X SDS electrophoresis buffer

	Final Concentration	Amount
10X SDS electrophoresis buffer	1X	100 ml
Distilled water	/	900 ml
	Total	1000 ml

10X SDS electrophoresis buffer

	Final Concentration	Amount
Tris	25 mM	30.3 g
Glycine	192 mM	144.0 g
SDS	0.1 % (w/v)	10.0 g
	Total	1000 ml

Prior to use dilution of 1X SDS electrophoresis buffer are prepared

4X SDS sample buffer

	Final Concentration	Amount
0.15 M Tris-HCl, pH 6.8	50 mM	50 ml
Glycerol	20 % (v/v)	40ml
Sodium dodecyl sulfate (SDS)	0.8 % (w/v)	0.8 g
β-mercaptoethanol	4 % (v/v)	4 ml
Bromophenol blue	0.001 % (w/v)	0.001 g
	Total	100 ml

Silver destain solution

	Final Concentration	Amount
Potassium ferricyanide	1 % (w/v)	0.5 g
Sodium thiosulfate	1.6 % (w/v)	0.8 g
25 mM ammonium carbonate	/	50 ml
	Total	50 ml

Freshly prepared

Spleen washing medium

	Final Concentration	Amount
RPMI 1640 medium	/	490 ml
Acid-citrate dextrose (ACD)	10 % (v/v)	/
10000 units/ml penicillin	100 units/ ml	5 ml
10000 µg/ml streptomycin	100 µg/ml	5 ml
	Total	500 ml

Store at 4℃

ACD was freshly added before use (i.e. 1 ml ACD + 9 ml medium)

Splenocyte preparation medium

	Final Concentration	Amount
RPMI 1640 medium	/	500 ml
10000 units/ml penicillin	100 units/ ml	5 ml
10000 µg/ml streptomycin	100 µg/ml	5 ml
	Total	50 ml

RPMI 1640 medium - store at 4℃

Sorensen buffer, pH 6.5

	Final Concentration	Amount
di-sodium monophosphate	66.6 mM	4.5 g
Potassium di-phosphate	66.6 mM	4.5 g
	Total	500 ml

Solution was filtered with 0.45 µm membrane

TA solution

	Final Concentration	Amount
100 % Acetonitrile (ACN)	33% (v/v)	500 µl
0.1 % Trifluoracetic acid (TFA)	66 % (v/v)	1000 µl
	Total	1500 µl

Transfer buffer

	Final Concentration	Amount
Tris	25 mM	15.1 g
Glycine	190 mM	72.0 g
Methanol	10 % (v/v)	500 ml
	Total	5000 ml

Store at 4℃

20 mM Tris-buffer saline (TBS), pH 7.6

	Final Concentration	Amount
Tris	20 mM	9.68g
Sodium chloride	137 mM	32.04
Distilled water	/	4000 ml
	Total	4000 ml

Adjust pH with 0.1N HCl to 7.6
Store at 4℃

20 mM Tris-buffer saline Tween (TBST), pH 7.6

	Final Concentration	Amount
Tris	20 mM	9.68g
Sodium chloride	137 mM	32.04
25 % Tween-20	0.05 % (v/v)	8 ml
Distilled water	/	4000 ml
	Total	4000 ml

Adjust pH with 0.1N HCl to pH 7.6 prior to addition of Tween-20
Store at 4℃

0.15 M Tris-HCl, pH 6.8

	Final Concentration	Amount
Tris	0.15 M	18.17 g
Distilled water	/	80 ml
	Total	100 ml

Adjust pH with 0.1 N HCl to pH 6.8
Store at 4℃

0.5 M Tris-HCl, pH 6.8

	Final Concentration	Amount
Tris	0.5 M	30.28 g
Distilled water	/	400 ml
	Total	500 ml

Adjust pH with 0.1 N HCl to pH 6.8
Store at 4℃

1.5 M Tris-HCl, pH 8.8

	Final Concentration	Amount
Tris	1.5 M	90.85 g
Distilled water	/	400 ml
	Total	500 ml

Adjust pH with 0.1 N HCl to pH 8.8
Store at 4℃

Trypan blue exclusion dye

	Final Concentration	Amount
Trypan blue exclusion powder	0.4 % (w/v)	0.4 g
Phosphate buffer saline (PBS), pH 7.4	/	100 ml
	Total	100 ml

Solution was filtered with 0.45 μm syringe filter

Wright-Sorensen working solution

	Final Concentration	Amount
Wright stain solution	0.05 % (w/v)	25 ml
Sorensen buffer pH 6.5	/	125 ml
	Total	150 ml

