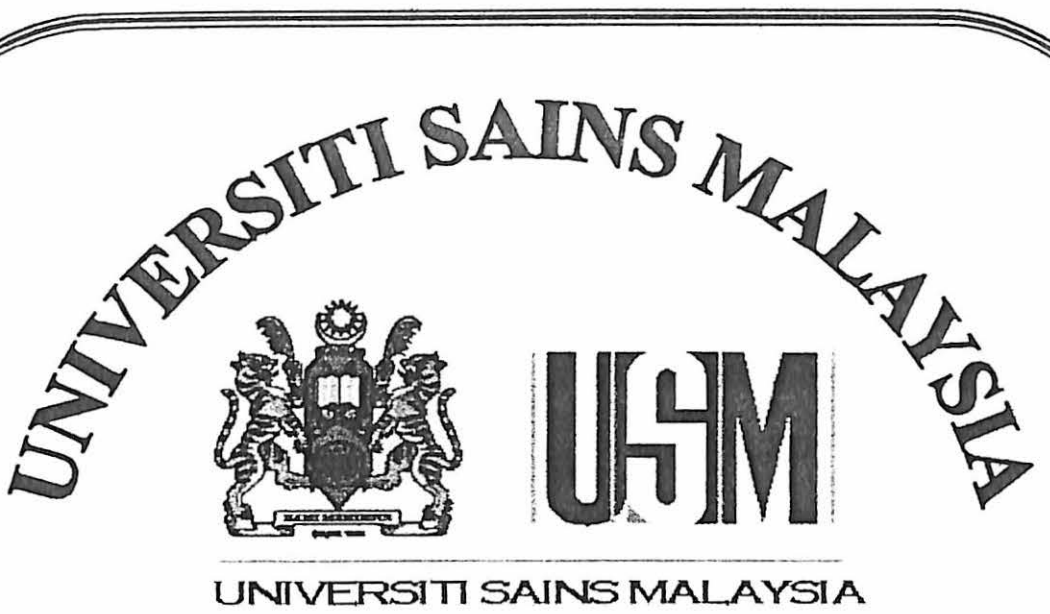

UNIVERSITI SAINS MALAYSIA

Expression of the transcription factor, PPARs in immune cells of balb/c mice

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Health Science (Biomedicine)

Mohana Letchumanan

School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150, Kubang Kerian, Kelantan
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CERTIFICATE

This is to certify that the dissertation entitled

"Expression of the transcription factor, PPARs in immune cells of balb/c mice"

is the bona fide record of research work done by

Ms Mohana Letchumanan

during the period September 2003 to March 2004

under our supervision.

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Date: 5/4/04

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Date: 5/4/04

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ABSTRACT

Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. Despite significant involvement in the lipid metabolism, little is known about the expression pattern and the role of different PPARs in immune regulation. Emerging evidence suggests that PPARs may act as negative immunomodulators. To provide further insight into the role of PPARs in the immune system, the expression patterns of PPARα, PPARγ1 and PPARγ2 in peritoneal macrophages and splenocyte-derived T lymphocyte subsets, CD4+ and CD8+ cells of balb/c mice were studied.

Total RNA from each of the cell subsets was extracted, reverse-transcribed to cDNA and the expression of PPARα, PPARγ1 and PPARγ2 quantified by Real-Time PCR using specific primers and probes. The expression levels were then calculated per μg total RNA and statistically analyzed using statistical software. In addition, cytokine expression profiles in the selected immune cells were also assessed using multiplex PCR method.

The results obtained in the present study demonstrate that all the immune cell subtypes predominantly express PPARγ1 suggesting the probable involvement of this transcription factor in immune response or other biological functions of these cells. The study also further confirms that lymphocytes express PPARα. CD8+ lymphocytes showed the lowest expression of all PPAR isoforms compared to other immune cells suggesting minimal involvement of the PPARs in the immune response mediated by these cells. In addition baseline expression levels of various cytokines in immune cell subsets in young adult mice also were showed in the current study.

