



**Elucidation of genes up-regulated by hypoxia and  
lipopolysaccharide in human macrophages**

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

In The Name Of Allah, The Most Merciful The Most  
Compassionate

اللَّهُمَّ صَلِّ وَسَلِّمْ عَلَى  
رَسُولِكَ مُحَمَّدٍ

May Allah`s Blessings And Peace Be Upon Mohammed And His  
Progeny

## *Dedication*

This work is dedicated to my late father who passed away while I was doing this project

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# Abstract

Macrophages accumulate in inflamed tissues such as atherosclerotic plaques, malignant tumours, and wounds, which are characterised by hypoxia, and respond by altering expression of genes which enhance adaptation to hypoxia. In addition to hypoxia, lipopolysaccharide (LPS) can be present in infected tissues due to contamination by microbes.

The hypothesis in this thesis was that synergy between hypoxia and LPS could affect macrophage gene expression in sites such as infected wounds which contain both factors. Using cDNA microarrays, investigation was carried out into which genes were induced by hypoxia and LPS in primary human macrophages. Gene expression profiles changed markedly: 55 genes were up-regulated by hypoxia, 277 by LPS and 384 by hypoxia plus LPS, suggesting significant cross talk or synergy between hypoxia and LPS. Two synergistically regulated genes, TRAIL (TNF-related apoptosis-inducing ligand) and DDIT4 (DNA-damage-inducible transcript 4) were studied in more detail. Proximal TRAIL promoter luciferase reporter constructs were prepared, but were not inducible, suggesting involvement of more distal promoter elements.

The hypoxia-inducible factor HIF-stabilizing agents desferrioxamine and cobalt chloride were used to investigate the role of HIF-1 in up-regulation of DDIT4 mRNA. Both agents up-regulated DDIT4 expression, and were capable of synergistic up-regulation when combined with LPS, suggesting that, for DDIT4, HIF-1 is responsible for the hypoxia and LPS synergy. This report also shows for the first time that DDIT4 is induced by LPS alone. DDIT4 mRNA induction by hypoxia was slightly down-regulated by the PI3-kinase inhibitors LY294002 and wortmannin, also suggesting a role for PI3-K. Immunoblotting showed DDIT4 protein up-regulation in response to hypoxia and LPS, which was consistent with mRNA levels determined by arrays and RT-PCR.

In conclusion, this thesis suggests that hypoxia may also be able to synergise with a variety of stimuli to alter macrophage gene expression in a range of pathological conditions.

# Abbreviations

$\alpha$ - Alpha

ADM- Adrenomedullin

ADORA2A- adenosine A2a receptor

ALDA- Aldolase A

Amp- Ampicillin

AP-1- Activator Protein-1

APCs- Antigen Presenting Cells

APS- Ammonium Persulphate

AREs- Adenylate Uridylate Rich Elements

ARNT- Aryl hydrocarbon Receptor Nuclear Translocator

ASMCs- Arterial Smooth Muscle Cells

ATF- Activating Transcription Factor

ATP- Adenosine Triphosphate

$\beta$ - Beta

$\beta$ -2M-  $\beta$ -2 microglobulin

bFGF- basic Fibroblast Growth Factor

BNIP3- BCL2/adenovirus E1B-interacting protein1 NIP3

bp- base pair

BSA- Bovine Serum Albumin

CA12- Carbonic anhydrase 12

CCL1- Chemokine (C-C motif) ligand 1

CCL8- Chemokine (C-C motif) ligand 8

CCL5- Chemokine (C-C motif) ligand 5

CCL20- Chemokine (C-C motif) ligand 20

CXCL1- Chemokine (C-X-C motif) ligand 1

°C- Celsius

CBP- CREB Binding Protein

CBP/P300- CREB Binding Protein p300

CD- Cluster of Differentiation

cDNA- Complementary Deoxyribonucleic acid

ChIP- Chromatin Immunoprecipitation

CNS- Central Nervous System

CO<sub>2</sub>- Carbon dioxide

COX-2- Cyclooxygenase 2

CREB- CAMP Response Element Binding protein

CS- Chondroitin Sulphate

CSF- Colony Stimulating Factor

CSPGs- Chondroitin Sulphate Proteoglycans

DC- Dendritic cell

DEPC- Diethylpyrocarbonate

DFO- Desferrioxamine

dH<sub>2</sub>O- Deionised Water

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic acid

dNTPs- Deoxynucleotide Triphosphates

D-PBS- Degassed Phosphate Buffered Saline

DTT- Dithiothreitol

E.coli- Escherichia coli

ECM- Extracellular Matrix

EDTA- Ethylenediaminetetraacetic acid

eEF2- eukaryotic Elongation Factor-2

EGF- Epidermal Growth Factor

ELISA- Enzyme-Linked Immunosorbent Assay

EMSA- Electrophoretic Mobility Shift Assay

EPO- Erythropoietin

ER- Endoplasmic Reticulum

ERK- Extracellular Regulated Kinase

ET-1- Endothelin-1

EtBr- Ethidium Bromide

FACS- Fluorescence Activated Cell Sorting

FCS- Fetal Calf Serum

FGF2- Fibroblast Growth Factor 2

FIH- Factor Inhibiting HIF

FITC- Fluorescein Isothiocyanate

FSC- Forward Scatter

$\gamma$ - Gamma

g- Gram or gravitational acceleration

G3PDH- Glyceraldehyde 3-Phosphate Dehydrogenase

GAG- Glycosaminoglycans

GC- Golgi Complex

GLUT-1- Glucose transporter 1

GM-CSF- Granulocyte/Macrophage-Colony Stimulating Factor

GSK- Glycogen Synthase Kinase

H<sub>2</sub>O- Water

HBSS- Hank's Balanced Salt Solution

HCl- Hydrochloride acid

HIF-1 Hypoxia-Inducible Factor 1

HMDM- Human Monocyte-Derived Macrophages

HRE- Hypoxia Response Elements

HRP- Horse Redish Peroxide

IAP-2 Inhibitors of Apoptosis Protein 2

IFN- Interferon

Ig- Immunoglobulin

IL- Interleukin

ID- Inhibitory domain

IKK- Inhibitory kabba B kinase

iNOS- inducible Nitric Oxide Synthase

IFN $\gamma$ - Interferon  $\gamma$

Kb- Kilo-base

KCL- Potassium Chloride

kDa- Kilo Dalton

LAR- Luciferase Assay Reagent

LB- Luria Bertani Medium

LDHA- Lactate Dehydrogenase A

LDL- Low Density Lipoprotein

LPS- Lipopolysaccharide

MAPK- Mitogen Activated Protein Kinase

MCP-1- Monocyte Chemoattractant Protein 1

M-CSF- Monocyte Colony--Stimulating Factor

MCT2- Monocarboxylate Transporter 2

MDMs- Monocyte-derived macrophages

MIF- Migration Inhibitory Factor

MM6- Human Monocytic cell line

MMP-7- Matrix Metalloproteinase-7

MMP-2- Matrix Metalloproteinase-2

MMP-9- Matrix Metalloproteinase-9

MR- Mannos Receptors

MyD88- Myeloid differentiation primary response gene 88

mRNA- Messenger Ribonucleic Acid

N<sub>2</sub>- Nitrogen

NaCl- Sodium Chloride

NF-κB- Nuclear Factor-Kappa Binding

NO- Nitric Oxide

NOS- Nitric Oxide Synthase

O<sub>2</sub>- Oxygen

ODD- Oxygen Dependent Degradation Domain

PAI-1- Plasminogen Activator Inhibitor-1

PAMP- Pathogen Associated Molecular Patterns

PBMC- Peripheral Blood Mononuclear Cells

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PDGF- Platelet Derived Growth Factor

PEI- Polyethylenimine

PGK- Phosphoglycerate kinase

PHD- Prolyl Hydroxylase Enzymes

PI3K- Phosphoinositide 3-kinase

PKB- Protein Kinase B

PKC- Protein Kinase C

IP- Propidium iodide

PIMO- Pimonidazole hydrochloride

PRPs- Pattern Recognition Receptors

RelA v-rel- Reticuloendotheliosis viral oncogene homolog A

RelB v-rel- Reticuloendotheliosis viral oncogene homolog B

RISC- RNA-induced silencing complex

RLU- Relative Light Units

RNA- Ribonucleic Acid

RNAi- Ribonucleic Acid interference

RNase- Ribonuclease

rpm- Revolutions Per Minute

RT- Room Temperature or Reverse transcriptase

RT-PCR Real Time Polymerase Chain Reaction

SDS- Sodium Dodecyl Sulphate

SDS-PAGE- Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis

siRNA- small interfering Ribonucleic Acid

shRNA- small hairpin Ribonucleic Acid

SLC- Secondary Lymphoid tissue Chemokine

SNP- Single Nucleotide Polymorphisms

SP-1- Specific Protein-1

SR- Scavenger Receptors

SSC- Side Scatter

TAD- Transactivation Domains

TAMs- Tumour Associated Macrophages

TC- Tumour cells

TBE- Tris Borate EDTA buffer

TBS- Tris Buffered Saline

TCF/LEF- Transcription Factor Lymphoid Enhancer binding Factor

TESS- Transcription Element Search System

TGF- Transforming Growth Factor

THP-1- Human acute monocytic leukemia cell line

Th-T helper

TLR- Toll Like Receptor

TNF- Tumour Necrosis Factor

ULA- Ultra Low Attachment

UTR- Untranslated Region

UV- Ultraviolet light

U937- Human leukemic monocyte lymphoma cell line

VCAM1- Vascular cell adhesion molecule1

VEGF- Vascular Endothelial Growth Factor

VHL- Von Hippel Lindau protein

V- Volts

Wnt5a- Wingless-type MMTV integration site family, member 5A

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# CHAPTER 1: Introduction

## 1.1 Immune system

Mammals live in an environment containing vast numbers of microorganisms that they encounter every day. Nevertheless, these microorganisms rarely cause disease. This is because of the immune system, the body's defence against infectious organisms. Through a series of highly regulated steps called the immune response, the immune system attacks microorganisms. The immune system is made up of cells called white blood cells (leukocytes) which originate from a common precursor in bone marrow called hematopoietic stem cells (Seita & Weissman, 2010). Leukocytes consist of five main types of cells: neutrophils, basophils, eosinophils, lymphocytes and monocyte/macrophages, which have different functions and morphology (Stock & Hoffman, 2000). The leukocytes circulate through the body between the organs and lymph nodes via lymphatic vessels and blood vessels. The immune system works in a coordinated manner to monitor the body for microorganisms or substances that might cause diseases. There are two main types of immune responses used by the mammalian immune system; innate immunity (non-specific), considered as the first line of defence against invading organisms, and adaptive immunity (specific) which acts as a second line of defence (Vivier *et al*, 2011). In innate immunity, leukocytes such as neutrophils, monocytes, and macrophages respond to foreign organisms by rapidly engulfing and digesting them. Adaptive immunity is more complex, requires more time to respond, and needs leukocytes to be coordinated with each other to create highly specific immune responses against invaders (Medzhitov & Janeway, 1997).

## 1.2 Macrophages

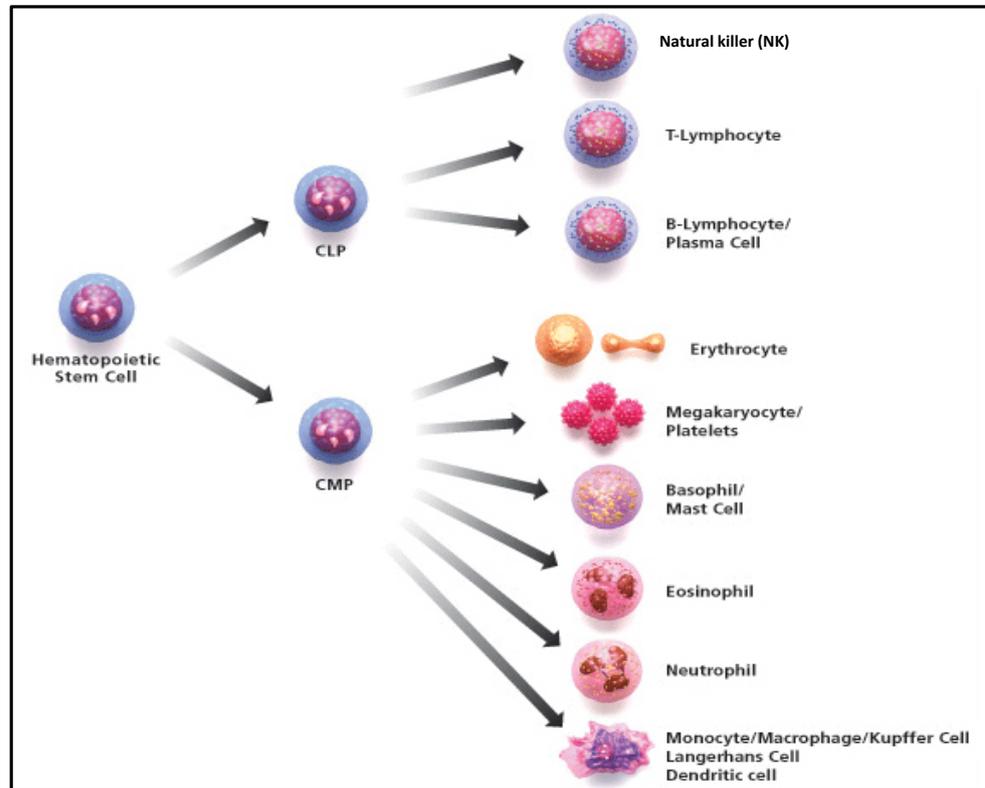
### 1.2.1 Origin of macrophages

The term “Macrophages” was first used by Russian zoologist Elie Metchnikoff over 100 years ago when he described the large mobile cells he observed in the larvae of starfish. He noticed that these cells engulf food (Tauber, 2003). Therefore, he hypothesized that these cells could ingest microbes and other foreign bodies as well. To test his hypothesis, Metchnikoff inserted small thorns from garden plants into the starfish larvae. The following day, he noted that the cells within the larvae were no longer moving around randomly, but were instead aggregated in high numbers around the foreign bodies (thorns), and he concluded that these phagocytic cells could protect the body from microorganisms (Gordon, 2008; Tan & Dee, 2009). Elie Metchnikoff won the Nobel Prize for his description of phagocytic cells (Mosser & Edwards, 2008).

Phagocytic cells are categorised into two main groups: polymorphonuclear and mononuclear phagocytes (van Furth *et al*, 1972). The mononuclear phagocytes include the tissue macrophages, the circulating monocytes in the blood, and the promonocytes, and their precursor cells in the bone marrow (Hume, 2008). Macrophages are large, irregular shaped cells measuring about 25-50 µm in diameter. They often have an eccentrically kidney-shaped or round nucleus with one or two prominent nucleoli (J. A. Ross & M. J. Auger; Chapter 1: The biology of Macrophages, 1993).