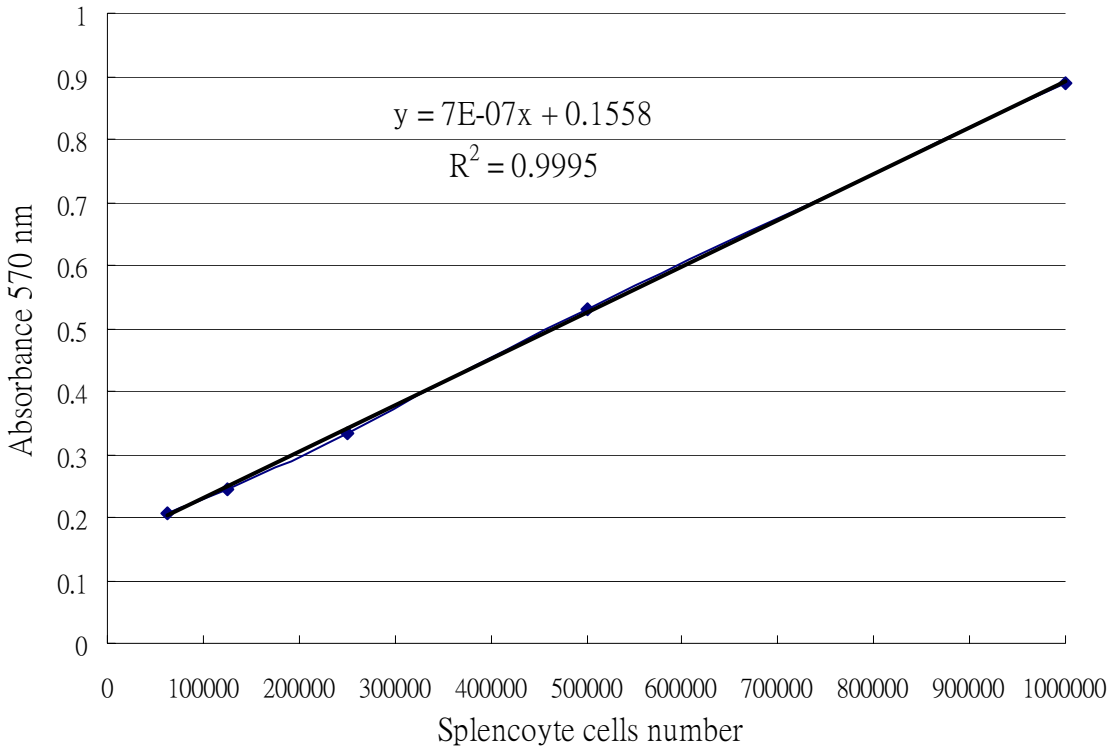
Wright stain solution

	Final Concentration	Amount
Wright stain powder	0.3 % (w/v)	0.3 g
100 % methanol	/	100 ml
	Total	100 ml

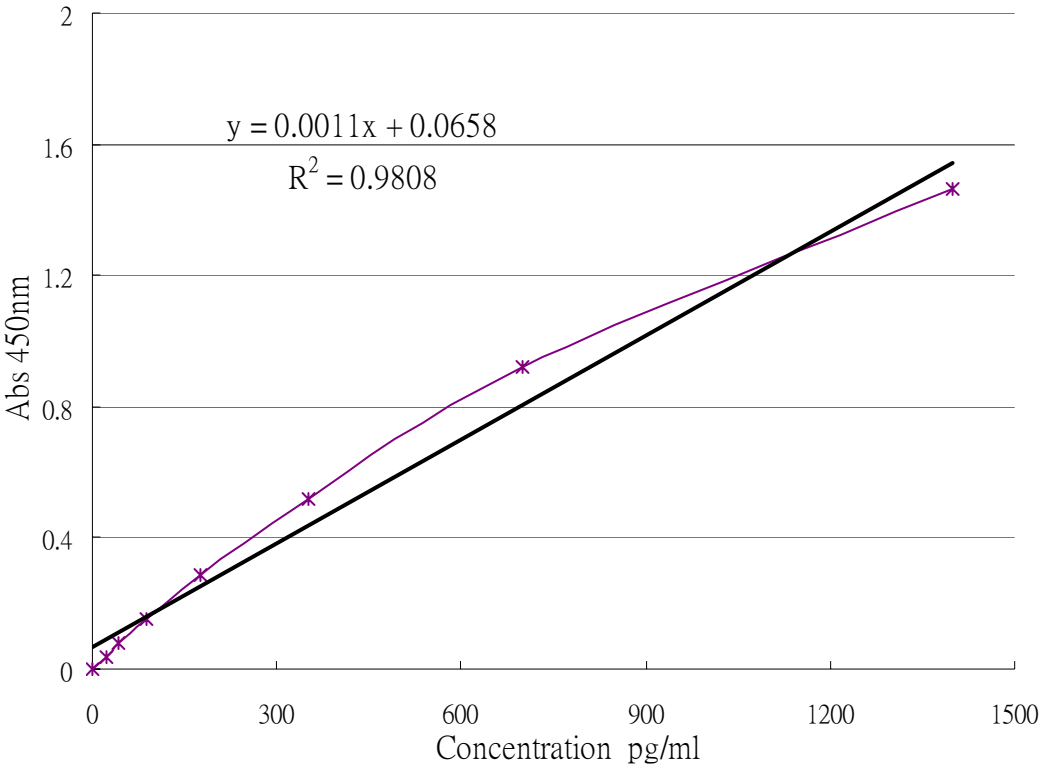
Solution was filtered with 0.45 filter membrane

Appendix II – Standard of MTT absorbance 570 nm with different number of splenocytes culture cells

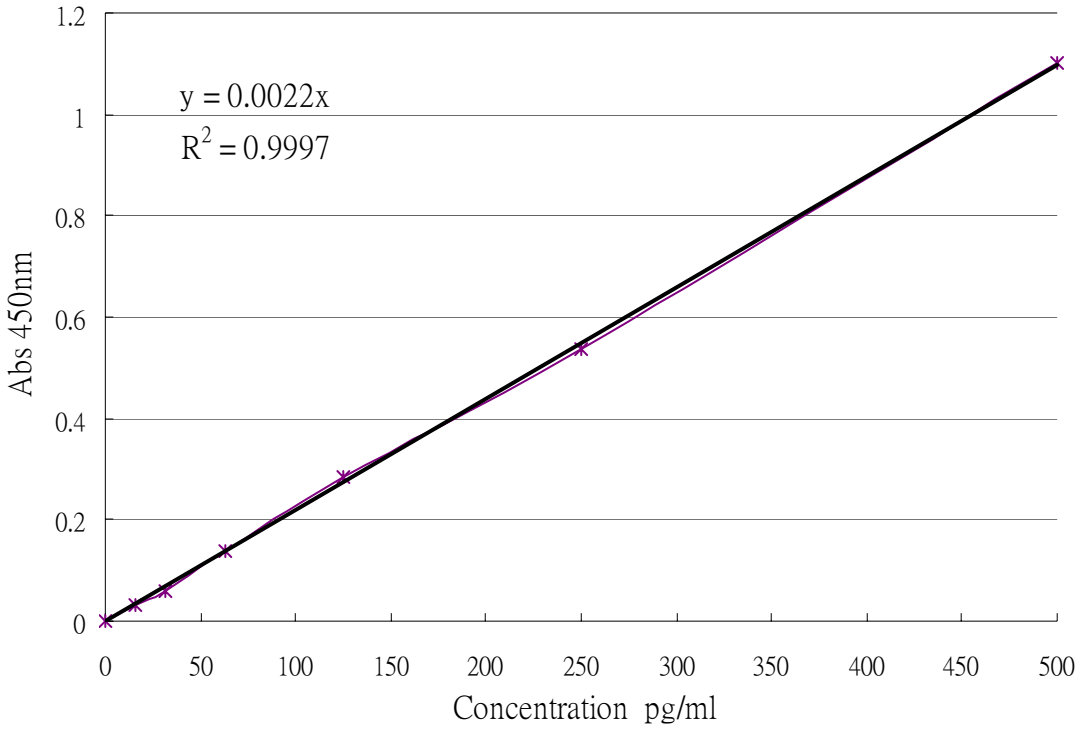
Splenocyte cell number	Abs 570 nm
1000000	0.889
500000	0.531
250000	0.332
125000	0.244
62500	0.207