INTRODUCTION

Peroxisome

Peroxisome is a subcellular organelle present in all human cells except mature red blood cells, as well as in many cells of animals, plants and fungi. There are more than 60 peroxisomal enzymes which catalyze various biochemical reactions within the cells, mainly the metabolism of hydrogen peroxide, lipid synthesis and β-oxidation of long and very long-chain fatty acids that cannot be oxidized by mitochondria. Thus, the importance of peroxisomal functions had been highlighted by the discovery of various peroxisomal disorders such as X-linked adrenoleukodystrophy, infantile Refsum's disease and Zellweger's syndrome (Wanders *et.al.*, 1996; Suzuki *et.al.*, 1996)

Peroxisome Proliferators

A number of structurally diverse substances were found to promote proliferation of peroxisomes. These substances are collectively named "peroxisome proliferators", which include fibrate hypolipidaemic drugs (e.g., bezafibrate, fenofibrate), non-fibrate hypolipidaemic drugs, some non-steroidal anti-inflammatory drugs (e.g., ibuprofen), thiazolidinediones and leukotriene D4 receptor antagonists (Chinetti *et al.*, 1998; Tedgui and Mallat, 2001). A rapid induction of gene expression, particularly those involved in fatty acid oxidation following peroxisome proliferator administration, had led to the discovery of receptor mediated mechanism of action. This receptor was identified and cloned by Issemann and Green, in 1990 from mouse liver and designated as, peroxisome proliferator activated receptor-α (PPAR-α) (Zhang *et al.*, 2002). Additional PPAR subtypes, PPAR-β and PPAR-γ were subsequently identified and cloned (Dreyer *et.al.*, 1992).

PPAR Structure, Function and Distribution

PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamiliy, which include receptors for thyroid hormone, steroid hormones and vitamin D (Boiter *et al.*, 2003). Basically, PPARs are composed of three distinct isoforms, namely PPARα, PPARβ (also known as PPARδ) and PPARγ. PPARγ has three splice variants termed PPARγ1, γ2 and γ3 (Willson *et al.*, 2001; Kintscher *et al.*, 2002). Each isoform is encoded by a separate gene (Tontonaz *et al.*, 1994; Zhu *et al.*, 1993; Mukherjee *et al.*, 1997).

PPARs like other nuclear hormone receptors, are typically organized into five to six structural regions (A, B, C, D, E and F) and four functional domains (A/B, C, D, E/F) (Vamecq and Latruffe, 1999) (Figure 1). The A/B domain at the amino-terminal is involved in transactivation. This region is poorly conserved and most variable in length between the three PPAR isotypes (Boiter *et al.*, 2003; Kersten, 2001). The C domain is the DNA binding domain. It is highly conserved and consists of two zinc finger-like structures.

P box in the first zinc-finger (as indicated in Figure 1) is involved in the recognition of the response element and the D box in the second zinc-finger is involved in dimerization (Wahli *et al.*, 1995). The D region encodes a flexible hinge region, thought to allow independent movement of the E/F domain relative to the C domain (Boiter *et al.*, 2003). The E/F domain is the ligand binding domain (LBD) and is responsible for ligand-binding which converts PPAR to an active form that binds DNA and modulates gene expression. In addition, the E region is essential for nuclear localization and dimerization of the receptor (Boiter *et al.*, 2003).

