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Expression of the transcription factor, PPAR in human monocytes

Dissertation submitted in partial fulfilment for the Degree of Bachelor of Health Sciences (Biomedicine)

Ku Sheau Jen

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

2004

CERTIFICATE

This is to certify that the dissertation entitled

"**Expression of the transcription factor, PPAR in human monocytes**"

is the bona fide record of research work done by

Ms Ku Sheau Jen

during the period from **29th July 2003** to **17th January 2004**.

under our supervision.

Signature of Supervisor:

Professor Dr. Norazmi Mohd. Nor,
Deputy Dean,
School of Health Sciences,
Health Campus,
Universiti Sains Malaysia.
Date: 22-03-2004

Signature of Co Supervisor:

Associate Professor Dr. Nik Soriani Yaacob,
Department of Chemical Pathology,
School of Medical Science,
Health Campus,
Universiti Sains Malaysia.
Date: 22-03-2004

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ABSTRACT

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of transcription factor that mediate ligand-dependent transcriptional activation and repression. They regulate genes associated with lipid and glucose metabolism. Recent evidence suggests that PPARs may also act as a negative immunomodulator. To investigate the potential role of PPARα, γ1 and γ2 in regulating inflammation mediated by monocyte, the expression of PPARα, γ1 and γ2 in lipopolysaccharide (LPS)-activated and non-activated human monocytes was quantified.

Monocytes secrete inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in response to LPS. To verify stimulation of monocytes by LPS, various cytokines including granulocyte/macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-1β, IL-6, IL-8 and transforming growth factor (TGF)-β expression of LPS-activated and non-activated human monocytes was analyzed by using multiplex PCR and their expression is normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. All these inflammatory cytokine expressions were increased in LPS-activated monocytes compared to non-activated monocytes.

Measurement of the gene expression levels of PPARα, PPARγ1 and PPARγ2 in both LPS-activated and non-activated monocytes was carried out using Real-Time PCR analysis. The study showed that LPS induced expression of both PPARα and PPARγ2 in isolated human monocytes with a preferential upregulation of PPARγ2. The PPARγ1 however was not expressed in both LPS-activated and non-activated

monocytes. The activation of PPARα and PPARγ2 by LPS indicates that they may play a role in the down-regulation of immune response evoked by LPS. In contrast, PPARγ1 may not be involved in normal functional of monocytes nor participate in modulating immune response induced by LPS. Human monocytes express PPARα as well as PPARγ2 with the amount of PPARγ2 lower compared to PPARα.

INTRODUCTION

Peroxisome proliferator–activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily. Three human PPAR isoforms have been identified to date: PPARα, δ/β and γ (Vamecq and Latruffe, 1999; Rosen and Spiegelman, 2001; Tedgui and Mallat, 2001). PPARγ has three splice variants termed PPARγ1, γ2 and γ3 (Willson *et al.*, 2001; Kintscher *et al.*, 2002).

Like other members of the steroid-receptor superfamily, PPARs have five structural regions (A-E) with four functional domains as shown in figure 1. The N-terminal or A/B domain encodes the transcriptional activation function 1 (AF1) domain, which contains a mitogen-activated protein kinase (MAPK) phosphorylation site. Phosphorylation at this site reduces the transcriptional activity of PPAR by reducing its ability to bind ligands (Willson *et al.*, 2001). The C domain is the DNA-binding domain (DBD). The D domain consists of a highly flexible hinge region (Boitier *et al.*, 2003). The E domain is the ligand-binding domain (LBD) which contains the activation function 2 (AF2) ligand-dependent activation domain (Vamecq and Latruffe, 1999; Boitier *et al.*, 2003). In addition, the E region is also important in nuclear localization and dimerization of the receptor. The three PPARs share 80% and 70% amino acid identity in their DBDs and LBDs, respectively as indicated in figure 2 (Willson *et al.*, 2001).