Macrophages are derived from precursor cells in bone marrow known as hematopoietic stem cells (figure 1.1) (van Furth & Cohn, 1968). In humans, the bone marrow contains the macrophage precursors, monoblasts and premonocytes. Premonocytes are released from bone marrow and circulate briefly in the blood

stream where they differentiate into mature monocytes. Then, within 24-48h, these monocytes migrate from blood into tissues where they further differentiate into resident macrophages (van Furth & Cohn, 1968).



**Figure 1.1: Schematic diagram showing Hematopoietic stem cells (HSCs) and their progeny.** HSCs give rise to all blood cell types; they differentiate into two main common cells, common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). Lymphocytes come from lymphoid progenitor cells and include B cells, T cells, and natural killer (NK) cells. Myeloid progenitor cells produce erythrocytes (red blood cells), megakaryocytes and platelets, granulocytes (including neutrophils, eosinophils and basophils) and monocytes, which differentiate into macrophages and dendritic cells.

<http://www.sigmaaldrich.com/etc/medialib/life-science/stem-cell-biology/hematopoietic-stem.Par.0001.Image.571.gif>

Macrophages in tissues are widely distributed throughout the whole body (Table 1.1) including bone marrow, liver, brain, lung, bone tissue, spleen, lymph nodes, serous cavities and in connective tissues, and have a particular phenotype in each area (Gordon & Taylor, 2005).

Specific macrophage name	Tissue	Main function
Bone marrow macrophages	Bone marrow	Erythropoiesis
Osteoclast	Bone	Bone marrow remodelling
Langerhans cell	Skin (Epidermis)	Immune surveillance
Lung	Alveolar macrophages	Immune surveillance
Microglial cell	Brain	Neural survival, help in connectivity, injury repair
Crypt macrophages	Intestine	Immune surveillance
Kupffer cell	Liver	Regenerate tissue after damage, and clearance of debris from blood and liver
Uterine macrophages	Uterine	Help cervical ripening
NA	Eye	Vascular remodelling
NA	Kidney	Ductal development
NA	Pancreas	Islet development
NA	Testes	Steroid hormone production
NA	Mammary gland	Branching morphogenesis and ductal development

**Table 1.1:** Diversity of resident macrophages in different tissues. NA=not applicable. Taken from trophic macrophages in development and disease. *Nat Rev Immunol* 9: 259-70. (Pollard, 2009).

### 1.2.2 Classification

Macrophages are a highly versatile cell type that can perform many functions depending on their stage of differentiation, environment and activation status (Gordon, 2003). This includes antigen presentation, anti-bacterial and antitumour activity, and the secretion of a wide variety of regulatory cytokines and enzymes (J. A. Ross & M. J. Auger; Chapter 1: The biology of Macrophages, 1993). Since they were discovered, many attempts have been made to classify macrophages. Some studies

indicate that macrophages can polarize into one of two types (M1 and M2) depending on the microbial and cytokine environment (Gordon, 2003; Mantovani *et al*, 2004). The first group is classical activated macrophages (M1) which are activated by interferon  $\gamma$  (IFN $\gamma$ ), lipopolysaccharide (LPS), and GM-CSF. These cells have a NO<sup>high</sup>, IL-12<sup>high</sup>, IL-23<sup>high</sup>, and IL-10<sup>low</sup> phenotype. They have anti-microbial and anti-tumour properties. The second group is alternatively activated macrophages (M2). These are activated by TGF $\beta$ , IL-10, IL-13, or IL-14. This group of cells have an IL-12<sup>low</sup>, IL-23<sup>LOW</sup>, and IL-10<sup>high</sup> phenotype (Mantovani *et al*, 2006; Mantovani *et al*, 2004).

A study by Mosser & Edwards suggested that macrophage classification should be based on the fundamental macrophage functions that are involved in maintaining homeostasis (Mosser & Edwards, 2008). They proposed three main functions: host defence, wound healing and immune regulation. However, this classification does not take into account the role of macrophages during development including embryonic macrophages (Rae *et al*, 2007) in which the differentiation of these macrophages is highly influenced by the microenvironment.

### 1.2.3 Function

One crucial role of the macrophage is to clear the tissues, blood, and lymph of microorganisms or foreign bodies, by phagocytosis. In addition to that, as scavengers, they remove necrotic or dead cell debris from the body (van Furth *et al*, 1972). When a macrophage ingests a pathogen, the pathogen becomes trapped in a phagosome, which then fuses with a lysosome. Within the phagolysosome, enzymes and highly reactive substances kill and digest the pathogen (Underhill & Ozinsky, 2002a). However, some bacteria, such as *Mycobacterium tuberculosis*, have evolved resistance

to these methods of digestion (Vandal *et al*, 2009). After ingestion, some material from engulfed microorganisms is not degraded completely; fragments of some antigens are retained and presented on the cell surface to lymphocytes through MHC (Major Histocompatibility Complex), and other co-stimulatory molecules such as CD40 and CD80/86 stimulate the adaptive immune response (Martinez-Pomares & Gordon, 2007; Unanue, 2002). Stimulated macrophages are capable of secreting over 100 immunomodulatory substances including chemokines, cytokines, complement components, coagulation factors, growth factors, and prostaglandin, which regulate the activation of other immune system cells in response to inflammation (J. A. Ross & M. J. Auger; Chapter 1: The biology of Macrophages, 1993). Macrophages play a central role in wound healing and building new blood vessels by regulating other cells such as neutrophils, fibroblasts, and endothelial cells. They are recruited to sites of tissue injury by cytokines released by resident macrophages and infiltrated neutrophils (Rodero & Khosrotehrani, 2010). An important early study showed that macrophage depletion in wounded guinea pigs resulted in a delayed infiltration of the wound by fibroblasts and decreased fibrosis, thereby delaying wound healing and tissue remodelling (Leibovich & Ross, 1975).

Macrophages are the main leukocyte recruited to tumour tissues and in some cases can constitute up to 50% of the tumour mass (Leek *et al*, 1994; Murdoch *et al*, 2004). There was a big debate whether the immune system suppresses or promotes tumour progression (Dunn *et al*, 2004). Several studies have reported that macrophages activated by inflammatory cytokines such as TNF $\alpha$  or granulocyte-macrophage colony stimulating factor (GM-CSF) are able to recognize and subsequently kill tumour cells (Klimp *et al*, 2002). They can recognize tumour cells via exposed phosphatidylserine on

the surface of the tumour cell (Utsugi *et al*, 1991). However, one macrophage type known as tumour-associated macrophages (TAMs) was found to promote tumour survival and expansion by enhancing tumour cell invasion and migration, and also to stimulate angiogenesis, therefore supplying tumour cells with oxygen and nutrition (Condeelis & Pollard, 2006).

### **1.3 Macrophages and inflamed tissues**

The description of the four cardinal signs of inflammation (redness, heat, swelling, and pain) described by Celsus over 2000 years ago are considered as the origin of the study of inflammation in biology. Inflammation is a process by which leukocytes protect animals from microorganisms, and it is obvious that this local inflammatory environment is very important to clear invading microbes. However, the immune system in some cases inappropriately triggers an inflammatory response when there are no foreign substances to deal with. These diseases, e.g. rheumatoid arthritis (RA), are called autoimmune diseases (Gierut *et al*, 2010). In RA, self antigen becomes a target for macrophages which are playing a central role in the development of this disease. Several studies have shown that apoptotic synoviocytes in the joint release cytokines and nuclear components that can activate macrophages, triggering secretion of pro-inflammatory cytokines, such as IL-6, IL-8, TNF $\alpha$  and IL-1 $\beta$ , which attract other cells to the site of inflammation, leading to exacerbation of inflammation (Rock & Kono, 2008; Liu & Pope, 2004). Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease are types of autoimmune disease mediated mainly by macrophages (Fatahzadeh, 2009). IBD is characterized by increased permeability of luminal epithelium tissues in the intestine, and thus it is possible for microbes to

spread into the mucosa and encounter macrophages. Continuous activation of macrophages produces pro-inflammatory cytokines including IL-6, IL-1 $\beta$  and TNF $\alpha$  which enhance inflammation in the mucosa. Microenvironment of inflamed tissue in autoimmune diseases and that infected by bacterial pathogens have similar cytokines and enzymes content produced from macrophages. Migration of monocytes and dendritic cells (DCs) into inflamed mucosa is mediated mainly by monocyte chemoattractant protein-1 (MCP-1) produced by activated macrophages (Mahida, 2000).

Lungs are the organs in the body most exposed to environmental toxins and a wide variety of pathogens. The lung is lined with a mucus barrier which can protect the lung by trapping pathogens. The mucus barrier contains anti-microbial peptides, lysozyme, and surfactant proteins which can bind with lipopolysaccharides to neutralize them. However, when this barrier is damaged or penetrated by microorganisms, a rapid pro-inflammatory response is induced by alveolar macrophages (Chaudhuri & Sabroe, 2008).

#### **1.4 Pattern recognition receptors**

One of the most remarkable features of leukocytes including macrophages is their capacity to recognize molecular structures of a huge number of microorganisms. Pattern recognition receptor (PRRs) is term used to describe a wide array of structurally unrelated molecules, which can be transmembrane, cytosolic, or soluble, and which bind specifically to microbes (Palm & Medzhitov, 2009). By using these PRRs, macrophages detect the presence of microorganisms through recognition of conserved microbial pathogen-associated molecular pattern (PAMPs). These PAMPs have three crucial characteristics. First, they are unique to microorganisms and absent

in host cells, so that they are considered as non-self antigens. Second, they are common in a wide array of microorganisms without much variation, so that a few PRRs can detect most infections. Third, they are essential for the microorganism's survival, so that they cannot be easily eliminated by mutation (Palm & Medzhitov, 2009; Medzhitov, 2001).

Transmembrane PRRs consists of two types, Toll-Like Receptors (TLRs) and C-type lectin. Dectin-1 is one C-type lectin family which can recognize  $\beta$ -glucans from fungal pathogens such as *Candida albicans* and induce adaptive immune responses through T helper cells. The TLR family consists of 11 members (Akira & Takeda, 2004) (Table 1.2). TLR1, TLR2, and TLR6 are structurally related to each other; therefore they can dimerize to recognise bacterial components such as lipoprotein/lipopeptides, peptidoglycan and glycolipid. These components are different from typical LPS which are recognized by mainly by TLR4 (Underhill & Ozinsky, 2002b). LPS is a very potent activator, so TLR4 can be activated by a very small amount. TLR3 and TLR5 are found to be activated by dsRNA of viruses and bacterial flagella, respectively. Both TLR7 and TLR8 show a high degree of similarity in their structure and are known to be involved in the recognition of guanosine- or uridine-rich single-stranded viral RNA such as that of HIV (Heil *et al*, 2004). TLR9 has been shown to recognize CpG motifs in bacterial DNA, and this component is a potent inducer for inflammatory cytokines such as IL-12, TNF- $\alpha$ , and IFN- $\alpha$ . TLR10 was detected in B cells from peripheral blood and in plasmacytoid DCs from tonsils, with no specific ligand yet identified for this receptor. TLR11 is the most recently identified receptor found in some bladder epithelial cells

which can recognize bacterial components (Moresco *et al*, 2011; Takeda & Akira, 2005)

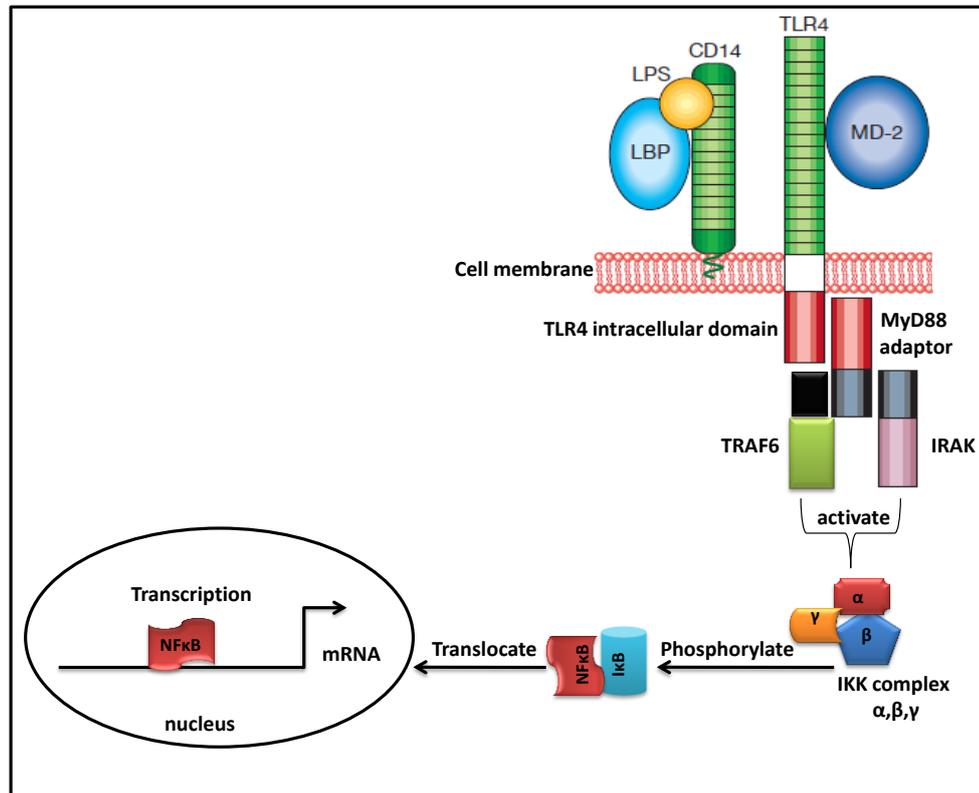
(Table 1.2).

Receptor	Ligand	Ligand origin
TLR 1	Triacyl lipopeptides, peptidoglycan	Bacteria
TLR 2	Glycolipids, peptidoglycan	Bacteria
TLR 3	dsRNA of viruses	viruses
TLR 4	Lipopolysaccharide	Gram-negative bacteria
TLR 5	Flagellin of bacteria	Bacteria
TLR 6	Lipoprotein, lipopeptides, glycolipids	Bacteria
TLR 7	Single-stranded viral RNA	viruses
TLR 8	Single-stranded viral RNA	viruses
TLR 9	CpG Oligodeoxynucleotide DNA	Bacteria
TLR 10	No specific ligand known	-
TLR 11	Multiple lipopeptides, glycolipids	Bacteria

**Table 1.2:** Toll-like receptors that bind with their different ligands.

The intracellular domains of TLRs and IL-1Rs have a region with high similarity to each other, and are termed a Toll/IL-1 receptor (TIR) domain but the extracellular structure is unrelated. The activation of leukocytes through TLRs needs distinct extra- and intracellular molecules. For example, the signalling of TLR4 is initiated by recognition and binding of PAMPs to TLR4, and this signalling needs help of other molecules like LBP (LPS binding protein), CD14 and MD-2 protein, to form a large complex. This complex leads to recruiting of the cytosolic adapter protein, MyD88 (myeloid differentiation primary response gene 88) to the receptor which interacts with the TIR domain. This allows activation of IRAKs (IL-1 receptor-associated kinases which subsequently lead to activation of TRAF6 (tumour-necrosis factor-receptor-associated factor 6) (figure 1.2).