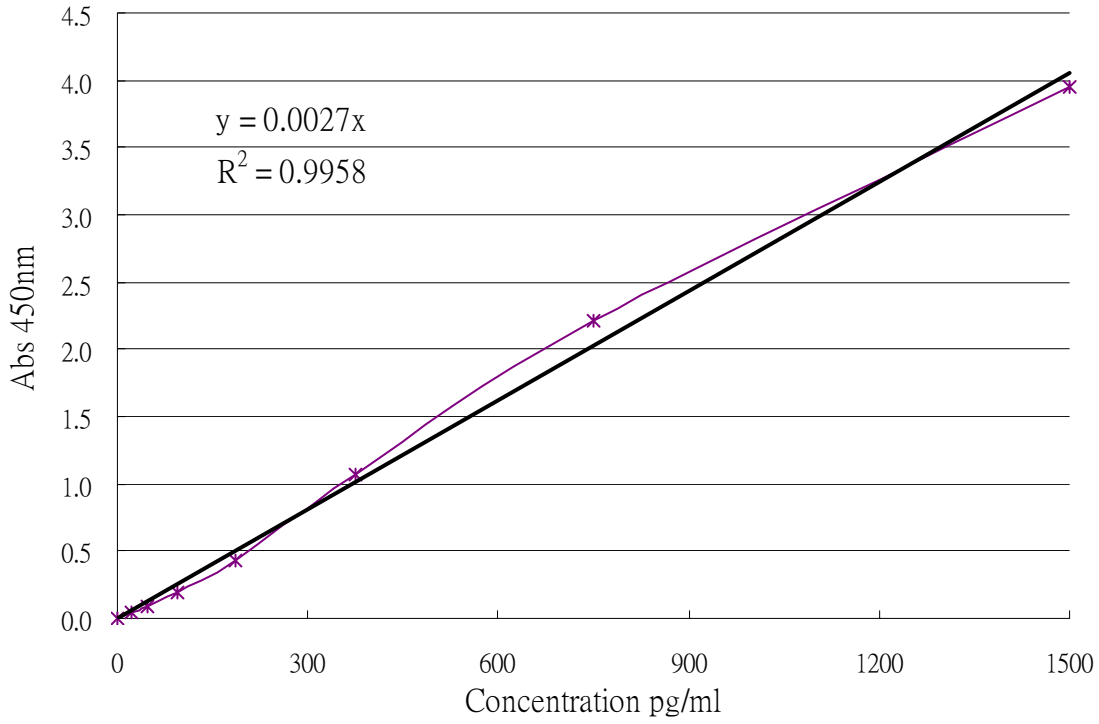
Appendix III – ELISA standard curve of interferon-gamma (IFN-γ)



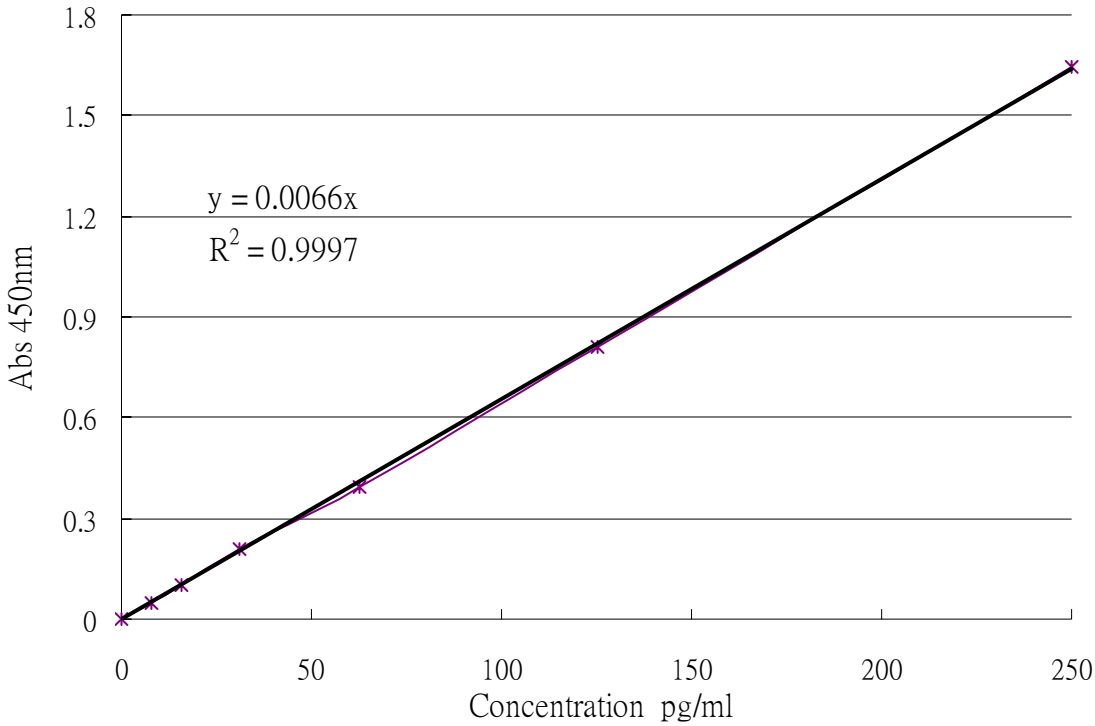
Appendix IV – ELISA standard curve of tumor necrosis factor-alpha (TNF-α)