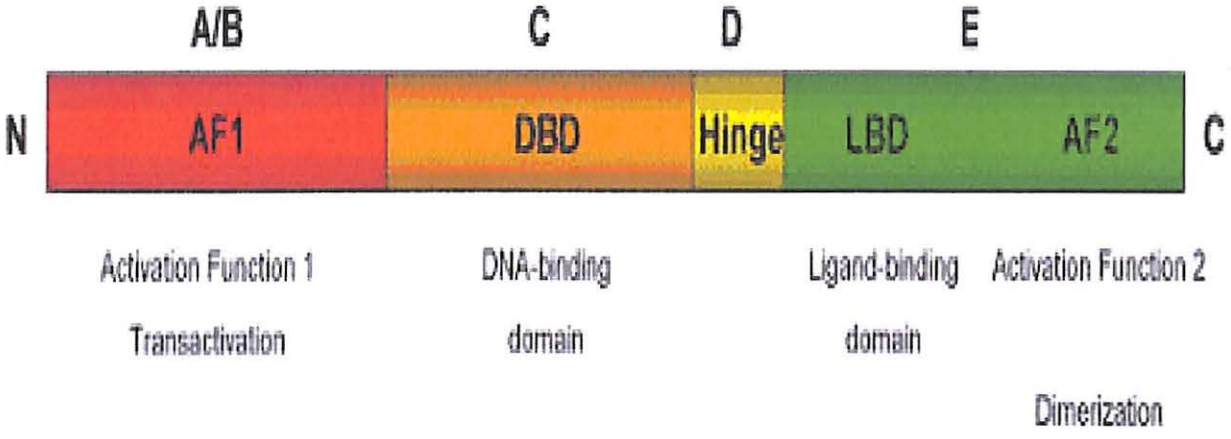

Figure 1. A schematic illustration of the domain structure of PPARs (Boitier *et al.*, 2003).

PPARs heterodimerize with the retinoid X receptor (RXR) (Figure 2). In the unliganded state, evidence indicates that the PPARs are associated with a nuclear receptor co-repressor. Upon activation by ligands for either PPAR or RXR, the PPARs undergo a conformational change that results in the dissociation from the co-repressor, enabling the PPAR to bind nuclear receptor co-activators and subsequent binding to specific peroxisome proliferator-responsive elements (PPRE), a direct repeat of the consensus sequence, AGGTCA, separated by a single nucleotide spacer, a so-called DR-1 motif located within the promoter region of target genes. (Chinetti *et al.*, 1998; Knethen and Brüne, 2001; Neve *et al.*, 2001; Tedgui and Mallat, 2001; Wang *et al.*, 2001). These co-activators then act to reorganize the chromatin templates allowing the basal transcription machinery to gain access to the promoter regions driving transcription or repression of target genes (Jones *et al.*, 2002).

Fatty acid derivatives and eicosanoids have been identified as natural ligands for PPARs. Furthermore, fibrates are synthetic ligands for PPARα that mediate the lipid-lowering activity of these drugs, whereas the antidiabetic thiazolidinediones and carbaprostacyclin are synthetic ligands for PPARγ and PPARδ, respectively (Chinetti *et al.*, 1998; Delerive *et al.*, 1999; Tedgui and Mallat, 2001).

Neve *et al.*, (2001) reported that human monocytes and macrophages express PPARα as well as PPARγ, although the amount of PPARγ compared with PPARα may be lower in these cells. In monocytes, expression of PPARα and PPARγ increases during differentiation (Chinetti *et al.*, 1998; Marx *et al.*, 2001; Neve *et al.*, 2001; Cunard *et al.*, 2002).