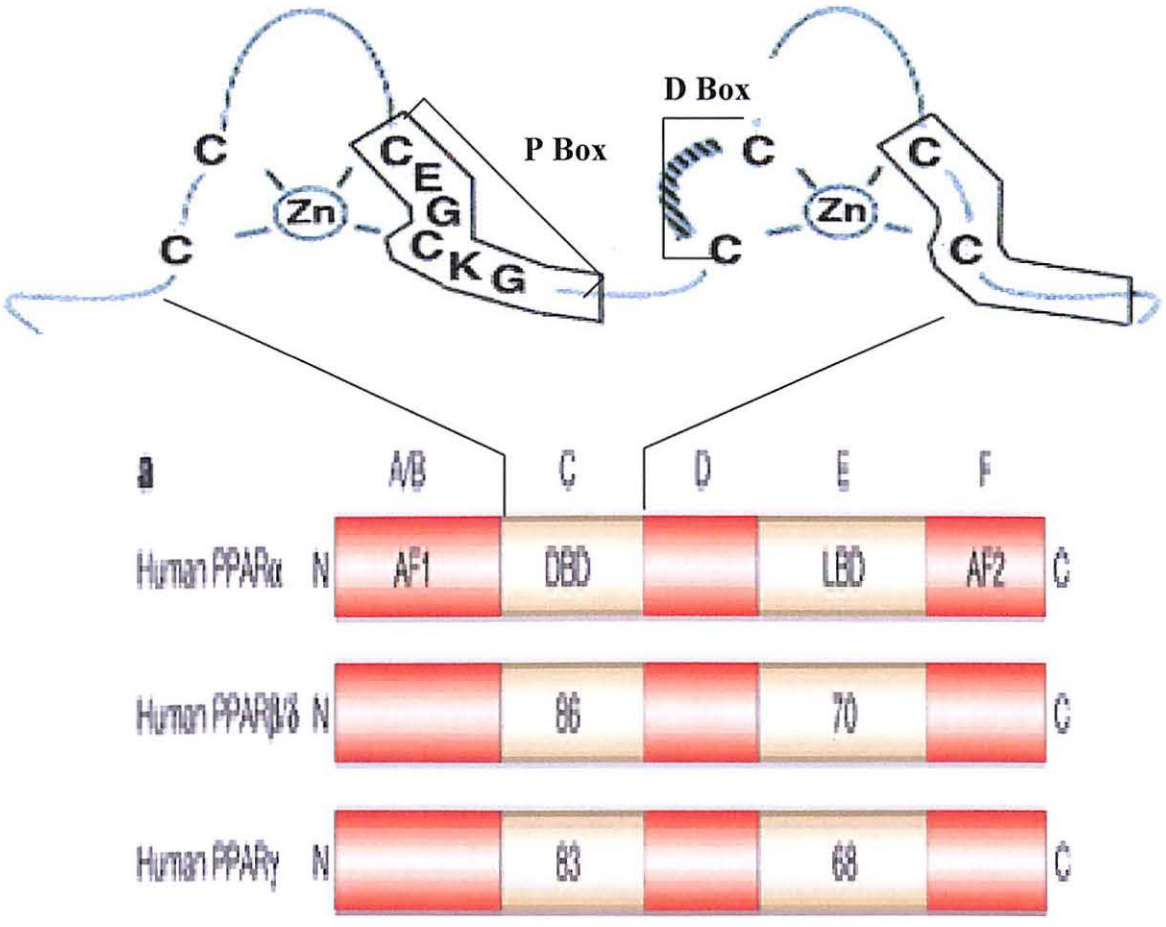

Figure 1

Schematic representation of the domain structures of various human PPARs.

The numbers denote total amino acid residues in the PPAR polypeptide.

(Wahli *et al.*, 1995)

PPARα is highly expressed in tissues with high rate of fatty acid catabolism, as in the brown adipose tissue, liver, kidney and heart (Braissant, 1996). It plays an important role in the hepatic lipid metabolism, bile acid synthesis and control of fatty acid transport and uptake (Boiter *et al.*, 2003). Meanwhile, PPARβ is ubiquitously expressed and its function has largely remained an enigma. It has been proposed that PPARβ regulates lipid metabolism in nerve cells and is a mediator of fatty acid-controlled differentiation (Kersten, 2001).

PPARγ isoform is present mainly in adipose tissue, colon and the immune system (Braissant, 1996), and plays an important role in adipocyte differentiation and in the regulation of macrophage activation (Chinetti *et al.*, 1998). In addition, it regulates the production of monocyte inflammatory responses by inhibiting the production of inflammatory cytokines such as IFNγ , a potent macrophage activator (Jiang *et al.*, 1998).

Like other members of the nuclear receptor superfamily, PPARs possess a central DNA-binding domain that recognizes DNA sequences, termed PPAR-response elements (PPREs), in the promoter regions of their target genes. PPARs heterodimerize with another member of the nuclear-receptor superfamily, the retinoid X receptors (RXR), and the transcription regulation of target genes by PPARs is actually achieved through the binding of these PPAR-RXR heterodimers to PPREs (Kliewer *et.al.*, 1992; Mangelsdorf *et.al.*, 1995; Mangelsdorf *et.al.*, 1990).

RXR also exists in multiple isoforms, RXRα, β, and γ, and like the PPARs, have a variable tissue distribution (Chambon *et.al.*, 1996; Mangelsdorf *et.al.*, 1992). RXR isoforms are activated by 9-cis retinoic acid (Kliewer *et.al.*, 1992). It is not known if any one of the

particular RXR isoforms preferentially binds one or more of the PPAR isoforms. RXR also forms heterodimers with other members of the nuclear receptor superfamily, and these interactions influence the PPAR-regulated transcriptional activation because of the competition among various RXR heterodimerization partners for RXR (Lemberger *et.al.*, 1996). In the presence of ligands for PPAR, the PPAR: RXR heterodimer does not require that 9-cis retinoic acid be present for transcriptional activation.

However, when combined as PPAR: RXR heterodimer, PPAR ligands and 9-cis retinoic acid can act synergistically on PPAR responses (Kliewer *et.al.*, 1992). The different heterodimers of RXR (e.g., PPAR: RXR) allow for specific responses by binding to highly specific sequences in the promoter regions of the genes they transactivate (Mangeldorf *et.al.*, 1995).