This leads to further activation of other proteins including inhibitor of NF- $\kappa$ B kinases (IKKs), resulting in the release of NF- $\kappa$ B and translocation to the nucleus to act as a transcription factor for inflammatory cytokine genes such as IL-12 and TNF- $\alpha$  (Akira & Takeda, 2004).



**Figure 1.2: Recognition of LPS on the surface of macrophages.** LPS binds to LPS binding protein (LBP) and binds to CD14 to form a complex which then activates TLR4 which in turn signals through the cytosolic adaptor proteins MyD88, IRAK, and TRAF6. IRAK and TRAF6 proteins lead to activation of inhibitory kappa kinase (IkK complex) which then phosphorylates the inhibitory kappa subunit (IkB) which targets it for proteasomal degradation allowing free NF $\kappa$ B transcription factor to translocate to the nucleus to induce expression of target genes. Adapted from (Aderem & Ulevitch, 2000).

## 1.5 Lipopolysaccharide (LPS)

### 1.5.1 Definition of LPS

Lipopolysaccharide (LPS) is a major component of the cell wall of Gram-negative bacteria. It contributes greatly to stabilizing the overall membrane structure of the bacteria by functioning as a barrier protecting them from heavy metals, lipid-disrupting

agent such bile salts, and lytic enzymes. LPS is also called endotoxin (referring to lipid A within its structure), a toxin contained in the cell walls of bacteria, that is released when the bacterium dies and the cell wall is autolysed (Hitchcock *et al*, 1986; Rietschel *et al*, 1982).

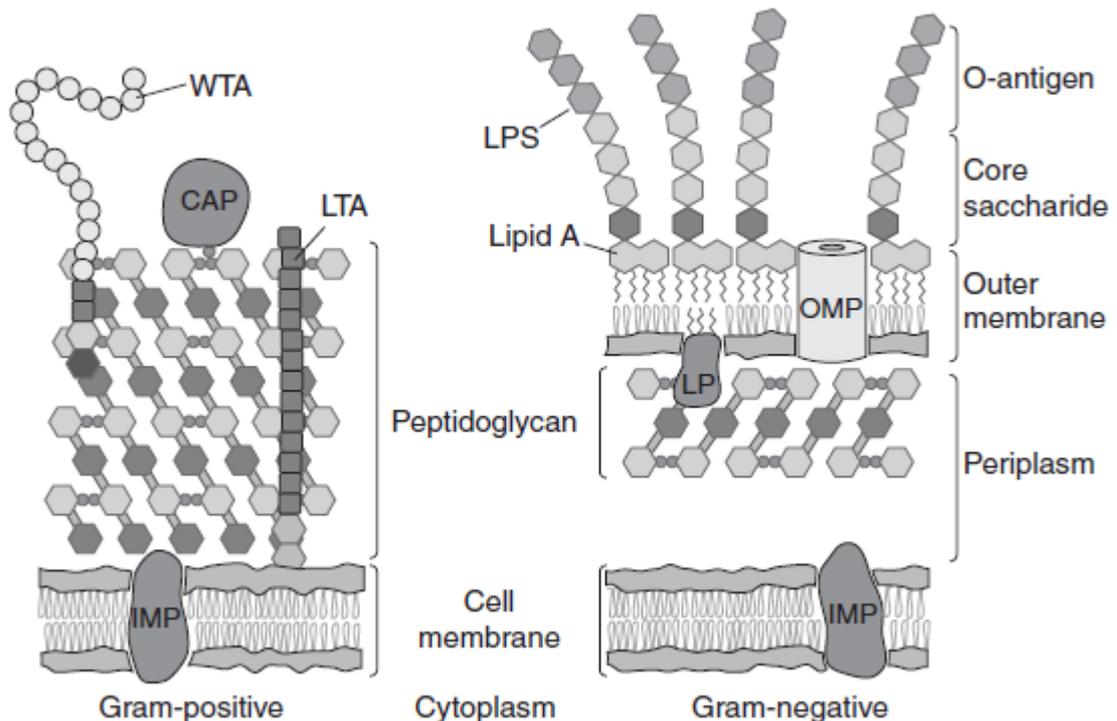
### **1.5.2 Lipopolysaccharide history**

The history of LPS research began in the eighteenth century with the search for substances that cause disease and fever. Early investigators hypothesized that the cause of fever was a physical entity and named it pyrogen, from the Greek root *pyr*, meaning fire. But they were unsure whether fever was a manifestation of disease or a host defence against developing disease. In 1892, Richard Pfeiffer, a co-worker of Robert Koch, published a proposal that *Vibrio cholerae* had a toxin closely associated with, and probably an integral part of, the bacterium. He later concluded that endotoxins were constituents of nearly all groups of bacteria (Beutler & Rietschel, 2003). Thus, Pfeiffer was the first scientist who used the word endotoxin to refer to LPS. The purification and structural characterization of endotoxin took many years of intensive research. Endotoxin was purified by Andre Boivin using a trichloroacetic acid (TCA)-based method (Rietschel *et al*, 1999). Boivin found that endotoxin was composed of lipid and polysaccharide with very little protein. Later in 1954, Otto Westphal and Otto Luderitz succeeded in isolating pure endotoxin using a phenol-water procedure, and were able to obtain highly purified, biologically active endotoxin from different Gram-negative bacteria. Their product contains no protein and was composed of just carbohydrate, fatty acids, and phosphorus. These researchers were the first to use the term lipopolysaccharide (LPS) to describe endotoxin (Westphal *et*

*al*, 1981; Hitchcock *et al*, 1986). It is now known that LPS is embedded in the outer leaflet of the outer membrane of Gram-negative bacteria, and is essential for bacterial survival (Beutler & Rietschel, 2003).

## 1.6 Gram-negative bacteria membranes

The cell envelop of Gram-negative bacteria consists of three main layers; the outer membrane (OM), periplasm, and the inner membrane (IM). The OM is characteristic of Gram-negative bacteria because Gram-positive bacteria lack this organelle. Basically, the OM is a lipid bi-layer with phospholipids and proteins forming the inner leaflet and lipopolysaccharides (LPS) forming the outer leaflet. Proteins and lipoproteins are inserted in the OM lipid bi-layer. Proteins in the OM function as channels and receptors for transportation and communication with the extracellular environment. The OM is attached covalently to the next layer, the periplasm, by different types of lipoproteins. The periplasm lies between the OM and the IM, and contains a hydrophilic peptidoglycan layer which forms a scaffolding structure that give the rigidity and flexibility of the bacterial cell envelop. The thickness of the peptidoglycan layer is variable in Gram-negative bacterial species, and it is much thicker in Gram-positive bacteria. The third layer of the bacterial envelop is the inner membrane (IM), also called the cytoplasmic membrane, which consists of a phospholipid bi-layer (figure 1.3). In contract to eukaryotic cells, bacterial cell do not have membrane-bound organelles, therefore, the IM plays an important role in allowing bacteria to perform their cellular processes. Membrane proteins and LPS are assembled in the IM and transported to the periplasm and OM (Hogg, 2005; Silhavy *et al*, 2010).



**Figure 1.3** Diagram show the details of Gram-positive and Gram-negative cell envelopes: CAP = covalently attached protein, WTA= wall teichoic acid, IMP= integral membrane protein, LPS= lipopolysaccharide, LP=lipoprotein, LTA= lipoteichoic acid, OMP=outer membrane protein. Taken from (Silhavy *et al*, 2010).

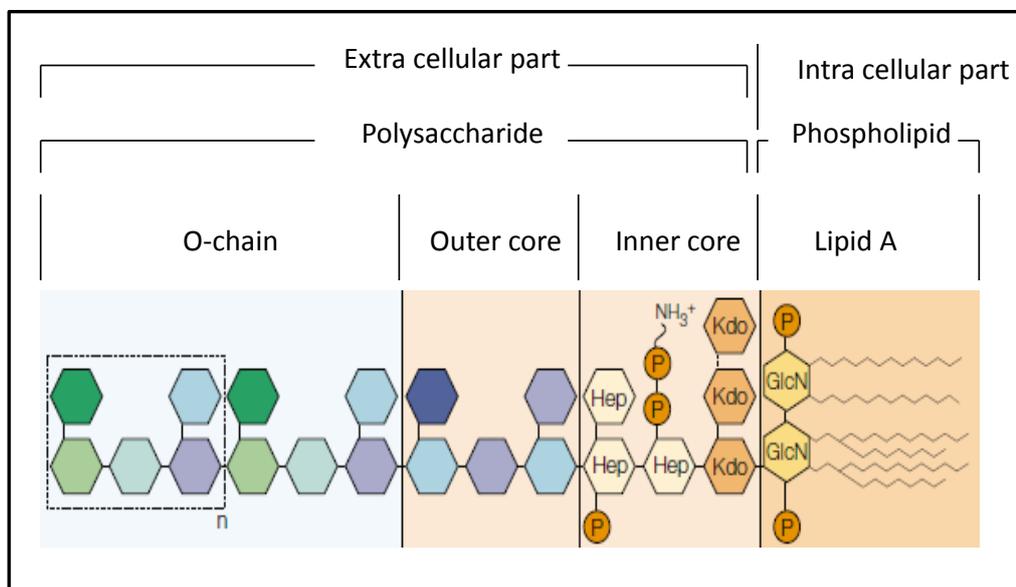
## 1.7 Lipopolysaccharide structure

### 1.7.1 O-antigen chain

The O-antigen part of LPS is a complex of repeated chains of an oligosaccharide polymer, composed of repeating units of five to eight monosaccharides (galactose, rhamnose, mannose, xylose, and robose) (figure 1.4). The structure and nature of the O-chain is extremely variable and diverse with different numbers of monosaccharides (Rietschel *et al*, 1994), therefore antisera raised against one species do not cross-react with other species. This allows classification of bacteria into serotypes. The O-antigen has several biological activities, such as nutrient transport, acting as a receptor for bacteriophages, and mediating interactions with host cells such as modulating the activation of the alternative complement pathway, and inhibiting the attachment to phagocytes (Heine *et al*, 2001).

### 1.7.2 Core oligosaccharide

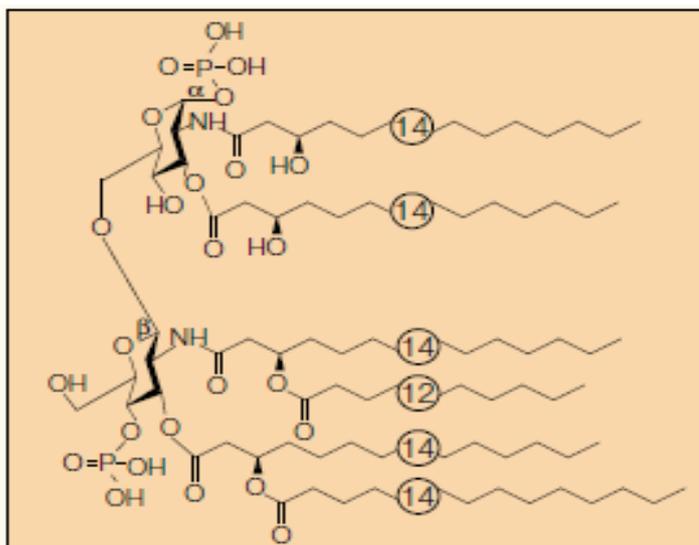
This glycosidic part of LPS is heterogeneous, and is composed of 10-12 monosaccharides such as heptose, glucose, galactose, and N-acetylglucosamine residues. The core oligosaccharide is divided into inner and outer parts. This core part is attached covalently to lipid A by an unusual acidic sugar called 3-deoxy-D-manno-octulosonic acid (KDOs) to form a bridge between the two parts (core and lipid A) (figure 1.4), and this sugar is essential for most endotoxins (Caroff & Karibian, 2003).



**Figure 1.4 Lipopolysaccharide structure:** Schematic diagram shows the details of LPS in each part. The polysaccharide portion of LPS, O-chain and core part are the exposed part of LPS, but lipid A is inserted in the outer membrane. (Adapted from Beutler & Rietschel, 2003).

### 1.7.3 Lipid A

Lipid A is a unique and distinctive phosphoglycolipid, which is highly conserved among species. It is the inner most of the three parts of LPS which is anchored in the outer membrane (OM) of Gram-negative bacteria (figure 1.5). All Gram-negative bacteria have certain common structures in lipid A, but differ in some details as discussed below (Beutler & Rietschel, 2003).



**Figure 1.5 Lipid A structure:** Diagram shows the primary structure of lipid A which is found in most Gram-negative bacteria. (Taken from Beutler & Rietschel, 2003).

Lipid A contains *D*-gluco-configured pyranosidic hexosamine residues (or 2,3-diamino-2,3-dideoxy-*D*-glucose), which are present as  $\beta(1\rightarrow6)$ -linked dimers. The disaccharide contains  $\alpha$ -glycosidic and non-glycosidic phosphoryl groups in the 1 and 4' positions, and (*R*)-3-hydroxy fatty acids at positions *O*-2, *O*-3, *O*-2' and *O*-3' in ester and amide linkages, of which two are usually further acylated at their 3-hydroxyl group (Banoub *et al*, 2010). However, variations in the fine structure can arise from the type of hexosamine present, the degree of phosphorylation, the presence of phosphate substituents, and importantly in the nature, chain length, number, and position of the acyl groups (Raetz *et al*, 2007). In the lipid A of the most studied organism, *Escherichia coli*, the hydroxy fatty acids are C<sub>14</sub> in chain length, and the hydroxy groups of the two (*R*)-3-hydroxy fatty acids of the distal GlcN-residue (GlcN II), and not those of the GlcN-residue at the reducing side (GlcN I), are acylated by non-hydroxy fatty acids (12:0 and 14:0) (Kabanov & Prokhorenko, 2010). Some molecular species contain an additional fatty acid attached to the amide-linked 3-hydroxy acid and the phosphate group may

be substituted with ethanolamine-phosphate (of GlcN I). There are a few important exceptions to this type of fatty acid pattern. For example, in the lipid A of *Helicobacter pylori* in comparison to that of *E. coli*, there are four rather than six fatty acids with a longer average chain-length (16-18) (Moran, 2007) .