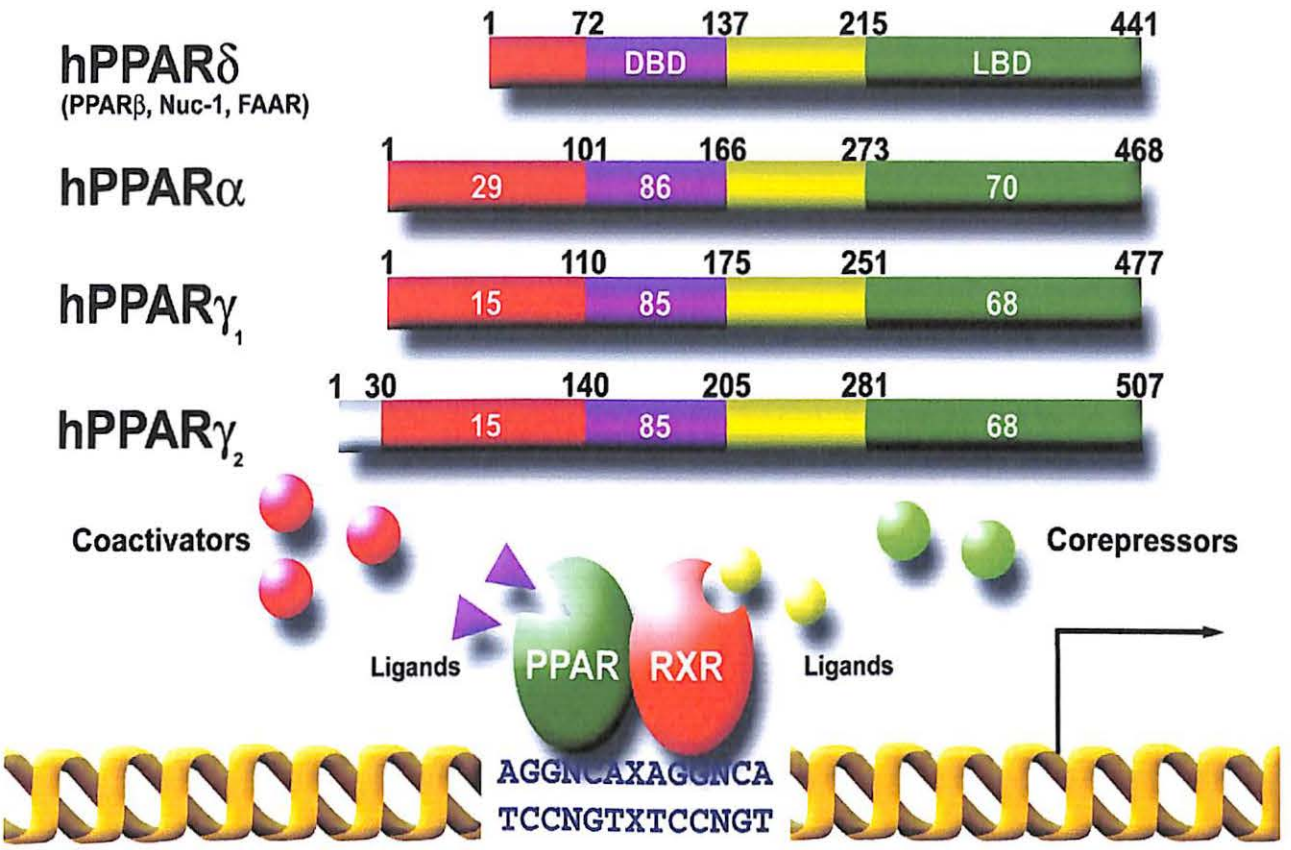

Figure 2. PPAR isoforms share a common domain structure and molecular mechanism of action. Amino acid numbers are indicated above each receptor, whereas percent identity at the amino acid level with PPARδ is displayed within each domain. In the lower half of the panel, a generic PPAR is shown binding to a PPRE as a heterodimer with RXR (Rosen and Spiegelman, 2001).

According to Wright *et al.*, (1990) and Maliszewski (1991), leukocytes including monocytes respond to lipopolysaccharide (LPS) at nanogram per milliliter concentrations with secretion of cytokines such as IL-1β, IL-6 and TNFα. LPS in the bloodstream rapidly binds to the serum protein, lipopolysaccharide binding protein (LBP) (Schumann *et al.*, 1990; Wright *et al.*, 1990). CD14, a differentiation antigen of monocytes and macrophages, then binds complexes of LPS and LBP (Wright *et al.*, 1990; Bažil and Strominger, 1991; Maliszewski, 1991). However, Wright *et al.*, (1990) reported that LPS in the concentration used (100ng/ml) can activate monocytes in the absence of either LBP or CD14.

PPARs have been shown to down-regulate inflammatory response (Jozkowicz *et al.*, 2000; Cunard *et al.*, 2002). PPARα activation negatively regulates cyclooxygenase type 2 (COX-2) activity (Vamecq and Latruffe, 1999), whereas PPARγ reduces monocyte secretion of inflammatory cytokines (Jiang *et al.*, 1998; Vamecq and Latruffe, 1999; Kersten *et al.*, 2000).

Little is known, however, about the induction of expression of different isoforms of PPARs in monocyte/macrophages upon activation by LPS. To get an initial insight into this question, the effects of LPS stimulation on PPARα, γ1 and γ2 expression in freshly isolated human monocytes were investigated. Activation of monocytes by LPS was verified through comparison of inflammatory cytokines expression in both LPS-activated and non-activated monocytes. Absolute quantification of PPARα, γ1 and γ2 expression was measured using Real time PCR.