Although the PPAR: RXR dimer is the focus for determining specific gene transcription on ligand activation, transactivation of a particular gene actually requires a large complex of proteins (Torchia *et.al.*, 1998). Thus, the regulation of PPAR-regulated transcriptional activation is made more complex by the involvement of coactivators and corepressors (Zhu *et.al.*, 1997; Zhu *et.al.*, 1996).

In the inactivated state, the PPARs are believed to be in complexes bound with corepressor proteins. In this state, in some but not all cell types, PPARs may have a cytoplasmic rather than a nuclear location (Chinetti *et.al.*, 1998; Bishop-Bailey *et.al.*, 1999). Upon ligand activation, PPARs dissociate from corepressors and recruit coactivators, including the PPAR-binding protein (Zhu *et.al.*, 1997) and the steroid receptor coactivator-1 (Zhu *et.al.*, 1996), and can translocate from the cytoplasm to the nucleus (Bishop-Bailey *et.al.*, 1999).

The earliest evidence suggesting the potential role of PPAR in immune regulation came from the study that PPARα^{-} deficient mice display a prolonged response to inflammatory stimuli. It was suggested that PPARα deficiency results in a reduced β-oxidation of these inflammatory fatty acid derivatives (Chinetti *et al.*, 2000).

PPARs were shown to inhibit the activation of inflammatory response genes such as IL-2, IL-6, IL-8 and TNFα by inhibiting NF-κB, STAT and AP-1 signaling pathways. The current study aims to quantify and compare the expression of PPARα, PPARγ1 and PPARγ2 in selected immune cells of balb/c mice and to study the relationship between cytokine levels and PPAR expression.

REVIEW OF LITERATURE

Since the discovery of the first member of PPAR family, the role of these receptors in the regulation of the metabolism of lipids and lipoproteins, and in immune and inflammatory responses as well as the mechanism of action involved have been extensively studied.

Jones *et.al* (2002), demonstrated that spleen-derived T cells (both $CD4^+$ and $CD8^+$) and B cells constitutively expressed PPARα. They found that PPARα is the predominant isoform expressed in lymphocytes whereas PPARγ dominates cells of the myeloid lineage (macrophages, dendritic cells and mast cells).

In addition, quantification of PPARα transcript in T lymphocytes isolated from peripheral lymph nodes, spleen, and Peyer's patches of normal C3H/HeN mice suggests that PPARα expression in T lymphocytes may be influenced by the microenvironment of lymphoid organ in which the lymphocytes reside, since Peyer's patch T cells expressed greater amount of PPARα compared to those isolated from peripheral lymphoid organs in the study (Jones *et.al.*, 2002).

In 1998, Tontonoz *et.al* reported that PPARγ might promote monocyte/macrophage differentiation and uptake of oxidized LDL, as the macrophages play a crucial role in vascular wall inflammation and atherosclerotic plug formation. The finding is further affirmed by Chinetti *et.al* (1998). They reported that both PPARα and PPARγ were expressed in differentiated human macrophages. The study in which, a qualitative RT-PCR analysis was performed using specific primers on RNA from freshly isolated human monocytes and macrophages at different stages of differentiation, revealed that PPARα

expression is already detectable in monocytes and increases along the differentiation process into macrophages.

By contrast, PPARγ expression is not detectable in monocytes, but is strongly induced upon differentiation into macrophages. Since PPARγ expression is undetectable in circulating human monocytes and appears only several hours after induction of differentiation, it is unlikely that PPARγ is involved in the initial differentiation process and appears to be an important component of further downstream processes in macrophage differentiation and function.

Various factors may influence the expression of different PPAR isoforms *in vivo*. It has been recently reported by Ricote *et.al* (1998), that the expression of PPARγ is up regulated during the differentiation of macrophage colony-stimulating factor (M-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) treated bone marrow progenitor cells.

PPARγ expression was detected in the adherent macrophage population that was induced by M-CSF. GM-CSF also induced PPARγ mRNA expression in the adherent macrophage population, although less strongly than M-CSF. Treatment of resident peritoneal macrophages with M-CSF and GM-CSF led to a marked increase in PPARγ protein levels (Ricote *et.al.*, 1998).

Furthermore, PPARγ was also found to be highly expressed in thioglycolate-elicited peritoneal macrophages (Ricote *et.al.*, 1998). These observations suggest that humoral factors (M-CSF and GM-CSF) that are produced during the inflammatory process

augment PPARγ expression. PPARγ mRNA expression was also upregulated in primary macrophages and THP-1 monocytic leukemia cells by the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (Ricote *et.al.*, 1998).