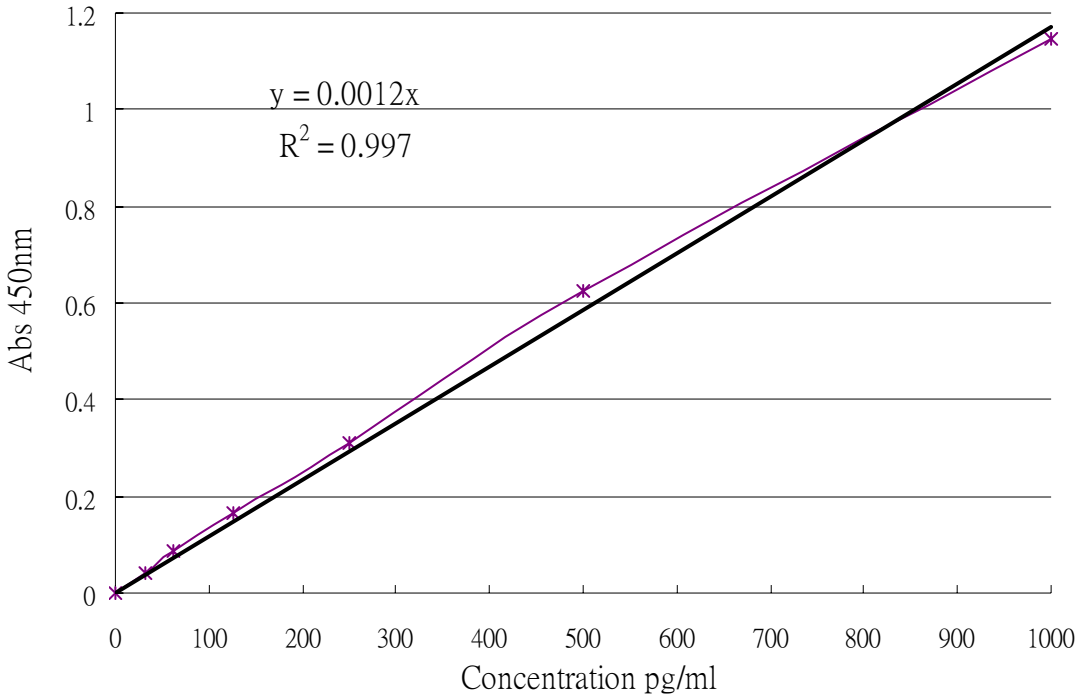
Appendix V – ELISA standard curve of interleukin-2 (IL-2)



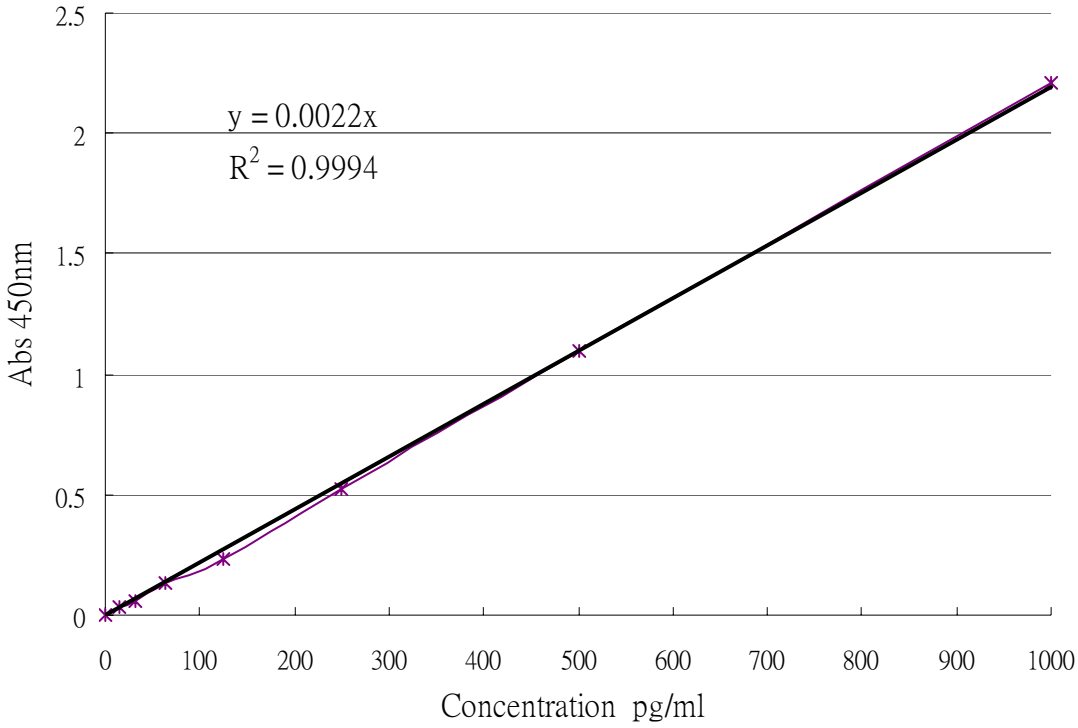
Appendix VI – ELISA standard curve of interleukin-1 alpha (IL-1α)



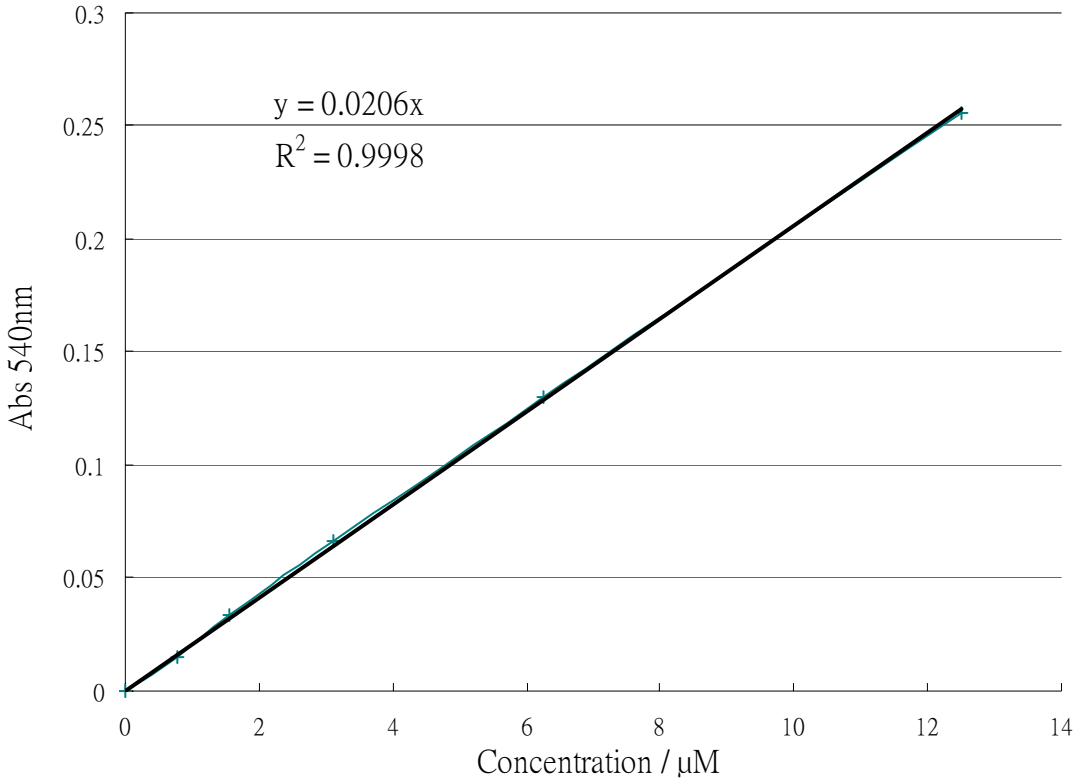
Appendix VII – ELISA standard curve of interleukin-6 (IL-6)