REVIEW OF LITERATURE

In 2001, Hsueh and Law showed that high doses of thiazolidinediones (TZD's), which are ligands for the PPARγ, reduced monocyte adhesion to endothelial cells *in vitro*, as well as inflammatory actions of macrophages, including their expression of IL-1, IL-6, TNFα, inducible nitric oxide synthethase (iNOS), and chemokine (C-C motif) receptor-2 (CCR-2). These data suggest that PPARγ ligands may attenuate inflammation and hence, atherosclerosis in the vessel wall (Hsueh and Law, 2001).

In 1999, Leininger *et al.* reported that induction of PPARγ coincided with or closely followed an endotoxin challenge and host responses to acute inflammation in peripheral porcine blood monocytes. However, Jiang *et al.* (1998) presented evidence that the inflammatory cytokine TNF-α which is rapidly produced by monocytes and macrophages in response to a number of stimuli such as endotoxins antagonizes the synthesis of PPARγ.

In the same year, Ricote *et al.* provided evidence that oxidized low density lipoprotein (oxLDL), macrophage colony-stimulating factor (M-CSF), and granulocyte/macrophage colony-stimulating factor (GM-CSF) which are known to be present in atherosclerotic lesions, stimulated PPARγ expression in primary macrophages and monocytic cell lines. 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. In contrast, treatment of

resident peritoneal macrophages with interferon (IFN)-γ or LPS did not stimulate PPARγ expression.

Recently, however, Knethen and Brüne (2001) provided evidence that the classic macrophage stimulators LPS/IFN-γ promote PPARγ activation. This is established by gel shift analysis, a supershift response in the presence of PPARγ but not PPARα antibodies, and a reporter gene assay coupled to luciferase activity. Activation of PPARγ was analyzed by electrophoretic mobility shift assays (EMSA) using a specific PPRE oligonucleotide derived from the human acyl-CoA synthase promoter. They proposed that LPS/IFN-γ induce a PPARγ response via production of activating ligands, which awaits further characterization. Thus, LPS/IFN-γ had been implicated in PPARγ activation in macrophages. But, there is still unclear whether the 3 PPARγ splice variants– PPARγ1, γ2 and γ3 are evenly expressed upon monocyte activation by LPS/IFN-γ.

In 2002, Kintscher *et al.* demonstrated that TGF-β_1, an essential and potent immune modulator induces transcriptionally active PPARγ1 and γ2 in THP-1 monocytes, a human monocytic leukemia cell line with a preferential up-regulation of PPARγ2. TGF-β_1 strongly induced PPARγ2 mRNA and protein expression with a lesser effect on PPARγ1. Transcription from a PPARγ2 promoter/luciferase reporter vector was activated approximately 3-fold by TGF-β_1. They also showed that mutation of two C/EBP recognition elements, a regulator of PPARγ2 promoter that is situated within the PPARγ2 promoter at -340 and -327 bp relative to the transcription start site reduced TGF-β_1-induced transcription by approximately 65%.

Few studies were done on PPARα as compared to PPARγ. In 1998, Poynter and Daynes reported that the nuclear factor (NF)-κB-driven cytokines – TNF-α, IL-1β, and IL-6 cause a reduction in the expression of PPARα. On the other hand, Neve *et al.* (2001) reported that LPS had no effect on the level of PPARα protein expression in monocytic leukemia THP-1 cells.

To date, there is still no report on the expression of PPARα in LPS-stimulated isolated human monocytes. Moreover, it is uncertain whether monocytes would differentially express the different isoforms of PPARs (PPARα, γ1 and γ2) upon stimulation by LPS.

OBJECTIVE OF THE STUDY

The objectives of this study are to:

1. Quantify PPARα, PPARγ1 and PPARγ2 expression in LPS-activated and non-activated human monocytes by Real-Time PCR.
2. Compare PPARα, PPARγ1 and PPARγ2 expression in LPS-activated human monocytes with their corresponding expression in non-activated human monocytes.