## **1.8 Hypoxia**

### **1.8.1 Definition of hypoxia**

Haemoglobin in red blood cells carries oxygen (O<sub>2</sub>) from the lungs and delivers it to the peripheral tissues to maintain the viability and proper metabolic activity of cells. The normal concentration of oxygen in the atmosphere at sea level is 21% (150 mm Hg); however in normal tissues it usually ranges from 2-9 % O<sub>2</sub> (20-80 mm Hg). Hypoxia often is defined as a reduction of oxygen as less than 2% O<sub>2</sub> (around 10 mm Hg) (Hockel & Vaupel, 2001; Semenza, 2010). Hypoxia in tissues occurs when an imbalance occurs between oxygen (O<sub>2</sub>) supply and consumption. This leads to improper function of cells and death if it is prolonged. Hypoxia is divided into two types: acute hypoxia (also called perfusion-limited hypoxia) where cells are exposed to transient hypoxia when blood vessels are occluded. The other type is chronic hypoxia, in which cells are exposed to low oxygen for long periods of time (Harris, 2002).

The ability of cells/tissues to adapt to hypoxia is crucial for their survival and development. Several studies have shown that hypoxia is naturally needed to control embryonic development of many tissues, including placenta, bone, cardio-vascular, and branching in trachea (Simon & Keith, 2008).

### 1.8.2 Role of hypoxia in pathological tissues

Recently, accumulated evidence has shown that hypoxia contributes to the progression of a number of diseases. In renal vascular disease, it was found that the interstitial capillary density is reduced in kidney due to an imbalance in the expression of pro- and anti-angiogenic factors, leading to hypoxia and tissue necrosis (Eckardt *et al*, 2003). Also hypoxia is a direct consequence in patients suffering brain stroke due to shortage of blood supply, which facilitates the pathogenesis of Alzheimer's disease by promoting the degeneration of neurons (Zhang & Le, 2010). Rheumatoid arthritis is characterized by synovial expansion in the inflamed joint, leading to increased demand for oxygen exhausting the supply, subsequently leading to synovial hypoxia (Hollander *et al*, 2001). This leads to induction of pro-inflammatory cytokines, such as interleukin-1 $\alpha$  (IL-1a), IL-1b, and tumour necrosis factor  $\alpha$ , via HIF (hypoxia inducible factor) activity in effector cells (Muz *et al*, 2009).

Hypoxia is a hallmark of solid tumours due to exponential cellular proliferation and an inefficient vascular supply. Most tumours with a volume greater than 1 mm<sup>3</sup> contain regions of hypoxia as a result of the aberrant blood vessel structure and limited oxygen diffusion found in tumour tissues (Shannon *et al*, 2003).

Vaupel and colleagues have demonstrated that human solid tumours contain different degrees of hypoxia, with significantly lower oxygen tension than that found in normal tissue. They found that oxygen tensions in human tumours show a range of 1.3 to 3.9% O<sub>2</sub> (10–30 mmHg), and in addition they showed low oxygen readings of around 0.01% (0.08 mmHg) in some tumours. Oxygen level in normal tissues are generally much higher, ranging from 3.1 to 8.7% O<sub>2</sub> (24–66 mmHg) (Vaupel *et al*, 1991). Another group showed that median O<sub>2</sub> in normal healthy prostate tissue is 31 mmHg while in tumour

tissue it is about 8 mmHg (Movsas *et al*, 1999). Furthermore, Höckel and colleagues showed that median O<sub>2</sub> in cervical tumours is about 13 mmHg, whereas in healthy normal cervical tissue it is more than 46 mm Hg (Hockel *et al*, 1991).

## **1.9 Macrophages in hypoxia**

### **1.9.1 Hypoxia-inducible factors (HIF-1, -2, and -3)**

#### **1.9.1.1 Discovery of HIFs**

Hypoxia-inducible factors (HIFs) are transcription factors that regulate oxygen homeostasis in tissues (Laboda *et al*, 2010). Studies analysing the molecular mechanisms regulating expression of the erythropoietin gene (EPO), which controls the production of red blood cells, lead to the discovery of HIF-1 by identifying a *cis*-acting hypoxia-response element (HRE) in the EPO gene (Semenza *et al*, 1991; Beck *et al*, 1993).

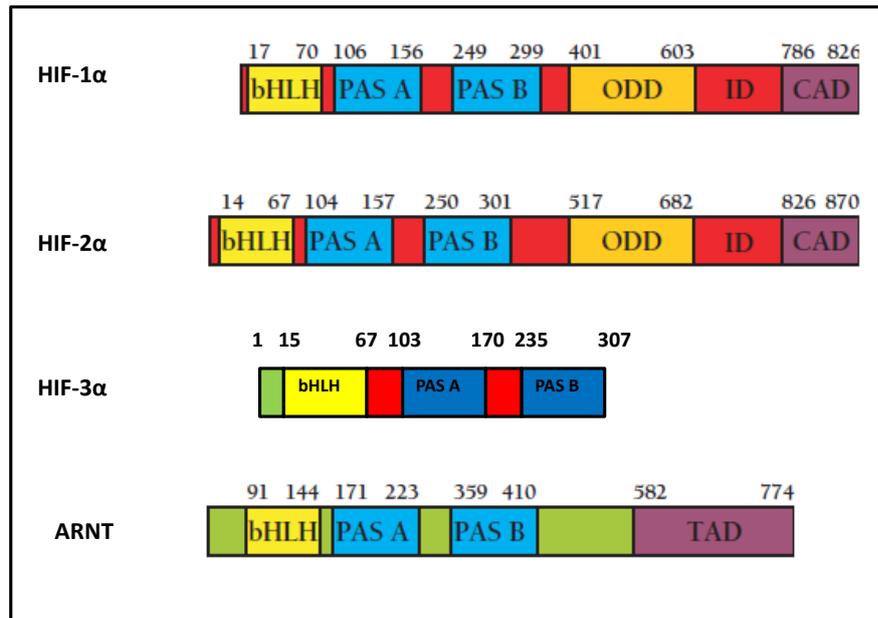
In addition to HIF-1, cells respond to hypoxia by activating and up-regulating a numbers of transcription factors (Cummins & Taylor, 2005) such as NF-κB (Leeper-Woodford & Detmer, 1999), HIF-2 (also known as EPAS1) (Endothelial Per-ARNT-Sim (PAS) Domain Protein 1) or HIF-like factor (HLF) (Sato *et al*, 2002), Ets-1 (Elbarghati *et al*, 2008), and Sp-1 (Lee *et al*, 2004). However, HIF-1 is the most well characterized transcription factor, and can regulate more than 100 genes; hence it has been referred to as a master regulator of oxygen homeostasis (Semenza, 2007).

#### **1.9.1.2 HIFs structure**

HIFs are heterodimeric proteins composed of one of three basic helix-loop-helix-PAS (bHLH-PAS) hypoxia-inducible alpha subunits (HIF-1α, HIF-2α, and HIF-3α) and a constitutively expressed beta subunit (HIF-1β) also known as the aryl-hydrocarbon

receptor nuclear translocator (ARNT) (Semenza, 2004). HIF-1 $\alpha$ :HIF-1 $\beta$  and HIF-2 $\alpha$ :HIF-1 $\beta$  heterodimers functions as a transcription factors for oxygen-sensitive genes, and they have overlapping target genes but there are specific target genes for each complex (Wang *et al*, 2005). HIF-1 $\alpha$  is expressed in most cells, however HIF-2 $\alpha$  is preferentially expressed in endothelial cells (Nagao & Oka, 2011). Structurally, they are closely related and share 48% amino acid homology (Sato *et al*, 2002). The role of the third protein (HIF-3 $\alpha$ ) is not well defined, but data has shown that it might function as an inhibitor of HIF-1 and HIF-2 (Makino *et al*, 2001; Makino *et al*, 2002).

As shown in figure 1.5, HIF-1 $\alpha$ , HIF-2 $\alpha$ , HIF-3 $\alpha$ , and ARNT are basic helix-loop-helix-Per-ARNT Sim PAS proteins. Each subunit contains two PAS domains (PAS A and B), and an N-terminus with a HLH domain which is required for dimerization and DNA binding. HIF-1 $\alpha$  and HIF-2 $\alpha$  contain oxygen-dependent degradation domains (ODD) that mediate oxygen-regulated stability and a C-terminal trans-activation domain (CAD) and inhibitory domain (ID). CAD is needed to activate transcription of hypoxia-inducible genes. The CAD domain is constitutively active but its transcriptional activity is inhibited by inhibitory domain (ID) under normoxia and induced by hypoxia independent of protein stability (Makino *et al*, 2001; Fedele *et al*, 2002).

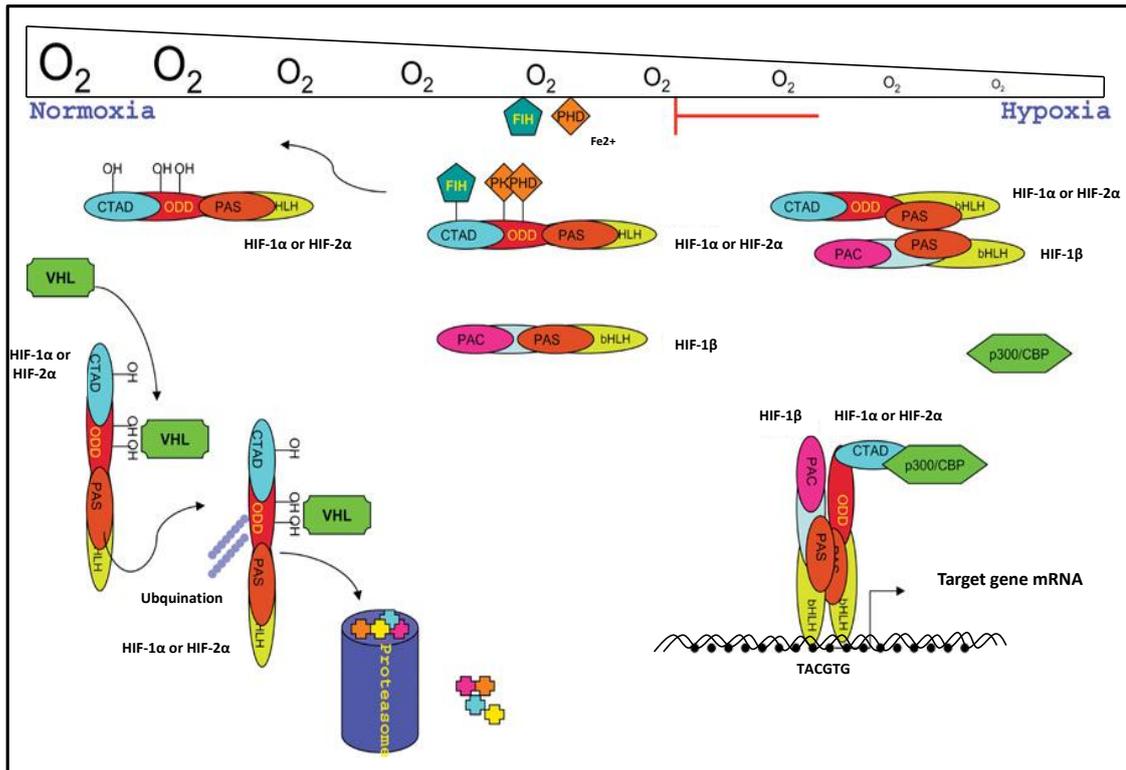


**Figure 1.6 Schematic representation of HIF-1 $\alpha$ , HIF-2 $\alpha$ , HIF-3 $\alpha$ , and ARNT:** HIF-1 $\alpha$ , HIF-2 $\alpha$ , HIF-3 $\alpha$ , and ARNT are basic-Helix-loop-Helix-Per-ARNT-Sim (bHLH-PAS) protein that contains an N-terminal bHLH domain and two Pas domains. HIF-1 $\alpha$  and HIF-2 $\alpha$  contain an oxygen-dependent degradation domain (ODD) that mediates oxygen-regulated stability, and also C-terminal trans-activation domain. Adapted from (Fedele *et al*, 2002) and (Makino *et al*, 2001).

### 1.9.1.3 Oxygen-dependent HIF activity

Oxygen-dependent enzymatic activity covalently modifies a domain of HIF-1 $\alpha$  known as the oxygen-dependent degradation (ODD) domain (Wenger, 2002). This modification is performed by prolyl hydroxylase (PHD) enzymes which continuously “sense” the oxygen levels and modify hypoxia-inducible transcription factor- $\alpha$  (HIF-1 $\alpha$ ) subunits (Schofield & Ratcliffe, 2004). In the presence of oxygen, the HIF-1/2 $\alpha$  subunits are hydroxylated by the prolyl hydroxylase enzymes (PHD 1-3), and this process requires 2-oxoglutarate (2-OG), ascorbate, and Fe<sup>2+</sup>. Hydroxylation occurs at proline residues P<sup>564</sup> and P<sup>531</sup> in the ODD for HIF-1 $\alpha$  and HIF-2 $\alpha$ , respectively. This is followed by binding of von Hippel-Lindau tumour suppressor protein (VHL) to the hydroxylated ODD in HIF- $\alpha$  subunits. VHL associates with Elongin B, Elongin C, RBX1, and cullin 2 to form a complex that recruits the E3 ubiquitin-protein ligase complex. This facilitates

ubiquitination of HIF-1/2 $\alpha$  and targets it for degradation by the 26S proteasome, leading to inhibition of HIF accumulation in normoxia resulting in halted gene transcription (figure 1.6) (Semenza, 2007). Another mechanism has proposed for HIF-1/2 $\alpha$  regulation, as shown in figure 1.5, HIF-1 $\alpha$  and HIF-2 $\alpha$  contain C-terminal trans-activation domain CAD that bind co-activators including CBP and p300 required to initiate the transcription activation, however FIH-1 (factor inhibiting HIF-1) blocks binding of these two co-activators (CBP and p300) by asparaginyl hydroxylation at N<sup>803</sup> and N<sup>851</sup> in HIF-1 $\alpha$  and HIF-2 $\alpha$  respectively (Hirota & Semenza, 2005; Fedele *et al*, 2002). In hydroxylation reactions, both PHD and FIH enzymes need to use oxygen and 2-OG as substrates, therefore, in hypoxia the reduced availability of oxygen inhibits the hydroxylation process by these two enzymes, and prevents HIF- $\alpha$  degradation (Cockman *et al*, 2009). This enables HIF-1/2 $\alpha$  subunits to translocate to the nucleus where they dimerize with ARNT (HIF- $\beta$ ) then bind hypoxia response elements (HRE) in the promoters of hypoxia-inducible genes such as VEGF, EPO and Glut-1, and interacts with the DNA polymerase II (Pol II) complex, initiating their transcription (figure 1.6) (Brahimi-Horn & Pouyssegur, 2009; Semenza, 2011).