Appendix VIII – ELISA standard curve of interleukin-10 (IL-10)



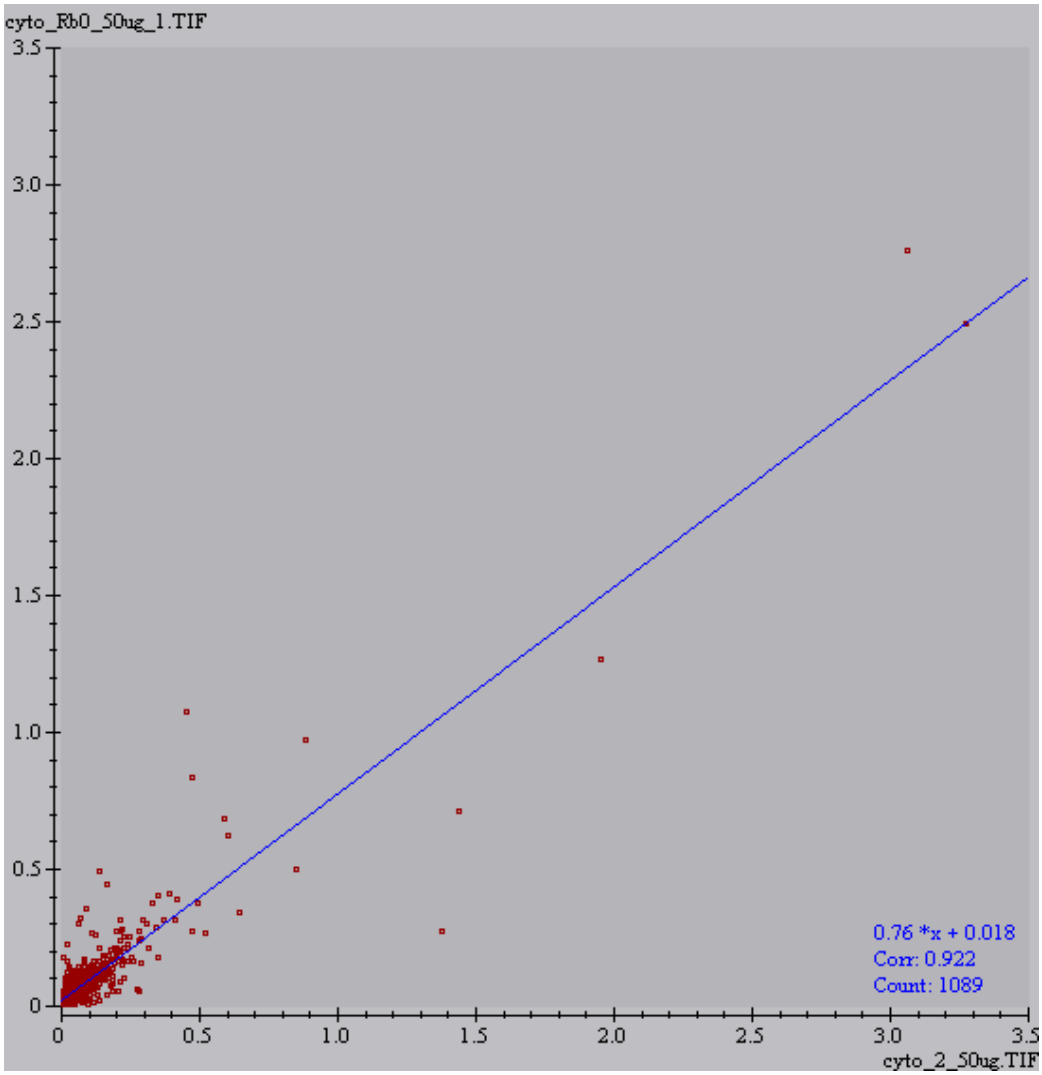
Appendix IX – Nitrite Standard Curve (Griess Reagent)



Appendix X – Mass spectrometry (MS) compatible silver stain protocol

Step	Purpose	Components	Volume	Time
1	Fixation	10 % Acetic acid 40 % Methanol 50 % Distilled water	250 ml	15 min
2	Fixation	10 % Acetic acid 40 % Methanol 50 % Distilled water	250 ml	15 min
3	Sensitization	17 g Sodium acetate 0.5 g Sodium thiosulphate 30 % Methanol	250 ml	30 min
4	Washing	Distilled water	250 ml	5 min
5	Washing	Distilled water	250 ml	5 min
6	Washing	Distilled water	250 ml	5min
7	Silvering	0.625 g Silver nitrate	250 ml	20 min
8	Washing	Distilled water	250 ml	1 min
9	Washing	Distilled water	250 ml	1min
10	Developing	6.25 g Sodium carbonate 100 µl Formaldehyde	250 ml	/
11	Stopping	3.74 g EDTA	250 ml	10 min
12	Washing	Distilled water	250 ml	∞

Appendix XI – Scatter plot of protein spots in 2D-map in terms of relative volume (calibration curve)



Appendix XII – Results of MASCOT searching

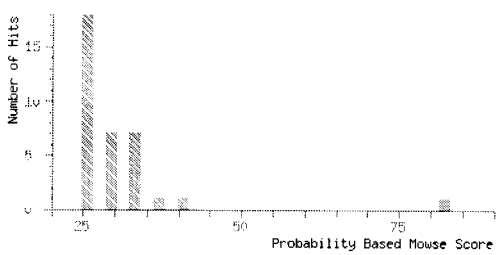
Spot 1

Mascot Search Results

```
User            : yoki
Email           : bcyoki@
Search title    :
Database        : NCBInr  (3103438 sequences; 1066605192 residues)
Taxonomy        : Rattus (34879 sequences)
Timestamp       : 5 Aug 2006 at 13:36:52 GMT
Top Score       : 82 for gi|51260019, Gelsolin [Rattus norvegicus]
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