OBJECTIVES

The objective of this project was to demonstrate the different expression pattern of PPARα, PPARγ1 and PPARγ2 in selected immune cells; peritoneal macrophages, CD4+ and CD8+ lymphocytes in the animal model of balb/c mice under normal condition. Specifically, this research approach was used to:

1. Quantify and compare the expression of the transcription factors, PPARα, PPARγ1 and PPARγ2 in selected immune cells of balb/c mice.
2. Study the relationship between cytokine levels and PPAR expression in selected immune cells of balb/c mice.

The study objective will be used to demonstrate the possible involvement of different PPAR isoforms namely, PPARα, PPARγ1 and PPARγ2 in selected immune cells; peritoneal macrophages, CD4+ and CD8+ lymphocytes.

MATERIALS AND METHODS

Materials

5.1 Experimental Animals

Male balb/c mice between 6 to 7 weeks old were used in the study. The animals were housed at the Universiti Sains Malaysia Animal House. All the mice were sacrificed by cervical dislocation

5.2 Reagents and Kits

Reagents and kits used in this study are listed below.

RNeasy® Mini Kit and Qiashredder (Qiagen Inc., USA), Dynabeads® Mouse CD4 [L3T4] and Dynabeads® Mouse CD8 [Lyt2] (Dynal Co., Norway), TaqMan® PCR Reagent Kit and 2X TaqMan® Universal Master Mix, TaqMan® Probes [AB] (Applied Biosystems Co., USA), RevertAid™ H Minus First Strand cDNA Synthetic Kit (Fermentas, USA), Mouse 18S rRNA Primer Set Kit (Maxim Biotech, USA), Human Monoclonal Antibodies: Control IgG1 PE, Control IgG1 FITC, anti-CD3 [Leu-4] FITC, anti-CD4 [L3T4] PE, anti-CD8 [Lyt2] PE, anti-CD14 FITC (Becton Dickson, USA), MPCR Kit for Human Inflammatory Gene Set 1(Maxim Biotech, USA), GeneAmp® Optical Caps, GeneAmp® Optical Tubes, GeneAmp® 96 Well Tray/Retainer Set, GeneAmp® Splash Free Support Base (Perkin Almer Cetus Corp, USA).

5.3 Solutions and Chemicals

Ethanol (Merck, Germany), $NaH_2PO_4.2H_2O$ (E. Merck, Germany), KCL (Becton Dickson, UK), NaCl (E. Merck, Germany), NH_4Cl (Becton Dickson, UK), KHCO (Becton Dickson, UK), Trypan Blue (Sigma, USA), Sodium Bicarbonate solution [7.5% w/v] (Sigma, USA), Tris base (Promega, USA), Diethyl pyrocarbonate water [DEPC] (Sigma, USA), EtBr powder (Sigma, USA), Agarose powder (Promega, USA), RPMI 1640 solution

5.4 Equipments

Dynal Magnetic Particle Concentrater [MPC-L] (Dynal Co, Denmark), Dynal Particle Mixer (Dynal Co, Denmark), Real-Time PCR (ABI PRISM® 5700 Sequence Detection System, Applied Biosystems,USA), 15 ml Falcon tube (Becton Dickinson Labware, USA), Haemacytometer (Assistant, Germany), Light microscope (Leica Microsystems, Germany), FACScan Flow Cytometer (Becton-Dickinson, USA), Spectrophotometer (Eppendorf, Germany), RNase-free quartz cuvette (Eppendorf, Germany), Agarose gel apparatus model MGU-202T (C.B.S. Scientific Co., California), Electrophoresis power supply (Amersham Pharmacia Biotech, USA), UV Transilluminator (Spectroline, Model TC-312A, USA), Digital Image Analyzer (Amersham Pharmacia Biotech, USA), Mini centrifuge (National Labnet Co., Woodbridge), Perkin Elmer GeneAmp® PCR System 2400 (Applied Biosystem, USA), ImageMaster TotalLab software (Amersham Pharmacia Biotech, USA), Perkin Elmer GeneAmp® PCR System 9700 (Applied Biosystem, USA), Primer Express Software (Applied Biosystem, USA).

Methods

5.5 Reagent Preparation

(a) 70% Ethanol

Prepared by dissolving 70ml ethanol in 30ml deionized water.

(b) 10X Phosphate Buffered Solution (PBS)

Prepared by dissolving 1.4 g $NaH_2PO_4.2H_2O$, 0.2g KCl and 8.1g NaCl in 800ml deionized water. The pH of the solution adjusted to 7.3-7.4 by 3M NaOH and made up to 1L by adding deionized water. The solution was then autoclaved at 121°C for 15 minutes and stored at room temperature. A working solution was prepared by adding in deionized water and stored at 4°C.