**Figure 1.7 Schematic presentation of the HIF pathway:** In the presence of  $O_2$ , HIF- $\alpha$  subunits are prolyl hydroxylated by prolyl hydroxylase-domain protein (PHDs) using  $Fe^{2+}$  which then causes HIF- $\alpha$  to interact with von Hippel-Lindue (VHL) complex which leads to ubiquitination (Ub), and HIF- $\alpha$  is degraded by the proteasome. In hypoxia, prolyl hydroxylases are inhibited, then stabilized HIF- $\alpha$  translocates to the nucleus where it interacts with ARNT (HIF-1 $\beta$ ) and bind to hypoxia-response element (HRE) and recruit p300/CBP and the DNA polymerase II (Pol II) complex to activate transcription of target genes. Modified from (Kenneth & Rocha, 2008).

#### 1.9.1.4 Oxygen-independent HIF activity

In the last few years, accumulating evidence has demonstrated that induction and activation of HIFs also occurs in response to many non-hypoxic stimuli (Dery *et al*, 2005). LPS is the most potent stimulator for macrophages leading to the activation of a number of hypoxia-inducible genes through NF- $\kappa$ B which are believed to be up-regulated independently of HIF-1 (Covert *et al*, 2005; Karin & Greten, 2005). Interesting a study by Blouin *et al* 2004 has shown that several hypoxia-inducible genes such as Glut-1, VEGF, and iNOS which are known to be regulated by HIF-1, also can be up-regulated by LPS (Blouin *et al*, 2004). Induction of these genes by LPS was found to

be mediated through increased HIF-1 $\alpha$  transcriptional under normal oxygen tension (Blouin *et al*, 2004; Frede *et al*, 2006). This increase is sufficient to shift the balance between synthesis and degradation in normoxia toward accumulation of HIF-1 $\alpha$  (Dery *et al*, 2005).

PI3-Kinase signalling pathway is involved in many cellular functions including proliferation, survival, cell growth, and motility (Fry, 2001; Katso *et al*, 2001). Abnormal signalling of PI3-k was found to be associated with many human disease processes such as inflammation and cancer (Stein, 2001; Liu *et al*, 2009). It was suggested that activation of the PI3-Kinase pathway could increase the rate of HIF-1 $\alpha$  translation in normoxia (Page *et al*, 2002). This pathway can be activated by growth factors and hormones which lead to the recruitment and activation of a downstream effector known the mammalian target of rapamycin (mTOR) (Alam *et al*, 2004). mTOR activation results in increased phosphorylation and inactivation of the 4E-binding protein 1(4E-BP), a eukaryotic translation initiation factor, and activation of p70-S6 kinase 1 which leads to increased synthesis of proteins including HIF-1 $\alpha$  (Zhong *et al*, 2000; Treins *et al*, 2002; Richard *et al*, 2000; Page *et al*, 2002).

Several studies have shown that HIF-1 $\alpha$  can be stabilized by CoCl<sub>2</sub> in normoxia (Maxwell *et al*, 1999). As mentioned previously, the hydroxylation by PHDs of HIF-1 $\alpha$  requires iron, so it was suggested that CoCl<sub>2</sub> may act as a competitor for iron-binding sites, thus halting hydroxylation of HIF-1 $\alpha$  (Epstein *et al*, 2001). Another study suggested that CoCl<sub>2</sub> can stabilize HIF-1 $\alpha$  by binding directly to the ODD, thereby preventing the interaction between HIF-1 $\alpha$  and VHL protein and subsequently inhibiting proteasomal degradation of HIF-1 $\alpha$  (Yuan *et al*, 2003).

The chelating agent desferrioxamine (DFO) also causes HIF-1 $\alpha$  accumulation under normoxia and induces hypoxia-inducible genes such as EPO (Wang & Semenza, 1993). A mechanism was proposed by Maxwell, who demonstrated that DFO disrupts pVHL-HIF-1 $\alpha$  complex formation which is required for ubiquitination and proteosomal degradation of HIF-1 $\alpha$  in normoxia (Maxwell *et al*, 1999).

Other factors such as virus infections also can enhance HIF-1 $\alpha$  protein stability in normoxia. Some viral infections cause expression of a certain oncoproteins which lead to expression and activation of HIF-1 through different signalling pathways. Latent membrane protein 1 (LMP1) is an oncoprotein produced as a result of Epstein-Barr virus infection which induces the production of reactive oxygen species (ROS) like hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) through the activation of p42/44 MAPK pathway, thus stabilizing the HIF-1 $\alpha$  protein (Wakisaka *et al*, 2004). Hepatitis B virus X (HBx) and viral IFN regulatory factor 3 (vIRF3) are two viral proteins that produced by hepatitis B (HBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) viruses, respectively, in infected tissues. These two proteins are involved in HIF-1 $\alpha$  protein stabilization in normoxia (Sodhi *et al*, 2000). They can bind to the bHLH/PAS domain of HIF-1 $\alpha$ , thereby preventing ubiquitin-dependent degradation by pVHL (Moon *et al*, 2004; Shin *et al*, 2008).

In addition, induction of HIF-1 $\alpha$  in normoxia can be seen in some tissues exposed to heat or mechanical stress. *In vivo* and *in vitro* studies have shown that increased temperature can cause production of heat shock protein such as HSP90 which are able to bind the bHLH domain of HIF-1 $\alpha$  protein, therefore shielding it from degradation (Katschinski *et al*, 2002). Mechanical pressure or stretching applied to cartilage and vascular smooth muscle cells promotes the induction of HIF-1 $\alpha$  mRNA and protein,

leading to expression of well characterized hypoxia-inducible genes such VEGF, MMP-1, MMP-3, and MMP-13 (Pufe *et al*, 2004; Chang *et al*, 2003).

#### **1.9.1.5 Role of HIFs in development**

Although HIF-1 and HIF-2 are similar in structure, binding the same HRE, and having similar functions, they appear to regulate different arrays of genes in different cell types (Loboda *et al*). Several studies on mutant mice have shown that HIF-1 $\alpha$  and HIF-2 $\alpha$  are essential for embryonic development, and gene deletion leads to death of the embryo between day 9 to 11 of gestational age (E day 9-11) (Iyer *et al*, 1998). It was found that these mice have cardiovascular malfunction, neural tube defects, and impaired embryonic angiogenesis (Compernelle *et al*, 2003; Ryan *et al*, 1998). However, hyperoxia was found to prolong the survival of HIF-1 $\alpha$  deficient mice with partial rescue of development stage cultured HIF-1 $\alpha$ <sup>-/-</sup> embryos (Compernelle *et al*, 2003). Similar to HIF-1 $\alpha$ , postnatal ablation of HIF-2 $\alpha$  was shown to have the crucial function of maintaining erythropoietin levels necessary for adequate red blood cell production (Gruber *et al*, 2007). Also, In HIF-2 $\alpha$  deficient embryos, small blood vessels in the optical vesicle and cephalic tissues fail to form larger branching vessels resulting in aberrant vascular remodelling (Peng *et al*, 2000). Other studies have also shown that deficient embryos die from circulatory failure during mid-gestational embryonic development (Tian *et al*, 1998). In addition to that, Compernelle and colleagues have shown that HIF-2 $\alpha$  and its downstream target VEGF are critical for foetal lung maturation, and deletion of the alpha subunit in HIF-2 causes respiratory distress syndrome (RDS) in neonatal mice (Compernelle *et al*, 2002).

As mentioned previously in section 1.9.1.2, HIF-3 $\alpha$  is thought to be an inhibitor of the other two HIFs (Makino *et al*, 2002). Hara and colleagues reported that HIF-3 $\alpha$  protein was expressed in human kidney cells; however a HIF-3 $\alpha$  -expression vector failed to up-regulate HRE-driven transcription in COS-7 cells (renal tumour cell) but rather suppressed an HRE-driven luciferase construct, suggesting that HIF3- $\alpha$  could form a complex with protein other than HIF-1- $\beta$  and that this new complex could be suppressive for HRE-driven genes (Hara *et al*, 2001). Makino and colleagues showed that expression of endothelin 1 and platelet-derived growth factor B was increased in lung endothelial cells of HIF-3 null mice. As HIF-3 $\alpha$  protein lacks the CTAD (C-terminal trans-activating domain), the activation of target gene transcription by HIF3- $\alpha$ - HIF-1- $\beta$  dimers is much weaker than that by HIF-1 $\alpha$ -HIF-1- $\beta$  or HIF-2 $\alpha$ -HIF-1- $\beta$  dimers, so HIF-3 might have the capacity to compete with HIF-1 and HIF-2, leading to repression of some cellular responses to hypoxia (Makino *et al*, 2002).

One *in situ* hybridization study has shown that HIF-1 $\alpha$  was expressed at high level in myocardial wall and the atrioventricular canal whereas HIF-2 $\alpha$  expression was ubiquitous in vasculature in the heart. In kidney, HIF-1 $\alpha$  expression was high in collecting ducts, but HIF-2 $\alpha$  was found expressed in the glomeruli and renal vessels. In the bladder HIF-1 $\alpha$  and HIF-1- $\beta$  were co-expressed in the transitional epithelium whereas HIF-2 $\alpha$  was expressed in the capillary of connective tissues (Jain *et al*, 1998).

In addition to HIF-1 $\alpha$  protein, other molecules were involved in HIF regulation of gene expression in hypoxia such as pVHL, HIF-1- $\beta$ , and PHDs. As mentioned previously, HIF-1- $\beta$  is needed to form active transcriptional complex with HIF-1, 2, 3  $\alpha$  and it was found that knockout of these genes in mice causes embryonic lethality associated with defects in neural tube closure (Doedens & Johnson, 2007). Also, knockout of pVHL or

PHD2 results in embryonic lethality (Doedens & Johnson, 2007; Maltepe *et al*, 1997). In summary, hypoxia induces transcription factors, HIF- $\alpha$ , HIF- $\beta$ , pVHL, PHDs, and all are required for essential mammalian development and function.

#### **1.9.1.6 Role of HIF in macrophage gene expression**

It has been mentioned previously that macrophages accumulated in several inflammatory tissues which are characterized by hypoxia. These include atherosclerosis plaques (Bjornheden *et al*, 1999), myocardial infarcts (Jurgensen *et al*, 2004), malignant tumours (Murdoch *et al*, 2005), rheumatoid arthritis (Hollander 2001), healing wounds (Stevens *et al*, 1991), and sites of bacterial infections (Pouyssegur *et al*, 2006; Blouin *et al*, 2004). Several studies have shown that macrophage mainly rely on HIFs under hypoxia for energy generation and activity by accumulation of HIF-1 $\alpha$  protein and subsequently expression of target genes such as VEGF and Glut-1 (Burke *et al*, 2002; Burke *et al*, 2003).

The accumulation of HIF-1 $\alpha$  protein was first reported in activated macrophages by (Talks *et al*, 2000) in tumour tissues, and later by (Hollander *et al*, 2001) in inflamed joints in patients with rheumatoid arthritis and by (Burke *et al*, 2002) in isolated hypoxic human primary macrophages *in vitro*. A study by (Albina *et al*, 2001) has shown increased levels of HIF-1 $\alpha$  in the inflammatory cells of healing wounds and it was suggested that this could be due to released inflammatory cytokines such as TNF- $\alpha$  in cells after injury, leading to increased expression of HIF-1 responsive genes such as VEGF which regulate the process of tissue repair (Albina *et al*, 2001). Another study conducted by Elbarghati and colleagues showed that primary human macrophages rapidly up-regulate HIF-1 $\alpha$  and HIF-2 $\alpha$  protein when exposed to acute hypoxia (0.1 %

O<sub>2</sub>) with different time periods, and these two proteins translocate to the nucleus for trans-activation of targeted genes. This study showed differences in the temporal expression of HIF-1 $\alpha$  and HIF-2 $\alpha$  in macrophages (Elbarghati *et al*, 2008).

An interesting study by Cramer and colleagues has shown that HIF-1 is an important regulator for energy and activity in myeloid cells (Cramer *et al*, 2003). They found that knockdown of HIF-1 $\alpha$  has great inhibitory effects on motility, invasiveness, and adhesion in isolated peritoneal macrophages. Moreover, the study demonstrated that acute and chronic inflammations rely heavily on HIF-1 regulation *in vivo* (Cramer *et al*, 2003).

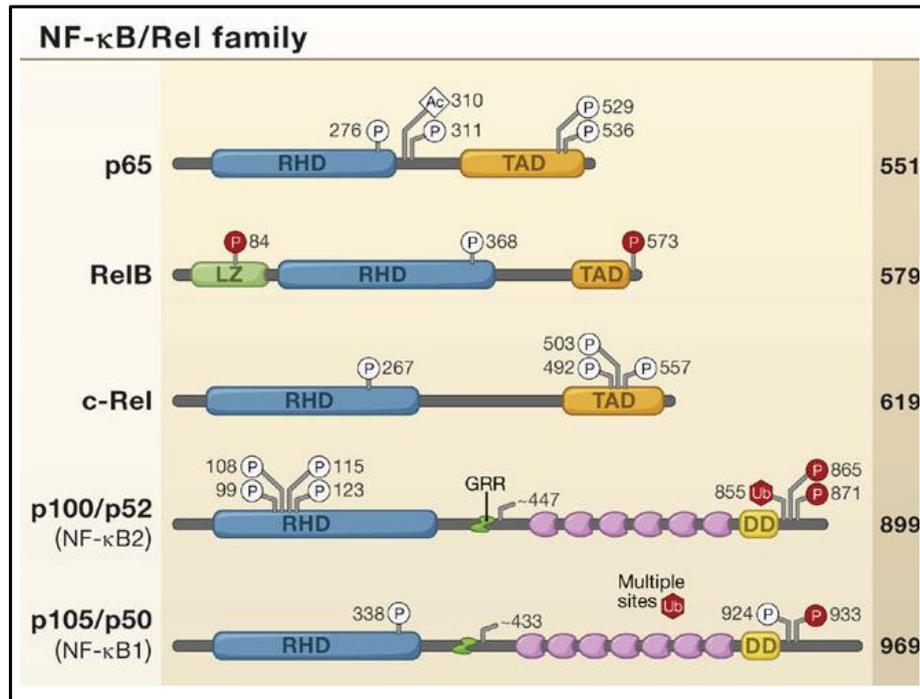
Another study by Peyssonnaud *et al* also shows the crucial role played by HIF-1 $\alpha$  in macrophage functions in knockdown animals (Peyssonnaud *et al*, 2005). These studies also provided preliminary *in vitro* evidence that deletion of HIF-1 $\alpha$  could impair macrophage bactericidal activity including phagocytic uptake of the bacterium, production of reactive oxygen species, activation of iNOS, and release of antimicrobial peptides. They showed that HIF-1 $\alpha$  helps macrophages in coordinating a proper innate immune response for killing of medically important bacterial species such as group A *Streptococcus*, methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa*, and Salmonella species which can trigger HIF-1 $\alpha$  expression in infected microenvironments (Peyssonnaud *et al*, 2005).