MATERIALS AND METHODS

SUMMARY OF EXPERIMENTAL PROTOCOL

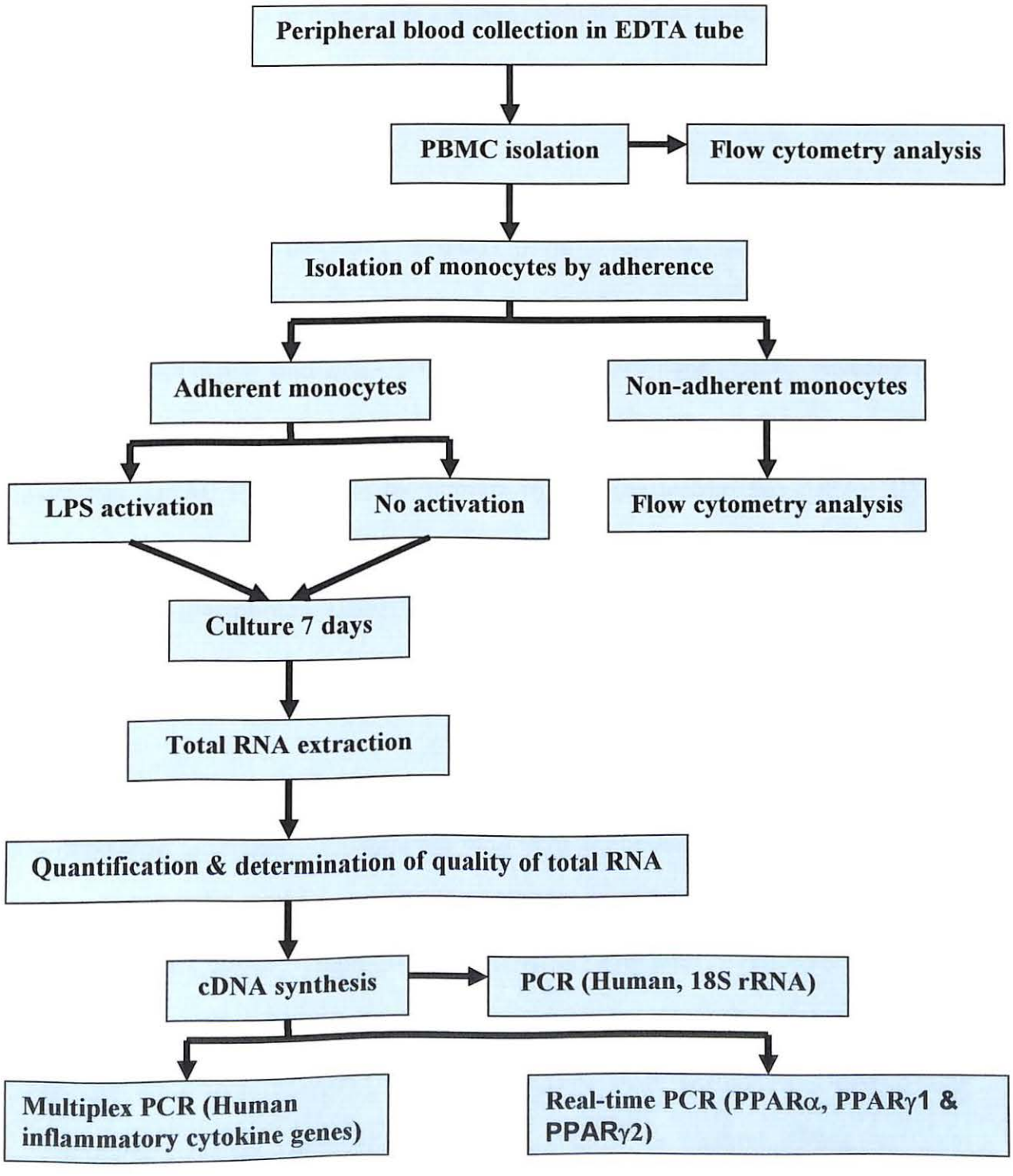

Figure 3. Flow chart of experimental protocol.

MATERIALS

Human Venous Blood Samples

Five healthy humans (3 females and 2 males) venous blood samples were collected for this experiment.

Reagents

Ficoll Histopaque-1077 (P=1.077 ± 0.001 g ml^{-1}) (Sigma, USA), LPSs (Sigma, Germany), human monoclonal antibodies– IgG1 FITC, IgG1 PE, anti-CD3 FITC, anti-CD4 PE, anti-CD8 PE and anti-CD14 PE (Becton Dickinson, USA), RNeasy RNA Extraction Kit (Qiagen, USA), RevertAid™ H Minus First Strand cDNA Synthesis Kit (Maxim Bio, USA), PCR set kit for human 18S rRNA (Maxim Bio, USA), 100 bp molecular weight marker (Invitrogen, USA), 6x loading buffer (Fermentas, USA), 100 bp DNA marker (Fermentas, USA), and MPCR kit for human inflammatory genes set 1 (Maxim Bio, USA).