(c) Lysis Buffer, 6X ACK

Prepared by dissolving 49.7g NH_4Cl, 100.1g KHCO and 0.222g EDTA in 800ml deionized water. The pH of the solution adjusted to 7.3-7.4 by 3M NaOH and made up to 1L by adding deionized water. To prepare a working solution, the 6X ACK stock solution diluted by adding deionized water and the 1X ACK was filtered through a 0.2 μm pore membrane filter to sterilize the solution.

(d) RPMI 1640 solution

Prepared by dissolving one packet of powdered media, 2.0 grams of NaHC03 and 4.4 ml lN HCl in 300 ml ddH20. The volume was made up to 1L using ddH20. The solution sterilized and stored at 4°C.

Agarose Gel Electrophoresis (RNA)

(e) 50x Tris-acetate buffer (TAE)

Prepared by dissolving 121g Tris base, 28.5ml glacial acetic acid and 50ml 0.5M EDTA pH 8.0 in diethyl pyrocarbonate (DEPC) water. The solution was then autoclaved at 121°C for 15 minutes and stored at room temperature. A 1x TAE running buffer and gel loading buffer prepared by diluting in DEPC water.

(f) RNA loading buffer for gel electrophoresis

Prepared by mixing 20g sucrose and 0.125g orange G in 50ml DEPC water.

(g) Ethidium bromide (EtBr)

10mg/ml EtBr solution was prepared by dissolving 0.1g EtBr powder in 10ml deionized water. The solution was kept in a dark bottle and stored at room temperature.

Agarose Gel Electrophoresis (DNA)

(h) 10x tris-borate EDTA (TBE) stock buffer

Prepared by dissolving 54g Tris base, 27.5g boric acid and 20ml 0.5M EDTA pH 8.0 in 500ml deionized water. The solution was then autoclaved at 121°C for 15 minutes and stored at room temperature. A working solution of 0.5x TBE buffer was made with deionized water.

(i) DNA loading buffer:

Prepared by dissolving 0.125g Orange G and 20g sucrose in 50ml deionized water.

5.6 Experimental Procedure

(a) Isolation of Mouse Peritoneal Macrophages and Splenocytes

Peritoneal macrophages and splenocytes were isolated from male balb/c mice of 6-7 weeks old.

Peritoneal Washing

Mouse was first sacrificed by cervical dislocation. Skin surrounding the peritoneal region was swabbed with 70% ethanol and was cut opened to reveal the abdominal cavity. Five milliliters of RPMI 1640 solution was then injected into the abdominal cavity using a sterile syringe and gently massaged in order to optimize the harvest. After 2 to 3 minutes, the RPMI solution was retrieved back into the syringe. The peritoneal washout was then put into a 10ml centrifuge tube and centrifuged at 1500 rpm for 5 minutes. The pellet formed was rinsed with 5 ml PBS and resuspended in 1 ml PBS. The cells were counted using a haemacytometer.

Splenocytes

After harvesting the peritoneal washout, the abdominal cavity was cut opened and the spleen removed and placed in a sterile petri dish containing five to six milliliters of RPMI 1640 solution. The spleen was then disrupted mechanically using a syringe plunger and the homogenate was filtered using sterile cotton wool placed in a syringe. The filtrate was collected into a new tube and centrifuged at 1500 rpm for 5 minutes. The pellet was then resuspended in 5 ml of ACK (1X) solution and incubated at room temperature for 5-10 minutes to enable the ACK solution lyse the red cells. The suspension was then

centrifuged at 1500 rpm for 5 minutes and was finally resuspended in one ml PBS. The total splenocyte population was counted manually using haemacytometer.

(b) Isolation of CD4+ and CD8+ Cells from Total Splenocytes

Estimation of Dynabeads Concentration

The antibody coated beads, Dynabeads® Mouse CD4 (L3T4) and Dynabeads® Mouse CD8 (Lyt2) used in the present study were obtained from Dynal Inc., Denmark. One hundred µl of the bead concentrate consists of 4×10^7 beads. The maximum binding capacity of the RNeasy column is 100 µg RNA and only 1.0×10^7 cells (maximum starting material) were used in order to obtain high yield and pure RNA. Therefore, in the experiment one hundred µl of the bead concentrate was used in the study. The volume of cell suspension to be added to the beads concentrate calculated.