```
1.    gi|51260019        Mass: 86413    Score: 82     Expect: 0.00021  Queries matched: 11
      Gelsolin [Rattus norvegicus]
      gi|385805          Mass: 2040     Score: 31     Expect: 25  Queries matched: 2
      protein-tyrosine phosphatase L1, PTPase L1 [rats, Peptide Partial, 19 aa, segment 2 of 13]
      gi|47576703        Mass: 36237    Score: 30     Expect: 35  Queries matched: 4
      olfactory receptor Olr171 [Rattus norvegicus]
      gi|18426662        Mass: 24985    Score: 28     Expect: 59  Queries matched: 4
      proteasome 26S non-ATPase subunit 9 [Rattus norvegicus]
      gi|695711          Mass: 19760    Score: 27     Expect: 68  Queries matched: 3
      pituitary adenylate cyclase activating polypeptide [Rattus rattus]
      gi|25742852        Mass: 19929    Score: 27     Expect: 73  Queries matched: 3
      regenerating islet-derived 3 alpha [Rattus norvegicus]
      gi|471156          Mass: 19800    Score: 27     Expect: 73  Queries matched: 3
      regenerating protein III (reg III) [Rattus norvegicus]
```

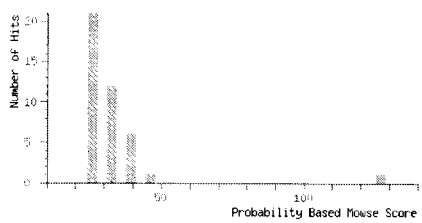
Spot 2

Mascot Search Results

User	:
Email	:
Search title	:
Database	: NCBInr (3103438 sequences; 1066605192 residues)
Taxonomy	: Rattus (34879 sequences)
Timestamp	: 5 Aug 2006 at 13:32:45 GMT
Top Score	: 127 for gi|55947, cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase [Rattus norvegicus]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05)



Concise Protein Summary Report

Format As [Concise Protein Summary] Help

Significance threshold p< [0.05] Max. number of hits [20]

[Re-Search All] [Search Unmatched]

1. gi|55947 **Mass:** 58025 **Score:** 127 **Expect:** 7e-009 **Queries matched:** 14
cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase [Rattus norvegicus]
gi|38566122 **Mass:** 36491 **Score:** 33 **Expect:** 20 **Queries matched:** 5
Enoyl coenzyme A hydratase 1, peroxisomal [Rattus norvegicus]
gi|12018256 **Mass:** 36521 **Score:** 33 **Expect:** 20 **Queries matched:** 5
enoyl coenzyme A hydratase 1, peroxisomal [Rattus norvegicus]

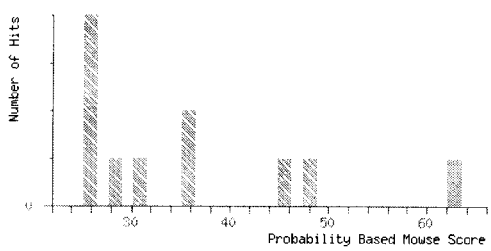
Spot 3

Mascot Search Results

User :
Email :
Search title :
Database : **NCBInr (3103438 sequences; 1066605192 residues)**
Taxonomy : **Rattus (34879 sequences)**
Timestamp : **5 Aug 2006 at 13:53:43 GMT**
Top Score : **63 for gi|25990263, mitochondrial aldehyde dehydrogenase [Rattus norvegicus]**

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|25990263 **Mass:** 53809 **Score:** 63 **Expect:** 0.018 **Queries matched:** 5
mitochondrial aldehyde dehydrogenase [Rattus norvegicus]
gi|45737864 **Mass:** 56051 **Score:** 62 **Expect:** 0.023 **Queries matched:** 5
mitochondrial aldehyde dehydrogenase precursor [Rattus norvegicus]
gi|45737868 **Mass:** 56078 **Score:** 62 **Expect:** 0.023 **Queries matched:** 5
mitochondrial aldehyde dehydrogenase precursor [Rattus norvegicus]
gi|45737866 **Mass:** 56079 **Score:** 62 **Expect:** 0.023 **Queries matched:** 5
mitochondrial aldehyde dehydrogenase precursor [Rattus norvegicus]
gi|14192933 **Mass:** 56966 **Score:** 62 **Expect:** 0.025 **Queries matched:** 5
aldehyde dehydrogenase 2 [Rattus norvegicus]
gi|16073616 **Mass:** 48639 **Score:** 60 **Expect:** 0.033 **Queries matched:** 5
aldehyde dehydrogenase [Rattus norvegicus]
gi|62658916 **Mass:** 39473 **Score:** 49 **Expect:** 0.43 **Queries matched:** 4
PREDICTED: hypothetical protein XP_579547 [Rattus norvegicus]

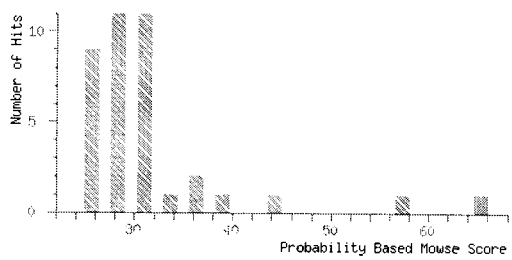
Spot 9

Mascot Search Results

User : yoki
Email : bcyoki@
Search title :
Database : NCBInr (3103438 sequences; 1066605192 residues)
Taxonomy : Rattus (34879 sequences)
Timestamp : 5 Aug 2006 at 13:46:53 GMT
Top Score : 65 for gi|11693176, acidic ribosomal phosphoprotein P0 [Rattus norvegicus]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|11693176 **Mass:** 34365 **Score:** 65 **Expect:** 0.01 **Queries matched:** 9
acidic ribosomal phosphoprotein P0 [Rattus norvegicus]
gi|57706 **Mass:** 34350 **Score:** 55 **Expect:** 0.12 **Queries matched:** 8
unnamed protein product [Rattus rattus]
gi|33086670 **Mass:** 15074 **Score:** 31 **Expect:** 30 **Queries matched:** 4
Cc2-17 [Rattus norvegicus]

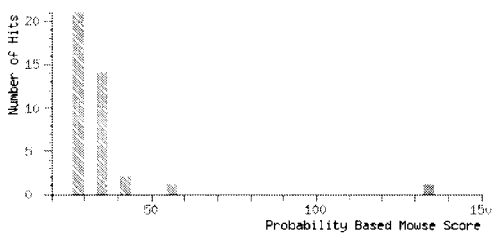
Spot 10

Mascot Search Results

User :
Email :
Search title :
Database : NCBInr (3103438 sequences; 1066605192 residues)
Taxonomy : Rattus (34879 sequences)
Timestamp : 5 Aug 2006 at 13:42:04 GMT
Top Score : 134 for gi|30410794, proteasome activator subunit 3 isoform 1 [Homo sapiens]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|30410794 **Mass:** 29602 **Score:** 134 **Expect:** 1.4e-009 **Queries matched:** 14
proteasome activator subunit 3 isoform 1 [Homo sapiens]
gi|13641168 **Mass:** 8194 **Score:** 45 **Expect:** 1 **Queries matched:** 4
adenylyl cyclase type VII [Rattus norvegicus]

2. gi|46237659 **Mass:** 119874 **Score:** 56 **Expect:** 0.094 **Queries matched:** 11
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 16 [Rattus norvegicus]

3. gi|33086600 **Mass:** 184955 **Score:** 40 **Expect:** 3.3 **Queries matched:** 13
Ab2-371 [Rattus norvegicus]

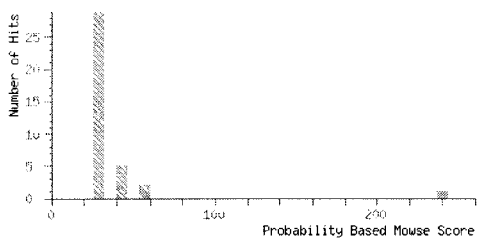
Spot 17

Mascot Search Results