## **1.9.2 The role of NF- $\kappa$ B in gene expression**

### **1.9.2.1 Nuclear factor- $\kappa$ B**

NF- $\kappa$ B was first identified and described by Sen and Baltimore in 1986 as a regulator of  $\kappa$ B light chain expression in mature B and plasma cells (Sen & Baltimore, 1986; Sen &

Baltimore, 2006). However, inducibility of its activity by exogenous stimuli was found in many cells types. Intensive studies have shown that NF- $\kappa$ B plays critical roles in regulation of cell responses and modulates transcription of many genes involved in cell survival, immune responses, differentiation, inflammation, and proliferation (Tak & Firestein, 2001). The NF- $\kappa$ B group of proteins in mammals consists of five members, NF- $\kappa$ B1 (p50 and its precursor p105), NF- $\kappa$ B2 (p52 and its precursor p100), RelA (also called p65), c-Rel, and RelB, encoded by the genes NF- $\kappa$ B1, NF- $\kappa$ B 2, RELA, REL, and RELB respectively. All these molecules have a common N-terminal Rel homology domain (RHD) that is responsible for sequence-specific DNA binding and homo- and hetero-dimerization (figure 1.7). Activated NF- $\kappa$ B dimmers bind to  $\kappa$ B sites within the promoter/enhancers region of target genes and regulate their transcription through the recruitment of co-activators and co-repressors. p65, RelB, and c-Rel have a transcription activation domain (TAD) that necessary for the positive regulation of gene expression, but the other two molecules, p52 and p50 have no (TAD) (figure 1.7), therefore rely on interactions with other molecules to positively regulate transcription or which it may work to repress transcription as a negative regulator (Hayden & Ghosh, 2008).



**Figure 1.8 Structure of the NF- $\kappa$ B protein family members:** Members of the NF- $\kappa$ B proteins are shown. The number of amino acids in each human protein is indicated on the right. Post-translational modifications that influence IKK activity or transcriptional activation are indicated with P for phosphorylation. Inhibitory events and phosphorylation sites on p100, p105, and I $\kappa$ B proteins that mediate proteasomal degradation are indicated with red Ps. RHD, Rel homology domain; TAD, transcription domain; LZ, leucine zipper domain; GRR, glycine-rich region; and DD, death domain. Taken from (Hayden & Ghosh, 2008).

### 1.9.2.2 Nuclear factor- $\kappa$ B activation pathway

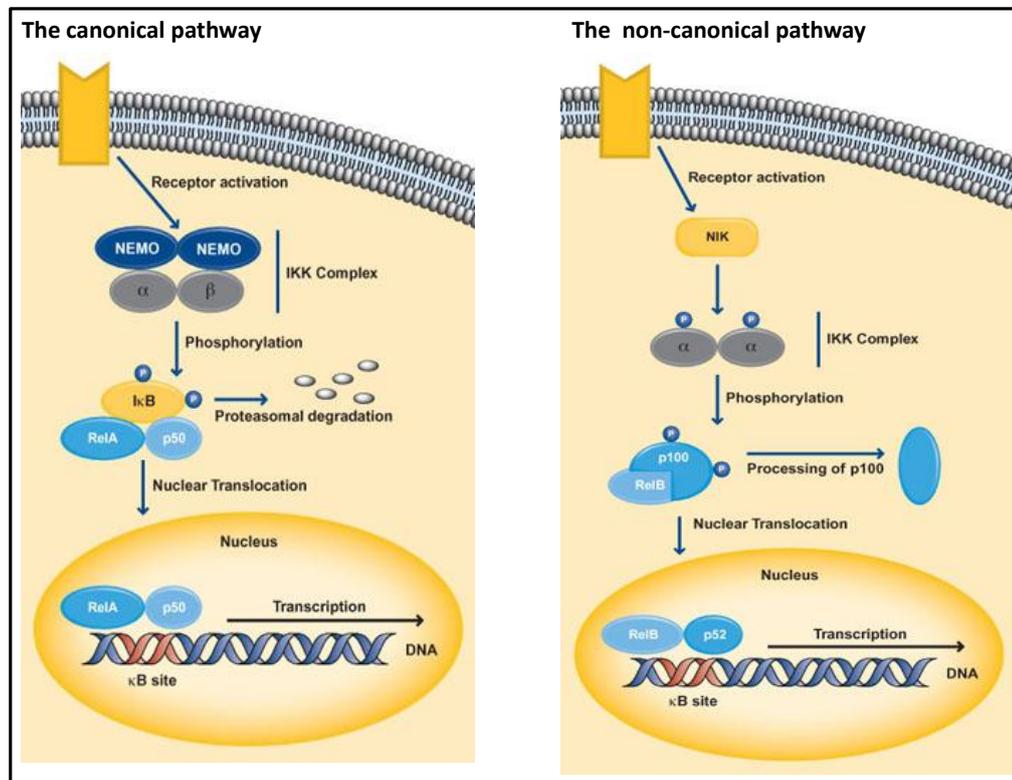
In unstimulated cells, NF- $\kappa$ B dimers are retained in the cytoplasm by being bound to NF- $\kappa$ B inhibitory proteins (I $\kappa$ B) (Gilmore, 2006). There are two major pathways that lead to activation and translocation of NF- $\kappa$ B dimers from the cytoplasm to the nucleus; the classic/canonical which is used in response to most stimuli or the alternative/non-canonical pathway (see figure 1.8) (Bonizzi & Karin, 2004). The classic NF- $\kappa$ B pathway is activated by pathogen-associated molecular patterns (PAMPs) such as LPS or pro-inflammatory cytokines like TNF- $\alpha$ , working through binding to different receptors belong to Toll-like receptor (TLRs) or the TNF (TNFR) and IL-1 receptors (IL-1R) (Sun & Ley, 2008). Engagement of these receptors with their ligands leads to

activation phosphorylation events which cause activation of an I $\kappa$ B kinase (IKK) complex consisting of catalytic kinase subunits (IKK $\alpha$  and/or IKK $\beta$ ) and the regulatory non-enzymatic scaffold protein NEMO (NF-kappa B essential modulator also known as IKK $\gamma$ ). The activation of IKK complex then catalyzes the phosphorylation I $\kappa$ B inhibitor, targeting it for poly-ubiquitination and then degradation by proteasomal complex, releasing the p50-p65 (RelA) dimer which then translocate to the nucleus where it binds to the promoter region of targeted genes and activates their transcription. NF $\kappa$ B activation leads to the expression of the I $\kappa$ B $\alpha$  gene, which consequently sequesters NF $\kappa$ B subunits and terminates transcriptional activity and work through feedback inhibition (Pomerantz & Baltimore, 2002; Gilmore, 2006).

The alternative NF- $\kappa$ B pathway is activated through stimuli which induce proteolysis of the NF- $\kappa$ B2 p100 subunit (Hayden & Ghosh, 2004). This pathway occurs during the development and maintenance of lymphoid organs responsible for the generation of B and T lymphocytes. Limited number of agonists such as lymphotoxin- $\beta$  receptor (LT $\beta$ R), CD40, and B-cell-activating factor belonging to the TNF family (BAFF) were known to induce processing of p100 to p52 through phosphorylation and activation of IKK $\alpha$  by NF $\kappa$ B-inducing kinase (NIK), this leads to processing and liberation of p52-RelB, which translocates to the nucleus to regulate target genes. Some studies have shown that the canonical NF- $\kappa$ B pathway may also be linked to the alternative NF- $\kappa$ B pathway and may influence the range and duration of its activation (Bonizzi & Karin, 2004; Hayden & Ghosh, 2004).

NF- $\kappa$ B is highly regulated and important in controlling lymphocyte and macrophage functions in immune and inflammatory responses (Beinke & Ley, 2004). Improper regulation of NF- $\kappa$ B can lead to the constitutive overproduction of pro-inflammatory

cytokines such as TNF- $\alpha$  and IL-1, which are associated with a number of chronic inflammatory disorders, including rheumatoid arthritis and Crohn's disease (Feldmann & Maini, 2001; Girardin *et al*, 2003).



**Figure 1.9 Activation of NF- $\kappa$ B by the classic/canonical and atypical/alternative pathways:** The canonical NF- $\kappa$ B pathway (left-hand panel) and the non-canonical NF- $\kappa$ B pathway (right-hand panel). Both pathways are activated by a variety of signals. The binding of ligands to the receptors lead to the recruitment and activation of many proteins resulting in processing and liberation of p50/RelA and p52/RelB from the canonical and non-canonical pathways respectively. These heterodimers subsequently translocate to the nucleus to activate target genes by binding to DNA sequence  $\kappa$ B site.

<http://www.abcam.com/index.html?pageconfig=resource&rid=11255&pid=10629>

### 1.9.2.3 The relationship between NF- $\kappa$ B and HIF in non-macrophage cells

As stated above, NF- $\kappa$ B activation up-regulates genes involved in normal cell functions, such as cell proliferation and survival, migration, differentiation, and apoptosis, as well as in inflammation and immune responses (Vallabhapurapu & Karin, 2009). It was found that NF- $\kappa$ B is constitutively activated in many diseased tissues including several types of tumours such as prostate cancer (Suh & Rabson, 2004; Rayet & Gelinas, 1999).

The inflammation degree and the types of inflammatory/immune cells present in inflamed or tumour tissues impact on their progression and regression (Battaglia *et al*, 2008). Several studies have shown the activation of classical and/or alternative NF- $\kappa$ B signaling plays important roles in bone differentiation and maintenance (Boyce *et al*). Franzoso and colleagues reported (Franzoso *et al*, 1997) that NF- $\kappa$ B knockout in mice caused failure of tooth eruption.

Many studies have shown the important role for the NF- $\kappa$ B in the regulation of cardiac myocyte survival through repression of apoptotic cell death triggered by hypoxia or ischaemic myocardial injury (Mustapha *et al*, 2000). For example, transgenic mice harbouring a cardiac-specific expression of a NF- $\kappa$ B inhibitor (I $\kappa$ B $\alpha$ ) display a 50% greater infarct tissue size with significantly higher levels of post-infarct apoptosis (Misra *et al*, 2003). These studies strongly suggest a protective role for NF- $\kappa$ B during pathological remodeling of the heart following acute cardiac injury.

Accumulating evidence has shown that inflammation and cancer are linked together in different ways (Karin, 2009). NF- $\kappa$ B was reported to induce several cellular alterations associated with tumorigenesis and invasion of cells (Karin *et al*, 2002). For example, NF- $\kappa$ B was found to promote angiogenesis through inducing VEGF and IL-8 expression (Huang *et al*, 2000). Constitutive NF- $\kappa$ B activation pathways often observed in cancer cells may be promoted by either microenvironment signals, including hypoxia, secreted cytokines, and by reactive oxygen intermediates (ROI), or by genetic alteration (Staudt, 2010; Karin *et al*, 2002).

HIFs and NF- $\kappa$ B have been linked with cell responses to hypoxia and inflammation in various cell types (Schmedtje *et al*, 1997; Zampetaki *et al*, 2004; Matsui *et al*, 1999; Elbarghati *et al*, 2008). There is some evidence to suggest that there is interplay

between these two transcription factors in response to hypoxia or inflammation. Inducible nitric oxide (iNOS) is an enzyme responsible for the production of NO, which has been implicated in many biological functions such as vasodilatation, septic shock and angiogenesis. Mi and colleagues have reported that HIF-1 $\alpha$  and NF- $\kappa$ B binding sites are required for the synergistic expression of iNOS mRNA in murine macrophages stimulated with low concentrations of LPS (1–10 ng/ml) when cultured under hypoxia for 18h compared to normoxic conditions. This study indicated that there is a cooperative interaction between HIF-1 $\alpha$  and NF- $\kappa$ B in the modulation of HIF-1 $\alpha$  transcriptional activity by an inflammatory signal (LPS) that could have important role on macrophage infiltration of tumour tissues (Mi *et al*, 2008).

A study by Bonello and colleagues has identified a functional binding site for NF- $\kappa$ B in the HIF-1 $\alpha$  promoter at (-197/-188 bp). They found that human pulmonary artery smooth muscle cells (PASMC) stimulated by the reactive oxygen species-mediated HIF-1 $\alpha$  induction occurred at the transcriptional level and was dependent on NF- $\kappa$ B (Bonello *et al*, 2007). This suggested that NF- $\kappa$ B can regulate HIF-1 $\alpha$  promoter activity under normoxic conditions. This suggestion was supported by data showing that HIF-1 $\alpha$  promoter luciferase reporter activity induced by H<sub>2</sub>O<sub>2</sub> or thrombin was inhibited by dominant-negative I $\kappa$ B $\alpha$  in PASMC and HEK293 cells (Bonello *et al*, 2007). Another study conducted by Belaiba and colleagues showed that short-term (1h) hypoxia-induced HIF-1 $\alpha$  transcriptional activity was through NF- $\kappa$ B binding site within the HIF-1 $\alpha$  promoter in PASMC cells (Belaiba *et al*, 2007).

However, other studies showed that HIF-1 $\alpha$  might mediate NF- $\kappa$ B activity, and these two transcription factors may both have independent pathways in regulating hypoxia-induced genes expression. For example, transgenic mice with constitutive HIF-1 $\alpha$

expression in basal keratocytes demonstrated HIF-1-mediated NF- $\kappa$ B activation, enhancing production of chemokines and cytokines. HIF-mediated NF- $\kappa$ B activation was mediated by I $\kappa$ B phosphorylation (Scortegagna *et al*, 2008). Furthermore, it was found that activation of NF- $\kappa$ B and IKK $\alpha$  gene transcription in neutrophils exposed to hypoxia was dependent on HIF-1 $\alpha$  (Walmsley *et al*, 2005). All these studies suggest that HIF can mediate NF- $\kappa$ B activation, and also suggest that both HIF and NF- $\kappa$ B may have independent pathways or roles in regulating hypoxia-induced genes.

#### **1.9.2.4 The relationship between NF- $\kappa$ B and HIF in macrophages**

##### **1.9.2.4.1 Non-hypoxic signalling in macrophages**

Several studies have shown that non-hypoxic stimuli are able to stimulate accumulation of HIF-1 $\alpha$  and NF- $\kappa$ B in macrophages (Karin *et al*, 2002). It was reported that LPS does not prolong HIF-1 $\alpha$  protein half-life but induces protein translation and accumulation in the nucleus and that this activation was dependent on ROS activation of the Akt-mTOR-S6 kinase pathway through Toll-Like receptor 4 (TLR4) as seen in differentiated THP-1 cells (Jawahir *et al*, 2008).