Washing and Preparation of the Dynabeads

One hundred µl of Dynabeads concentrate was pipetted into a tube and was placed in the Dynal Magnetic Particle Concentrator (Dynal Inc., Denmark). The beads were allowed to settle for 2 minutes a room temperature. The supernatant was removed carefully to avoid removal of the beads from the tube. One ml of PBS was then added to the beads and again allowed to settle for 2 minutes. The supernatant was discarded and the splenocyte suspension added into the tube.

Extraction of CD4+ and CD8+ Lymphocytes

A constant amount of 3.5 x 10^7 splenocytes was used for sorting T-helper (CD4+) and T-cytotoxic (CD8+) cells using dynabeads (positive selection). The volume of splenocytes containing 3.5 x 10^7 cells was calculated from the total spelenocyte population and pipetted into washed Dynabeads® Mouse CD4 (L3T4) concentrate in a tube. The mixture was mixed well and was then incubated for 1 hour in the cold room (on a rotor).

After 1 hour, the tube was placed in the Dynal Magnetic Particle Concentrator and allowed to settle for 2 minutes (CD4+ cells in the suspension will specifically bind to the Dynabeads® Mouse CD4 (L3T4) beads).

The supernatant was carefully transferred to avoid the removal of bead -CD4+ complexes and unbound beads into new a tube. The pellet (bead-CD4+ complexes and unbound beads) was washed with one ml of PBS and centrifuged at 1500 rpm for 5 minutes. The pellet was then resuspended in one ml PBS and kept on ice or refrigerated. Cells in the supernatant removed earlier was counted using haemacytometer. The supernatant was then added to washed Dynabeads® Mouse CD8 (Lyt2) concentrate. The mixture was incubated for 1 hour in the cold room (on a rotor).

After 1 hour, the tube was placed in the Dynal Magnetic Particle Concentrator and allowed to settle for 2 minutes (CD8+ cells in the suspension will specifically bind to the Dynabeads® Mouse CD8 (Lyt2) beads). The supernatant was carefully transferred to avoid the removal of bead –CD8+ complexes and unbound beads into new tube. The pellet

(bead-CD8+ complexes and unbound beads) was washed with one ml of PBS and centrifuged at 1500 rpm for 5 minutes. The pellet was then resuspended in one ml PBS and kept on ice or refrigerated. Once again, cell count was performed using haemacytometer. The suspension was then kept on ice or refrigerated.

(c) Flowcytometry Analysis

Flowcytometry analysis was employed to enumerate the percentage of CD4-positive and CD8-positive lymphocytes from the total splenocytes harvested. Flow cytometry analysis was carried out using monoclonal antibodies labeled with fluorescent dyes specific for mononuclear cell surface antigens. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labeled monoclonal antibodies that emit green and orange fluorescence respectively were used. A total of 10 µl antibodies conjugated with FITC or PE were added into labeled 5 ml polystyrene round-bottom tube as shown in Table 1. Fifty µl of cell suspension was added into each tube, mixed well and incubated in the dark for 30 minutes. All 4 tubes were centrifuged at 1500 rpm for 5 minutes. After centrifugation, supernatants were then discarded and the pellets were washed with 2 ml PBS. The cells were fixed in 200 µl PBS and analyzed using the FACScan flowcytometer (Becton-Dickson, USA).

Tube	FITC	PE
1	Ig G1	Ig G1
2	CD3	CD4
3	CD3	CD8
4		CD14

Table 1

Fluorescence dye labeled mouse monoclonal antibodies used in flowcytometry analysis

(d) Total RNA extraction

The RNA extraction procedure was performed in a RNAse-minimized environment using reagents supplied in the commercial kit, RNeasy Mini Kit., (Qiagen, USA). A maximum of 100 μg of RNA longer than 200 bases can be extracted using the RNeasy Mini Kit. The kit consists of 3 reaction buffers namely RLT buffer, RPE buffer and RW1 buffer together with RNeasy mini spin columns.

The RNeasy mini spin columns contain silica gel-based membrane that facilitate total RNA binding during the extraction. Before starting the procedure, β-mercaptoethanol was added into the RLT buffer (10μl of β-mercaptoethanol per ml buffer and ethanol in the ratio of 1:4 was added to the RPE buffer).

The peritoneal macrophages, CD4+ and CD8+ lymphocytes harvested were lysed directly by adding RLT buffer (600 μl for 5 x 10^6-1 x 10^7 cells, 350 μl for cells <5 x 10^6) with added β-mercaptoethanol. The lysates were then passed through a 21-G needle fitted to a 3 ml syringe for about 5-10 times and were then transferred into separate 1.5 ml collection tubes. Following to this, one volume of 70% (v/v) ethanol was added to the homogenized lysate and mixed well by pipetting.