Chemicals

Ethanol (Merck, Germany), ethylene diamine tetra acetic acid (EDTA) (Sigma, USA), $NaH_2PO_4.2H_2O$ (E. Merck, Germany), KCl (BDH Lab, UK), NaCl (E. Merck, Germany), NH_4Cl (BDH, England), KHCO (BDH, England), Trypan blue (Sigma, USA), Dulbecco's Modified Eagle's Medium (DMEM) powder (Sigma, USA), sodium bicarbonate solution (7.5%w/v) (Sigma, USA), Tris base (Promega, USA), diethyl pyrocarbonate (DEPC) water (Sigma, USA), orange G (Sigma, USA), ethidium bromide (EtBr) powder (Sigma,USA), agarose powder (Promega, USA), and boric

acid (Promega, USA). All chemicals were of the highest grade of purity and commercially available.

Equipments

Haemacytometer (Assistant, Germany), light microscope (Leica Microsystems, Germany), 25-cm^2 cell culture flasks (Costar, USA), CO_2-water jacketed incubator (Nuaire, USA), 5 ml polystyrene round-bottom tube (Becton Dickinson, USA), 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 Biosystems, USA), ImageMaster TotalLab software (Amersham Pharmacia Biotech, USA), Perkin Elmer GeneAmp PCR system 9700 (Applied Biosystems, USA), ABI PRISM® 5700 Sequence Detector (Applied Biosystems, USA), and Primer Express software (Applied Biosystems, USA).

Statistical Analysis

Each experiment was performed at least five times and statistical analysis was performed using the two tailed Student's t-test using the SPSS software version 11.0 (SPSS Science Inc., USA).

GENERAL REAGENTS PREPARATION

a) 70% ethanol solution

To prepare 70% ethanol solution, 70 ml ethanol was mixed with 30 ml deionized water.

b) 0.5 M EDTA pH 8.0

A 0.5 M EDTA solution was prepared by dissolving 93.06 g EDTA in 300 ml deionized water. The pH of the solution was adjusted to 8.0 since EDTA would only dissolve at pH 8.0. When EDTA was completely dissolved, the final volume was made up to 500 ml by adding deionized water.

MEDIA PREPARATION

a) 10x phosphate-buffered saline (PBS)

In preparing 10x PBS stock solution, 1.4 g $NaH_2PO_4.2H_2O$, 0.2 g KCl and 8.1 g NaCl were dissolved in 800 ml deionized water and the pH of the solution was adjusted to 7.4 using 3 M NaOH. The solution was then made up to 1 L by adding deionized water. The final stock solution was autoclaved at 121°C for 15 minutes and stored at room temperature. To prepare 1x PBS working solution, the 10x PBS stock solution was further diluted by adding deionized water. This working solution was stored at 4°C.

b) Lysis buffer, 6x ammonium chloride solution (ACK)

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

c) Dulbecco's Modified Eagle's Medium (DMEM)

DMEM (containing 4 mM L-glutamine) was prepared by dissolving 10 g DMEM powder in 800 ml deionized water. For each liter of medium, 49.3 ml of sodium bicarbonate solution (7.5%w/v) was added. The pH of the medium was adjusted to 7.0 by using 1N HCl or 1N NaOH. Then additional deionized water was added to bring the solution to the final volume. DMEM (containing 2 mM L-glutamine) was used for culturing human monocytes by the addition of an equal volume of deionized water. To bring to a concentration of 50 μg gentamycin/ml DMEM, 0.1 g of gentamycin powder was added to DMEM. This medium was then filtered through 0.2 μm pore membrane filter and stored at 4°C.

d) LPS stock solution preparation

Lyophilized LPS was reconstituted with 1 ml sterile Hank's Balanced Salt Solution (HBSS) and further diluted to the working concentration of 10 ng/ml DMEM using HBSS (Bažil and Strominger (1991)) and stored at -20°C.

PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

Twenty ml venous blood from healthy donors was collected into tubes containing EDTA. Experiments were initiated on the day blood was collected and all manipulations were carried out under sterile conditions. PBMC was isolated by density gradient centrifugation. The blood was diluted 1:2 with PBS in four 15 ml Falcon tubes and layered slowly onto a 3 ml Ficoll Histopaque-1077 (P=1.077 ± 0.001 g ml^{-1}) per 10 ml blood by using a sterile Pasteur pipette. The preparation was then centrifuged at 1500 rpm (400 x g) for 30 min. The interphase containing peripheral blood mononuclear cells was obtained after centrifugation.