VEGF gene expression is mediated by the transcription factor HIF-1 in hypoxia in several cell types including macrophages (Burke *et al*, 2003). However, a study done by Ramanathan and colleagues has shown that VEGF can be induced by non-hypoxic signals, also through the HIF-1 pathway. They showed that HIF-1 $\alpha$  mRNA activation in macrophages is induced by synergistic action of LPS and adenosine A<sub>2A</sub> receptor agonists in non-hypoxic conditions. In addition, they found levels of HIF-1 $\alpha$  protein, HIF-1 $\alpha$  DNA-binding activity and VEGF expression were increased in murine RAW 264.7 macrophage cells treated with LPS and adenosine A<sub>2A</sub> receptor agonist. However, both

HIF-1 $\alpha$  protein and DNA-binding activity was found to be higher in cells cultured under hypoxia than in LPS-treated cells. Deletion of the NF- $\kappa$ B binding site in the VEGF promoter had little effect on its activation by LPS and A<sub>2A</sub> receptor agonists. In addition, using the NF- $\kappa$ B inhibitor (BAY11-7085) had a little effect on LPS and A<sub>2A</sub> receptor agonists induced VEGF expression, suggesting that activation of NF- $\kappa$ B may not play a crucial role in up-regulation of VEGF expression by LPS and A<sub>2A</sub> receptor agonists in those cells (Ramanathan *et al*, 2007).

The link between HIF-1 $\alpha$  and NF- $\kappa$ B related family member signaling has also been investigated using knockout mice. As shown in the study by Rius and colleagues, treatment of murine bone-marrow derived macrophages (BMDM) with Gram-positive group A streptococcus bacteria, was able to activate HIF-1 $\alpha$  and subsequently the HIF-1 target gene COX-2 was expressed in an IKK- $\beta$ -dependent manner, suggesting that HIF-1 $\alpha$  expression was mediated by NF- $\kappa$ B when cells were exposed to these bacteria (Rius *et al*, 2008).

#### **1.9.2.4.2 Hypoxic signalling in macrophages**

The activation of NF- $\kappa$ B in response to hypoxia was documented in several studies (Ryan *et al*, 2005). For example Battaglia and colleagues showed that NF- $\kappa$ B binding activity was increased when monocytes were exposed to short-term hypoxia, and identified NF- $\kappa$ B p50 as the important transcriptional activator for the CCL20 gene (Battaglia *et al*, 2008).

Rius and colleagues reported that short-term hypoxia (4 hrs) induced phosphorylation of the two Inhibitory proteins, Kappa B kinase- $\alpha/\beta$  (IKK $\alpha/\beta$ ) which binds NF- $\kappa$ B, which leads to increase nuclear accumulation of NF- $\kappa$ B and subsequently upregulation of its

target gene HIF-1 $\alpha$  in RAW264.7 cells. Hypoxia also induced the nuclear translocation of RelA, which preceded HIF-1 $\alpha$  accumulation in these cells. In IKK $\beta$ -deficient macrophages, HIF-1 $\alpha$  protein expression was moderately down-regulated when cells were exposed to hypoxia or the hypoxia-mimetic desferrioxamine (DFX). The expression of hypoxia inducible genes dependent on HIF-1 $\alpha$  such as those encoding vascular endothelial growth factor (VEGF) and GLUT-1, was nearly abolished in IKK- $\beta$ -deficient macrophages, which indicate that NF- $\kappa$ B controls HIF-1 $\alpha$  mRNA expression, thereby serving as a regulator of the hypoxic response. The induction and regulation of HIF-1 $\alpha$  by NF- $\kappa$ B is essential for cell survival to provide the cells and tissues exposed to hypoxia with sufficient energy supplies and allow them to resist cell death (Rius *et al*, 2008).

The signalling pathway of transcription factors provides cells the ability to sense and respond to hypoxia. A number of studies have shown that the adaptive responses of cells to hypoxia are mediated by the activation of specific genes controlled mainly by the hypoxia-inducible transcription factors HIF-1 and HIF-2 (Semenza, 1998). Tumour-associated macrophages (TAMs) are present in large numbers in most types of human tumour and originate mainly from circulating monocytes rather than tissue macrophages (Yamashiro *et al.*, 1994). These cells regulate processes of tumour biology such as angiogenesis, glucose/energy metabolism, proliferation, apoptosis and metastasis (Harris, 2002). In addition to HIF-1 $\alpha$  and HIF-2 $\alpha$ , macrophages can also up-regulate other transcription factors in response to hypoxia (Elbarghati *et al*, 2008) such as HIF-3 $\alpha$  (Heidbreder *et al*, 2003), activating transcription factor-4 (ATF-4) (Ameri *et al*, 2004), early growth response-1 (Egr-1) (Yan *et al*, 1999), nuclear factor  $\kappa$ B (NF $\kappa$ B) (Koong *et al*, 1994), CCAAT/ enhancer binding protein b (C/EBPb) (Hehlhans *et al*, 2001;

Yan *et al*, 1995) and erythroblastosis virus E26 oncogene homolog 1 (Ets-1) (Oikawa *et al*, 2001).

In the last few years, the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) has emerged as a central regulator of TAM function (Hoffmann *et al*, 2003) and reviewed by (Hagemann *et al*, 2009). NF- $\kappa$ B activation is critical for macrophage responses to microbial and inflammatory stimuli, including LPS, TNF- $\alpha$ , and IL-1 $\beta$  (Mantovani *et al*, 2004; Kawai & Akira, 2007). However, to reach maximal expression, many inflammatory cytokine genes require the activation of a number of transcription factors (Ozato *et al*, 2002). For example, gene such as NOS2, and CXCL10 require of signal transducer and activator of transcription (STATs) 1 and 2, interferon response factors (IRF1, 3, 5 7), AP-1, and NF- $\kappa$ B for their transcription (Colonna, 2007; Ohmori & Hamilton, 2001; Taniguchi *et al*, 2001).

Several studies have shown that the activation of NF- $\kappa$ B in macrophages is induced by hypoxia (Fang *et al*, 2009) and promotes tumour development in many inflammation-induced cancer models (Florian *et al*, 2004; Eli *et al*, 2004). It is thought that, during chronic inflammation, NF- $\kappa$ B activation in inflammatory macrophages results in the release of cytokines, such as TNF- $\alpha$  and IL-6 (Maeda *et al*, 2005; Lawrence *et al*, 2007) which trigger pro-survival signals by expressing anti-apoptotic genes, such as Bcl-XL and GADD45 $\beta$  in tumour cells (through NF- $\kappa$ B or STAT3 activation in these cells) and also support their growth and progression (Karin & Greten, 2005; Balkwill *et al*, 2005; Lin & Karin, 2007). Fang and colleagues showed that exposure to hypoxia for 18 hours activates a distinct set of transcription factors including HIFs-1 and -2, STAT4, STAT6 and NF- $\kappa$ B which markedly up-regulates a broad array of tumour-promoting genes in primary macrophages, such as VEGF, CXCL8, Glut-1, and ADM (Fang *et al*, 2009).

## 1.10 The effect of LPS on macrophage gene expression

LPS is the most potent and well characterised immune system-activating molecule from Gram-negative bacteria. The major cell surface receptor for LPS on macrophages is CD14, and the high-affinity binding of LPS to CD14 is greatly enhanced by the presence of the serum protein LPS-binding protein (LBP) (Schumann, 2011). The LPS / LBP complex interacts with CD14 together with Toll-like receptor (TLR) molecules to activate intracellular signal transduction pathways (Heumann & Roger, 2002). These signals in turn activate transcription factors, including NF- $\kappa$ B (Muller *et al*, 1993). As a result, LPS triggers a wide variety of cellular responses, including the production of cytokines and chemokines, release of arachidonic acid metabolites (such as prostaglandin E2), and generation of reactive oxygen and nitrogen intermediates that are responsible for pathophysiologic reactions (Bone *et al*, 1997). Macrophages play an essential role in infection and inflammation by producing these mediators.

Several intracellular signaling pathways have been discovered, and these pathways are activated as a result of LPS binding to TLRs. Activation of NF- $\kappa$ B is the most rapidly activated pathway and starts with activation of a cytosolic adapter protein called myeloid differentiation primary response gene 88 (MyD88) (Kawai *et al*, 1999). NF- $\kappa$ B protein is constrained in the cytoplasm by binding with inhibitory NF- $\kappa$ B molecules (I $\kappa$ Bs). To release NF- $\kappa$ B freely from I $\kappa$ Bs, I $\kappa$ Bs needs to be phosphorylated and degraded. The phosphorylation of I $\kappa$ Bs requires multiple molecules that play a role in LPS signaling in monocytes and macrophages, including interleukin-1 receptor-associated kinase 1 (IRAK), and TNF receptor-associated factor 6 (TRAF6) (Zhang *et al*, 1999). The two catalytic kinases responsible for phosphorylating I $\kappa$ Bs are known as the

I $\kappa$ B kinases, IKK $\alpha$  and IKK $\beta$ . The phosphorylation of I $\kappa$ Bs leads to their proteolytic breakdown, resulting in release of NF- $\kappa$ B which then translocates to the nucleus, binds DNA and activate gene transcription (May & Ghosh, 1999).

There are many other activators involved in LPS signaling. LPS stimulation of monocytes/macrophages induces the tyrosine phosphorylation of proteins, including protein kinase C and the src tyrosine kinase family members. These and other signalling molecules mediate activation of various mitogen-activated protein kinase (MAPK) pathways, including the ERK, JNK, and p38 pathways (Shapira *et al*, 1994). These pathways directly or indirectly phosphorylate and activate various transcription factors, including Elk-1 (member of ETS transcription factors), serum response factor (SRF), and cAMP responsive element binding protein (CREB), c-Jun (AP1, activator protein 1), c-Fos (member AP1, activator protein 1), activating transcription factor 1 and 2 (ATF-1 and -2) (Karin *et al*, 1997). These transcription factors mediate induction of numerous inflammatory cytokines and chemokines, including IL-1, IL-8, and TNF $\alpha$ .

Roach and colleagues performed a comprehensive study of transcription factors in human monocytes stimulated with 100 ng/ml of LPS, to enable better understanding of macrophage gene regulation after exposure to LPS (Roach *et al*, 2007). They assayed transcription factor expression with two independent technologies; massively parallel signature sequencing MPSS and microarray Gene-Chips. The data showed that 92 transcription factors (TFs) were significantly induced. About 49 of these TFs were known previously to be involved in the LPS response. The most significantly regulated transcription factors elucidated were AHR, BCL3, ETS2, MAFB, MAFF, MTF1, REL, ETV3, IRF4, IRF8, JUNB, KLF16, MAZ, MYBL2, and THRA. An interesting finding of this study

was that most TF expression levels were changed within 2 hours of LPS stimulation (Roach *et al*, 2007).

The regulation of many transcription factors by LPS subsequently leads to induction of a huge array of genes. Suzuki and colleagues analysed the abundance of a large number of transcripts in human LPS-stimulated monocytes (Suzuki *et al*, 2000). The study determined that many cytokine and chemokine genes were up-regulated. As expected, the well-known pro-inflammatory cytokines IL-1, IL-6, and TNF, were expressed at higher levels in LPS-stimulated monocytes than resting cells, confirming that these cytokines play a central role in the initiation of immune responses. The study also showed significant regulation of CC chemokines, including MIP-1b, LARC, MIP-1a, TARC, MDC, RANTES, monocyte chemoattractant protein-1 (MCP-1), and MCP-2. These CC chemokines are believed to attract monocytes, basophils, eosinophils, and lymphocytes to the infected sites. CXC chemokines were also up-regulated by LPS stimulus, including MIP-2 $\beta$ , MIP-2 $\alpha$ , and IL-8. These CXC chemokines belong to the subgroup called ELR (glutamate-leucine-arginine) chemokines. These ELR CXC chemokines have similar functions such as neutrophil chemoattraction and activation, endothelial cell chemoattraction, and angiogenesis, which indicate that monocytes are involved in inflammatory angiogenesis. Other known inflammation-related transcripts such as MMP-9 and inducible cyclooxygenase (COX2) were increased on LPS stimulation. These data suggest that the proteinase MMP-9 may be involved in local remodelling and repair, and the enzyme COX2 may play a critical role in inflammation and modulation of the systemic reaction.

### **1.11 Previous microarray studies on hypoxic macrophage gene expression**

Over the last ten years, gene expression profiling using microarray technologies became a powerful approach to understand complex biological systems and the pathogenesis of diseases (Russo *et al*, 2003). This technique has achieved a robust analytical performance, enabling its use for analysing the whole transcriptome for screening thousands of genes simultaneously in a single experiment (Murphy, 2002). In the field of hypoxia research, a number of genome-wide and non-genome wide profiling studies have been published. The following section will discuss their findings.

Burke and colleagues used nylon arrays representing 1185 genes to examine mRNA levels. Two microarray experiments were conducted using RNA samples obtained from two different donors in different occasions. They reported that primary human macrophages responded to 16hrs of hypoxia (0.5 % O<sub>2</sub>) by up-regulating the mRNAs of a number of genes (Burke *et al*, 2003). Two of up-regulated genes, Glut-1 and VEGF, were previously known to be hypoxia-inducible genes. This study showed for the first time three novel genes up-regulated in human macrophages; MMP-7, neuromedin B receptor (NMBR), and DNA-binding protein inhibitor (Id2) with 9, 5.8, and 3.3 fold change respectively. In addition, several genes were found to shown down-regulated, including IL-1 $\beta$ , RAB7 (a member of the RAS oncogene family), and cathepsin C. The real-time RT-PCR data in this study does not exactly match the array data. For example, MMP-7 showed less fold induction by RT-PCR compared with array data (RT-PCR=6 fold increase) however, RT-PCR of Glut-1 is found to be higher than array data (RT-PCR=17 fold increase). Another microarray study, studied the responses of murine macrophage-like RAW 264.7 cells to hypoxia (Fong *et al*, 2007). Similar to Burke's

work, they used a microarray with a limited number of genes (1000 mouse genes) involved in apoptosis and survival, cell cycle, stress responses, metabolism and the structural matrix. The study showed twenty-six genes were differentially expressed in RAW 264.7 cells when exposed to hypoxia (1 % O<sub>2</sub>) for 24 hours. Five genes were confirmed by real time PCR to validate the microarray data. The PCR data showed some variability but the pattern of all five genes remained consistent with the array data. Immediate early response 3 (IER3) is one of eight genes found to be up-regulated in microarray data and this gene is involved in the NF-kappa B activation pathway. Acute hypoxia also up-regulated metallothioneins MT-I and MT-II, which were believed to play a protective role by suppressing apoptosis. Eighteen genes were down-regulated by hypoxia, and several of these were involved in apoptosis and cell growth arrest. Cathepsin C and L are proteases and may be involved in apoptosis and cell damage. In addition, Cdc42, Cyclin B, Cdk4 were down-regulated in RAW264.7 macrophages and these genes associated with many macrophages functions such as cell growth arrest (Fong *et al*, 2007).