```
User            :
Email           :
Search title    :
Database        : NCBInr  (3103438 sequences; 1066605192 residues)
Taxonomy        : Rattus (34879 sequences)
Timestamp       : 5 Aug 2006 at 14:20:32 GMT
Top Score       : 240 for gi|71534287, myxovirus (influenza virus) resistance 1 [Rattus norvegicus]
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

```
1.    gi|71534287       Mass: 75085    Score: 240    Expect: 3.5e-020  Queries matched: 25
      myxovirus (influenza virus) resistance 1 [Rattus norvegicus]
      gi|56721          Mass: 75050    Score: 230    Expect: 3.5e-017  Queries matched: 23
      unnamed protein product [Rattus norvegicus]
      gi|56723          Mass: 75597    Score: 32     Expect: 23  Queries matched: 7
      unnamed protein product [Rattus norvegicus]
      gi|62660050       Mass: 17295    Score: 32     Expect: 25  Queries matched: 5
      PREDICTED: hypothetical protein XP 214644 [Rattus norvegicus]
```

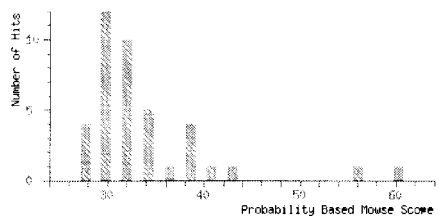
Spot 19

Mascot Search Results

```
User            :
Email           :
Search title    :
Database        : NCBInr  (3103438 sequences; 1066605192 residues)
Taxonomy        : Rattus (34879 sequences)
Timestamp       : 5 Aug 2006 at 14:14:39 GMT
Top Score       : 60 for gi|62660921, PREDICTED: similar to reticuloendotheliosis oncogene [Rattus norvegicus]
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05)



Concise Protein Summary Report

Format As: Concise Protein Summary Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All Search Unmatched

1. gi|62660921 **Mass:** 68948 **Score:** 60 **Expect:** 0.033 **Queries matched:** 8
   PREDICTED: similar to reticuloendotheliosis oncogene [Rattus norvegicus]
   gi|52546859 **Mass:** 24690 **Score:** 28 **Expect:** 55 **Queries matched:** 3
   PREDICTED: similar to LRRGT00028 [Rattus norvegicus]

2. gi|55250432 **Mass:** 46520 **Score:** 55 **Expect:** 0.11 **Queries matched:** 6
   Adipose differentiation-related protein [Rattus norvegicus]

3. gi|52353308 **Mass:** 28806 **Score:** 44 **Expect:** 1.6 **Queries matched:** 5
   tropomyosin 3, gamma isoform 1 [Rattus norvegicus]
   gi|438360 **Mass:** 28760 **Score:** 31 **Expect:** 26 **Queries matched:** 4
   tropomyosin

4. gi|37654272 **Mass:** 25320 **Score:** 41 **Expect:** 3 **Queries matched:** 4
   LRRGT00028 [Rattus norvegicus]

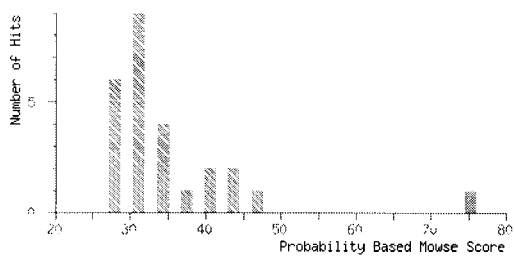
Spot 25

Mascot Search Results

User :
Email :
Search title :
Database : NCBInr (3103438 sequences; 1066605192 residues)
Taxonomy : Rattus (34879 sequences)
Timestamp : 5 Aug 2006 at 14:11:57 GMT
Top Score : 75 for gi|91997, aspartate transaminase (EC 2.6.1.1), cytosolic - rat

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|91997 **Mass:** 46627 **Score:** 75 **Expect:** 0.0011 **Queries matched:** 9
aspartate transaminase (EC 2.6.1.1), cytosolic - rat
gi|38197390 **Mass:** 46628 **Score:** 75 **Expect:** 0.0011 **Queries matched:** 9
Glutamate oxaloacetate transaminase 1 [Rattus norvegicus]
gi|6980970 **Mass:** 46528 **Score:** 52 **Expect:** 0.24 **Queries matched:** 7
glutamate oxaloacetate transaminase 1 [Rattus norvegicus]

2. gi|62657466 **Mass:** 125621 **Score:** 48 **Expect:** 0.59 **Queries matched:** 11
PREDICTED: similar to pre-acrosome localization protein [Rattus norvegicus]

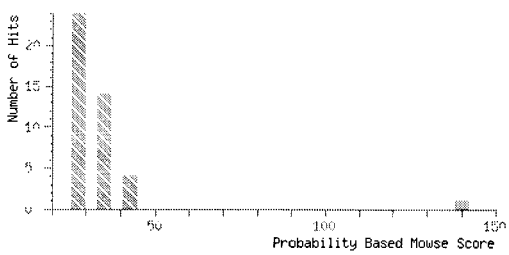
Spot 27

Mascot Search Results