The mononuclear cell layer was transferred to a fresh tube, mixed with 3 volumes PBS and centrifuged for 5 min at 1500 rpm. The supernatant was removed and if the cell pellet is contaminated with red blood cells, 1 ml ACK was added to the pellet and mixed well to lyse red blood cells for 5 min at room temperature. After 5 min, the cell suspension was centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was removed and the cell pellet washed with 10 ml PBS and the cell suspension centrifuged again at 1500 rpm (400 x g) for 5 min. Mononuclear cells were resuspended in 1 ml PBS and counted by using haemacytometer under light microscopy. Before counting, 5 µl cell suspension was mixed with 5 µl 0.2% Trypan blue. Flow cytometry analysis was carried out on this cells suspension to determine the percentage of monocytes in PBMC.

ISOLATION OF MONOCYTES BY ADHERENCE AND LPS ACTIVATION

Monocytes were isolated from mononuclear cells by exploiting their ability to adhere to glass or plastic. PBMC was suspended in serum-free DMEM which is supplemented with 2 mM L-glutamine and 50 μg/ml gentamycin at 2 x 10^6 cells/ml. Five ml of the cell suspension was added to two 25-cm^2 cell culture flasks and incubated for 1 hour in a humidified 37°C, 5% CO_2 incubator.

After 1 hour, the non-adherent cells were decanted into two 15 ml Falcon tubes and counted by using haemacytometer after adding 0.2% Trypan blue in 1:2 dilution. Flow cytometry analysis was performed on these cell suspensions to estimate the percentage of monocytes that have adhered to the flasks. The cell culture flasks were washed twice with 5 ml serum-free DMEM to remove any residual non-adherent cells and 5 ml fresh DMEM was replaced into each flask.

To induce activation of monocytes, 10 ng/ml LPS was added into one of the flasks and the other used as the control. The flasks were incubated for 7 days in a humidified 37°C, 5% CO_2 incubator.

FLOW CYTOMETRY

Flow cytometry analysis was carried out using monoclonal antibodies labeled with fluorescent dyes specific for mononuclear cells (Th cells, Tc cells and monocytes) surface antigens. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labeled monoclonal antibodies which emit green and orange fluorescence respectively were used.

A total of 10 μl antibodies conjugated with FITC or PE was added into each 5 ml polystyrene round-bottom tube as shown in table 1. Fifty μl total PBMC or 100 μl non-adherent PBMC was added into each tube, mixed well and placed in the dark for 30 minutes. All 4 tubes were centrifuged at 1500 rpm (400 x g) for 5 minutes. After centrifugation, supernatants were removed and 2 ml PBS was added into each tube and centrifugation step was repeated. The cells were fixed and analyzed by using FACScan flow cytometer.

TOTAL RNA EXTRACTION

After the 7-day incubation period, total RNA was extracted from both activated and non-activated monocytes by using the RNeasy RNA Extraction Kit. The monocytes were disrupted directly by addition of 350 μl Buffer RLT with β-mercaptoethanol (β-ME) (10 μl β-ME per 1 ml Buffer RLT) after completely removing the cell culture medium. The lysate was then transferred onto a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at maximum speed (12,000 rpm) to homogenize the sample. One volume (350 μl) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting.

Tube	FITC	PE	Purpose
1	Ig G1	Ig G1	As control to set marker.
2	CD3	CD4	To determine the percentage of Th cells.
3	CD3	CD8	To determine the percentage of Tc cells.
4		CD14	To determine the percentage of monocytes.

Table 1. Human monoclonal antibodies employed in flow cytometry analysis.

The sample was then applied to an RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 s at 10,000 rpm. The flow-through was discarded and 700 µl Buffer RW1 was added to the RNeasy column and centrifuged for 15 s at 10,000 rpm to wash the column. The flow-through together with collection tube was discarded. The column was then transferred into a new 2 ml collection tube and washed by pipetting 500 µl Buffer RPE with 4 volumes of 100% ethanol and centrifuged for 15 s at 10,000 rpm. The flow-through was discarded and another 500 µl Buffer RPE was added to the RNeasy silica-gel membrane but was centrifuged at 10,000 rpm for 2 min to dry the membrane. To eliminate any chance of possible Buffer RPE carryover, the column was placed in a new 2 ml collection tube and was centrifuged at full speed for 1 min. Finally, to elute the RNA into a 1.5 ml collection tube, 20 µl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and centrifuged for 1 min at 10,000 rpm. Purified RNA was stored at -40°C.