The mixtures were then applied onto the RNeasy mini spin columns placed in a 2 ml collection tube and centrifuged for 15 second at 10 000g. The flow-through in the collection tube was discarded and 700 μl of RW1 buffer was added to the RNeasy mini spin columns and centrifuged for 15 second at 10 000 rpm to wash the columns. The flow-through together with the collection tube was then discarded. The RNeasy columns were

then transferred into a new 2 ml collection tube and 500 µl of RPE buffer was pipetted into the RNeasy column and centrifuged for 15 second at 10 000 rpm. The flow-through was discarded and another 500 µl of RPE buffer was then added into the RNeasy column and centrifuged for 2 minutes at maximum speed (12 000 rpm).

Following centrifugation, the RNeasy column transferred into a new 1.5 ml collection tube. Finally, to elute the total RNA that was bound to the gel membrane of the spin column, 40µl of RNase-free water was directly pipetted onto the RNeasy membrane and centrifuged for 1 minute at 10 000 rpm. Purified RNA stored at -70°C until used.

(e) Agarose Gel Electrophoresis (RNA Analysis)

The integrity and size distribution of total RNA can be checked by agarose gel electrophoresis and ethidium bromide staining. The respective ribosomal bands should appear as sharp bands on the stained gel. If the ribosomal bands are not sharp, but appear as a smear of smaller sized RNA's, it is likely that the RNA sample suffered major degradation during preparation. One % agarose gel was prepared by dissolving 0.4g agarose powder in 40ml 1x TAE buffer and boiled in a microwave oven. The gel solution was then cooled to 55°C in the water bath and subsequently 2µl of 10mg/ml EtBr was added to the mixture. The gel mixture was poured on washed (DEPC water) gel caster that had been assembled with a comb and left to harden at room temperature for 20-30 minutes. Meanwhile, RNA sample was prepared by mixing 1µl RNA 1X loading buffer with 5µl total RNA and loaded into the wells on the gel.

The gel was run at 70 volt for about 1½ hour in 1X TAE buffer. Two sharp and clear RNA bands that are 28S rRNA and 18S rRNA respectively should appear on the stained gel. The bands formed were visualized under the UV light (UV Translluminator, Spectroline, USA) and the image was captured using digital image analyzer.

(f) Spectrophotometry Analysis

Quantitation of RNA

The concentration of the total RNA in aqueous solutions was determined by measuring the absorbance at 260 nm (A_{260}) using a spectrophotometer (Biophotometer., Eppendorf, Germany). An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml. The RNA sample was first diluted with Rnase-free water (2µl RNA in 99µl RNase free-water). The readings should be greater than 0.18.

Purity of RNA

The ratio of readings at 260 nm and 280 nm (A_{260}/ A_{280}) gives an estimate of the purity of RNA with respect to DNA and protein contaminants. Pure solution of RNA has a peak absorbance at 260 nm and a 260:280 absorbance ratio within the range 1.8-2.0 (Sambrook *et al.* 2000). However, if protein or DNA, or both are present in the sample, the ratio will drop to about 1 or lower. If the impurities were acceptably small, the RNA could be used in the next steps.

(g) First strand cDNA synthesis

Extracted RNA samples in this study, were reverse-transcribed to full-length first strand cDNA using the RevertAid^{TM} H Minus First Strand cDNA Synthetic Kit (MBI Fermentas, USA). The kit enabled reverse-transcription of up to 5 μg of total RNA. The kit consist of the following reagents:

- RevertAid™ H Minus M-MuLV Reverse Transcriptase (200u/μl)
- Ribonuclease Inhibitor (20u/μl)
- 5X Reaction Buffer
- 10mM dNTP Mix
- Oligo$(dT)_{18}$ Primer
- Control RNA
- Control Primer
- Deionized Water, nuclease free

Oligo$(dT)_{18}$ primer was used as primer in this study to enable only mRNA's with 3'-poly (A) tail to act as templates for cDNA synthesis, since the primer is complementary to the 3'-end of poly(A) mRNA. Initially, the following mixture of 5μg - 2 μg of total RNA, 0.5 μg oligo$(dT)_{18}$ primer and nuclease-free deionized water were prepared in a RNase-free 1.5 ml microcentrigfuge tube on ice.

The mixture was gently mixed and spun down for about 3-5 seconds. The mixture was then incubated at 70°C for 5 minutes. The mixture was chilled on ice and briefly centrifuged. Four μl of 5X reaction buffer, 1 μl of ribonuclease inhibitor and 2 μl of