```
User            : yoki
Email           : bcyoki@
Search title     :
Database        : NCBInr  (3103438 sequences; 1066605192 residues)
Taxonomy        : Rattus (34879 sequences)
Timestamp       : 5 Aug 2006 at 13:21:01 GMT
Top Score       : 140 for gi|142491330, LIM and SH3 protein 1 [Rattus norvegicus]
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|142491330 **Mass:** 30351 **Score:** 140 **Expect:** 3.5e-010 **Queries matched:** 14
   LIM and SH3 protein 1 [Rattus norvegicus]

2. gi|74047736 **Mass:** 75703 **Score:** 43 **Expect:** 1.8 **Queries matched:** 9
   ProSAPiP1 protein [Rattus norvegicus]

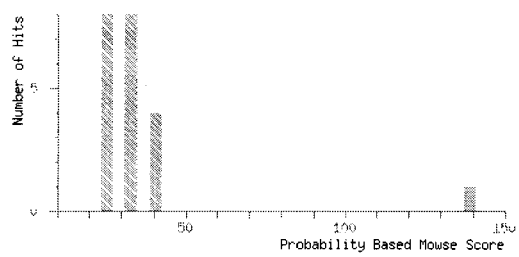
Spot 29

Mascot Search Results

User :
Email :
Search title :
Database : NCBInr (3103438 sequences; 1066605192 residues)
Taxonomy : Rattus (34879 sequences)
Timestamp : 5 Aug 2006 at 14:42:05 GMT
Top Score : 139 for gi|47940150, LIM protein [Rattus norvegicus]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|47940150 **Mass:** 36018 **Score:** 139 **Expect:** 4.4e-010 **Queries matched:** 10
LIM protein [Rattus norvegicus]
gi|8393153 **Mass:** 35959 **Score:** 139 **Expect:** 4.4e-010 **Queries matched:** 10
LIM protein [Rattus norvegicus]
gi|34865432 **Mass:** 16894 **Score:** 30 **Expect:** 33 **Queries matched:** 3
PREDICTED: hypothetical protein XP_347076 [Rattus norvegicus]
gi|37231603 **Mass:** 15557 **Score:** 29 **Expect:** 41 **Queries matched:** 3
Ribosomal protein S17 [Rattus norvegicus]

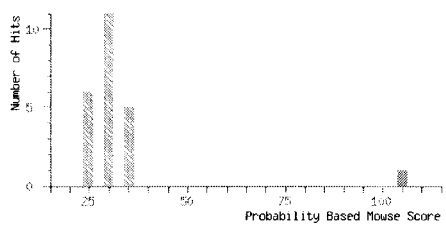
Spot 30

Mascot Search Results

User : yoki
Email : hcyoki@
Search title :
Database : NCBInr (3103438 sequences; 1066605192 residues)
Taxonomy : Rattus (34879 sequences)
Timestamp : 5 Aug 2006 at 13:25:55 GMT
Top Score : 105 for gi|57527447, ribose-phosphate pyrophosphokinase I -like [Rattus norvegicus]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|57527447 **Mass:** 35297 **Score:** 105 **Expect:** 1.1e-006 **Queries matched:** 8
ribose-phosphate pyrophosphokinase I -like [Rattus norvegicus]
gi|76673634 **Mass:** 35325 **Score:** 105 **Expect:** 1.1e-006 **Queries matched:** 8
PREDICTED: similar to Ribose-phosphate pyrophosphokinase I (Phosphoribosyl pyrophosphate synthetase
gi|206429 **Mass:** 35176 **Score:** 56 **Expect:** 0.084 **Queries matched:** 5
phosphoribosylphosphate synthetase (PRPS2) precursor
gi|56079 **Mass:** 35190 **Score:** 55 **Expect:** 0.11 **Queries matched:** 5
unnamed protein product [Rattus norvegicus]
gi|27662798 **Mass:** 35343 **Score:** 39 **Expect:** 4.3 **Queries matched:** 4
PREDICTED: similar to phosphoribosyl pyrophosphate synthetase 1-like 1 [Rattus norvegicus]
gi|62666219 **Mass:** 22840 **Score:** 30 **Expect:** 34 **Queries matched:** 3

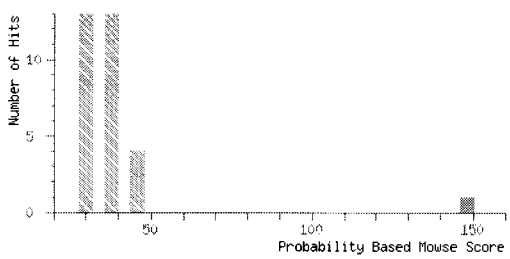
Spot 31

Mascot Search Results

User :
Email :
Search title :
Database : NCBInr (3103438 sequences; 1066605192 residues)
Taxonomy : Rattus (34879 sequences)
Timestamp : 5 Aug 2006 at 14:05:00 GMT
Top Score : 148 for gi|38512111, Tpi1 protein [Rattus norvegicus]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|38512111 **Mass:** 27214 **Score:** 148 **Expect:** 5.5e-011 **Queries matched:** 12
Tpi1 protein [Rattus norvegicus]
gi|62647962 **Mass:** 27345 **Score:** 148 **Expect:** 5.5e-011 **Queries matched:** 12
PREDICTED: triosephosphate isomerase 1 [Rattus norvegicus]
gi|12621074 **Mass:** 27417 **Score:** 130 **Expect:** 3.5e-009 **Queries matched:** 11
triosephosphate isomerase 1 [Rattus norvegicus]
gi|62663437 **Mass:** 27304 **Score:** 128 **Expect:** 5.5e-009 **Queries matched:** 11
PREDICTED: similar to Tpi1 protein [Rattus norvegicus]
gi|74271820 **Mass:** 27306 **Score:** 82 **Expect:** 0.0002 **Queries matched:** 8
triosephosphate isomerase [Rattus norvegicus]
gi|30027734 **Mass:** 5197 **Score:** 34 **Expect:** 16 **Queries matched:** 3
orf2 [Rattus sp.]

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