QUANTIFICATION AND DETERMINATION OF QUALITY OF TOTAL RNA

Quantification of RNA

The concentration of the RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml. This relation is valid only for measurements in water. Therefore, RNA sample was diluted by using RNase-free water (1 µl RNA in 49 µl RNase-free water) in an RNase-free quartz cuvette.

Purity of RNA

The purity of the samples was then checked to determine if the RNA samples had been contaminated by DNA or protein. Proteins absorb light at 280nm, and DNA absorbs more weakly than RNA, so to find relative sample purity, the ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) was determined. Pure RNA has an A_{260}/A_{280} ratio of 1.8-2.1 in RNase-free water. However, if protein or DNA, or both are present in the sample, then the value of the ratio will drop to about 1 or lower. If the impurities were acceptably small the RNA could be used in the next steps.

Integrity of RNA

Agarose gel electrophoresis reagents preparation

a) 50x Tris-acetate buffer (TAE)

A 50x TAE buffer for RNA product analysis was prepared by dissolving 121 g Tris base, 28.5 ml glacial acetic acid and 50 ml 0.5 M EDTA pH 8.0 in DEPC water in a final volume of 500 ml. The buffer was autoclaved at 121°C for 15 min for sterilization. By diluting 50x TAE buffer with DEPC water, 1x TAE running buffer and gel loading buffer can be prepared. The stock as well as working solution was stored at room temperature.

b) RNA loading buffer

RNA loading buffer was prepared by dissolving 20 g sucrose and 0.125 g orange G in 50 ml DEPC water. This buffer was stored at 4°C.

c) Ethidium bromide (EtBr) solution

To prepare 10 mg/ml stock EtBr solution, 0.1 g EtBr powder was dissolved in 10 ml deionized water and stored in a dark or foil-wrapped bottle at room temperature.

Agarose gel electrophoresis

The integrity and size distribution of total RNA can be checked by agarose gel electrophoresis and EtBr staining. The respective ribosomal bands (28S rRNA and 18S rRNA) should appear as sharp bands on the stained gel with 28S rRNA bands present with an intensity approximately twice that of the 18S rRNA. If the ribosomal bands are not sharp, but appear as a smear of smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

The percentage of agarose gel being used was 1%. To prepare 1% agarose gel, 0.3 g agarose powder was dissolved in 30 ml 1x TAE buffer and boiled in a microwave oven. The gel solution was then cooled to 55°C in the water bath and 1 μl 10 mg/ml EtBr was added to the gel solution. The agarose solution was poured onto an appropriate gel tray that had been assembled with a comb and allowed to harden at room temperature for 20-30 min.

Agarose gel electrophoresis was run at 85 V for 1 h. One μl RNA loading buffer was mixed with 5 μl total RNA and the sample was loaded into the wells of the gel. UV transilluminator was used to visualize RNA in the gel. EtBr molecule intercalates RNA and causes RNA to fluoresce upon exposure of the bound RNA to UV light. The image of the RNA bands was then captured by using a digital image analyzer.

SYNTHESIS OF FIRST STRAND cDNA

RevertAid™ H Minus First Strand cDNA Synthesis Kit was used to synthesize full-length first strand cDNA from RNA templates. One μg total RNA was used for each synthesis. Oligo $(dT)_{18}$ primer (0.5 μg/μl) was selected as the primer in this reaction so that only mRNAs with 3'-poly(A) tails served as templates for cDNA synthesis since this primer is complementary to the 3'-end of poly(A)$^+$ mRNA. The reaction mixture which is composed of 1 μg total RNA, 1 μl Oligo $(dT)_{18}$ primer (0.5 μg/μl) and nuclease free deionized water was prepared in a sterile microcentrifuge tube on ice to bring the volume to 12 μl.

Before incubating the mixture at 70°C for 5 min, the mixture was mixed gently and centrifuged briefly in a mini centrifuge. The mixture was then chilled on ice, centrifuged briefly and 4 μl 5x reaction buffer, 1 μl ribonuclease inhibitor (20u/μl) and 2 μl 10mM dNTP mix were added to the mixture. The tube was mixed gently and centrifuged briefly before incubating at 37°C for 5 min. Finally, 1 μl RevertAid™ H Minus M-MuLV Reverse Transcriptase (200u/μl) was added to bring the final volume to 20 μl. The mixture was incubated at 42°C for 60 min and the reaction was stopped by heating at 70°C for 10 min. The cDNA produced was chilled on ice and diluted to 100 μl by adding RNase-free water. The cDNA sample was assigned into two aliquots before storing at -40°C for later use.