Gene expression profiling has demonstrated its utility in increasing understanding of transcriptomic changes in mammalian cells. White and colleagues used Affymetrix Human Genome U133A arrays to elucidate the effect of 16 hrs of hypoxia (0.1 % O<sub>2</sub>) on mRNA levels in human macrophages. This array contained 18,400 transcripts which include 14,500 well-characterized human genes including known angiogenic factors (White *et al*, 2004). The data have shown a comprehensive understanding of the proangiogenic phenotype of the hypoxic macrophage. The overexpression of HIF-2 $\alpha$  in normoxic human macrophages up-regulates various pro-angiogenic genes including VEGF, bFGF, IL-8 (CXCL8), COX2, HGF, VEGFR1, tissue factor (F3) and MMP12 (White *et*

*al*, 2004). This data is consistent with the previous work suggested that the HIF-2 $\alpha$  is expressed at higher level than HIF-1 $\alpha$  in macrophages (Griffiths *et al*, 2000). In contrast, other investigators have shown that human macrophages also markedly up-regulate HIF-1 $\alpha$  when exposed to hypoxia *in vitro* and in tumours suggesting a more important role of HIF-1 $\alpha$  than HIF-2 $\alpha$  (Burke *et al*, 2002).

Bosco and colleagues used the same array type used by White *et al*. to elucidate the transcriptome of human macrophages exposed to 1 % O<sub>2</sub> hypoxia for 16 hour. RNA isolated from 15 donors was randomly pooled into three subsets, each subset composed of RNA from five donors, and these subsets were used for three independent microarray experiments (Bosco *et al*, 2006). Microarray data analysis showed different functions of monocytes when exposed to acute hypoxia. It showed the induction of genes involved in cell growth or maintenance, signal transduction, and metabolism. The majority of up-regulated genes found in Bosco data were also regulated by hypoxia in previous studies. These genes related to angiogenesis, cell adhesion, transcription, and inflammatory response such as adrenomedullin (ADM), arginase-1 (ARG1), IL-1 $\alpha$  (IL-1A), TNF- $\alpha$ , matrix metalloproteinase-1 (MMP-1), and vascular endothelial growth factor, VEGF. Other hypoxic genes found in previous studies in cell types other than mononuclear phagocytes were also found to be up-regulated by hypoxia in monocytes in the Bosco study, such as glucose transporter 1 and 3 (GLUT-1, and 3), carbonic anhydrase XII (CA12), and DNA-damage inducible transcript-4 (DDIT4).

Many novel genes modulated in different directions by hypoxia in monocytes were presented in the above study. Some of the up-regulated genes are involved in lipid metabolism and transport, such as apolipoprotein B48 receptor, and fatty acid binding

protein 4 (FABP4). Hypoxia also up-regulated genes which are believed to play roles in the cytoskeleton and adhesion such as Galectins 8 (LGALS8), MMP-16, and tensin 1 (TNS1). Furthermore, the array data revealed a group of transcription regulatory proteins including activating transcription factor (ATF-2 and -5), Fos homology B (FOSB) and Fos-like Ag 2 (FRA2). However, the most prominent responses of monocytes in hypoxia in the above study are the immunological responses. These include genes encoding surface immune-regulatory signalling receptors such as early activation Ag (CD69), and triggering receptor expressed on myeloid cells 1 (TREM1). Another group of genes encoding cytokines/chemokines and their receptors including MIP-2 (CXCL2), MIP-2 $\beta$  (CXCL3), and ENA-78 (CXCL5), were also up-regulated by hypoxia. However, the novelty in this study was elucidating for the first time that MIP-3 $\alpha$  (CCL20) is up-regulated by hypoxia in primary human monocytes. MIP-3 $\alpha$  is an LPS-inducible chemokine, however this study revealed that the induction of this gene in monocytes by acute hypoxia is more than by LPS as showed by ELISA and immunocytochemical analysis (Bosco *et al*, 2006). Later, a study by Battaglia and colleagues showed that MIP-3 $\alpha$  is up-regulated by hypoxia in monocytes through activation of the NF- $\kappa$ B pathway (Battaglia *et al*, 2008).

Oxygen sensing and adaptations to hypoxia in mammalian cells are mainly mediated by the activation of specific genes through the actions of HIF-1 (Semenza, 2009) or HIF-2 (Wang *et al*, 2005). HIF-1 $\alpha$  and HIF-2 $\alpha$  have similarity in their amino acid sequences, and several studies have shown that HIF-1 and HIF-2 transcription factors can regulate a number of the same genes (Ema *et al*, 1997).

Previous reports have shown significant up-regulation of HIF-1 $\alpha$  and HIF-2 $\alpha$  proteins which indicate the role of these two transcription factors in macrophages exposed to

hypoxia (Burke *et al*, 2002; Fang *et al*, 2009). By using siRNA transfection, Fang and colleagues showed that both HIFs play a part in regulating the hypoxic induction of a number of known HIF target genes, VEGFA, GLUT1, CD184 (CXCR4), IL-8, and ADM, by MDMs. Furthermore, hypoxic induction of these genes was reduced but not lost in HIF-1 $\alpha$ -deficient murine macrophages at the mRNA and protein levels. In the Fang study, microarray technology was also used with two different platforms. The first one was the Affymetrix Human Genome U133A plus 2.0 gene chip array that detects around 20,000 human genes represented by a total of 47,000 transcripts on the gene chip. The second array type was the Sentrix HumanRef-8\_V2 Bead chip from Illumina which screens more than 22,000 transcripts. Both arrays were hybridized using RNA obtained from primary human macrophages incubated under hypoxia (0.5% O<sub>2</sub>) for 18 hours. Microarray data analysis of both arrays showed that hypoxia markedly up-regulated a broad array of chemotaxin genes which promote attraction of effector cells including monocytes (Fang *et al*, 2009), for example, genes which enhance trafficking of leukocytes and monocytes to hypoxic tissues such as IL-8, endothelin 1 (EDN1), MIP-3 (chemokine C-C motif ligand 20) (CCL20), colony-stimulating factor 2 (GM-CSF), chemokine (C-X-C motif) 1 and 2 (CXCL1/2), MIP-2 $\beta$  (chemokine (C-C motif) 3) and ENA-78 (CCL5). Many genes which promote angiogenesis in hypoxic tissues were also up-regulated by hypoxia in MDMs such as vascular endothelial growth factor A (VEGF), angiopoietin 2 (Ang-2), and adrenomedullin (ADM) (Fang *et al*, 2009). Microarray data showed some enzymes involved in metabolism were up-regulated in macrophages to maintain viability and survival such as enolase 2 (ENO2), neuroregulin 1 (NRG1), aldolase C, fructose-bisphosphate (ALDOC), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), and glucose transporters 1, 3, 5, and 6 (Glut-1,3,5, and 6).

This study showed for the first time that genes encoding two important transcription factors, STATs (signal transducers and activators of transcription) 4 and 6, were up-regulated by hypoxia in macrophages (Fang *et al*, 2009).

Gene expression in mammalian cells is a very complicated process and its regulation is highly controlled. This regulation can be at the transcriptional level or at the posttranslational level by changes in mRNA stability (Hollams *et al*, 2002; Abdelmohsen *et al*, 2007). Regulation of mRNA stability and degradation process is controlled by interactions between mRNA transcripts and endogenous proteins (Fabian *et al*).

Eliminating the stabilized mRNA in gene expression profiling is a useful approach to give a picture of transcribed mRNA in the nucleus before transfer to the cytoplasm, therefore Igwe and colleagues used a nuclear run-on assay (NRO) method to isolate RNA for their microarray study (Igwe *et al*, 2009). The difference between RNA isolated by the nuclear run-on assay versus most other methods is that the nuclear run-on assay provides a measure of the frequency of transcription and is largely independent of the effects of RNA stability. In other words, NRO is designed to look at the genes being transcribed in a cell nucleus at a specific time (Smale, 2009). The intention of the Igwe study was to identify hypoxia and/or nitric oxide-regulated genes in the mouse leukaemic monocyte macrophage cell line (RAW 264.7) after a short period (6h) of hypoxia. Utilizing the microarray technique, GeneChip mouse Genome arrays from Affymetrix were used, which represented 32,000 transcripts. The data showed that RAW 264.7 cells respond to hypoxia or nitric oxide (NO) or by combined treatment (hypoxia plus NO) by up-regulating 196, 85, and 292 genes respectively. However, these numbers in all conditions comprise just 1% of the overall numbers of transcripts in the array chip. The majority of genes were found induced by hypoxia comparing

with those regulated by NO suggesting that the effect of hypoxia is more potent on cells than NO, and this might be due to the activation of many transcription factors under hypoxia involved in gene expression (Cummins & Taylor, 2005). Only 14 genes (BNIP3, DDIT4, VEGF, TRIB3, ATF3, CDKN1A, SCD1, D4ERTD765E, SESN2, SON, NNT, LST1, HPS6, and FXD5) were common to all treatments and only two genes, DDIT4 and TRIB3, showed synergy between hypoxia and NO with 35.21 and 27.11 fold changes respectively. This suggests cross-talk between hypoxia and NO in RAW 264.7 cells (Igwe *et al*, 2009).

LPS is a potent inflammatory factor that has been implicated in the pathogenesis of septic shock (Li *et al*, 2011). LPS has been reported to induce HIF-1 $\alpha$  protein accumulation under nonhypoxic conditions in monocytes and macrophages (Blouin *et al*, 2004). Recently it was reported that in the mouse macrophage cell line RAW264.7, LPS-induced activation was enhanced by hypoxia, resulting in increased TNF- $\alpha$  secretion (Liu *et al*, 2008).

Liu and colleagues showed that increased TNF- $\alpha$  production under hypoxia is increased in the presence of LPS. This increase is mediated through enhanced expression of p38 MAPK activity, through the modulation of its substrate MK2 and HIF-1 $\alpha$ . HIF-1 $\alpha$  predominantly contributed to the up-regulation of TNF- $\alpha$ , especially *in vivo*, and is independent of p38 MAPK signalling. Moreover, the p38 MAPK inhibitor SB 203580 failed to reduce the activity of p38 MAPK under hypoxic conditions *in vivo*. The mechanisms underlying this effect may involve altered p38 activity or the expression of other factors that regulate TNF- $\alpha$  expression independent of p38 (Liu *et al*, 2008).

## 1.12 Co-localisation of hypoxia, LPS and macrophages in pathological / injured tissues

The skin is the largest organ and its primary function is to serve as a protective barrier against infection. After injury the skin needs to be repaired to maintain its function. The healing process of the injured tissues is an extremely complex process involving numerous cell types as well as growth factors, cytokines, and extracellular matrix (ECM) components (Breitkreutz *et al*, 2009).

The tissues contain several immune cells that can be activated by invading pathogens or skin damage. One of the most important immune cells involved in wound healing is the macrophage, which exhibits different immunological functions in the injured tissues, including phagocytosis and antigen-presentation. Furthermore, macrophages produce many cytokines and chemokines that stimulate new capillary growth, collagen synthesis and fibrosis (Mirza *et al*, 2009). Macrophages are thought to orchestrate the wound healing process throughout the different phases (Lucas *et al*, 2010). In any infected or injured tissues which are characterized by hypoxia, macrophages consist of two populations. The first is the 'resident' tissue macrophage that is present in tissues at all times. The other major population is monocytes, newly recruited from the bloodstream. Macrophages in wounds can display different functional phenotypes, and can be divided into two groups: M1 (classically activated) and M2 (alternatively activated) macrophages (Mantovani *et al*, 2002). There are several mediators that can stimulate macrophages to differentiate into M1 macrophages, the most important being bacterial products like lipopolysaccharide (LPS), and also interferon (IFN)- $\gamma$  (Mosser & Edwards, 2008; Gordon, 2003). M1 macrophages exhibit antimicrobial properties by release of inflammatory mediators such as TNF- $\alpha$ , NO and IL-6. In

contrast, macrophages activated by IL-4 and IL-13 develop into alternatively activated (M2) macrophages (Gordon, 2003), which suppress inflammatory reactions and adaptive immune responses. M2 macrophages have been reported to play an important role in wound healing, angiogenesis and defence against parasitic infections (Gordon, 2003).

Wound healing is a dynamic process consisting of several phases including inflammation, granulation tissue formation, and re-epithelialization. The inflammation phase initiated by the release of chemoattractants that attract phagocytic immune cells. Recruitment of immune cells is facilitated by chemokines, vasodilation and increase in blood vessel permeability by histamine. The first cells that infiltrate the wound are neutrophils, which remove foreign particles and bacteria from the wound area (Dovi *et al*, 2003). Neutrophils are a major source of several pro-inflammatory cytokines, such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , which can stimulate newly attracted monocytes to differentiate into M1 macrophages (Hubner *et al*, 1996; Werner & Grose, 2003).

The second type of leukocyte to accumulate at the site of injury is the monocyte. Monocytes migrate through the vessel wall in response to chemotactic stimuli using its surface integrins that interact with other receptors, like inter-cellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), expressed by endothelial cells in order to facilitate movement (Imhof & Aurrand-Lions, 2004). Monocytes migrate along defined chemotactic gradients and accumulate within hypoxic areas of the wound where they differentiate into macrophages and restore the altered blood supply by enhancing blood vessel formation through production of (VEGF, ANGPT-1, ANGPT-2, ANGPT-4, FGF-2, PGF, PDGF- $\beta$ ) (Crowther *et al*, 2001).

Macrophages are important in clearance of damaged cells and debris within the wound. In case of pathogens spreading in the wound bed, macrophages phagocytose these pathogens and present antigens to T-cells in order to initiate adaptive immune response. Macrophages involved in clearance of cells or dead tissue undergo apoptosis. Macrophages that survive and do not undergo apoptosis remain in the wound area and exert other functions that influence the wound healing process, like stimulation of collagen production, angiogenesis and re-epithelialisation (Baum & Arpey, 2005).

The second phase in wound healing is the proliferative phase, also called the granulation phase, and is characterized by active fibroplasia, epithelial regeneration, wound contraction and angiogenic sprouting, which are regulated mainly by macrophages (Lucas *et al*, 2010).

The third phase of wound healing is re-epithelialization includes the re-establishment of skin over the newly formed tissue. After injury, in the skin for example, keratinocyte organization is disturbed and has to be restored. This restoration process initiates within hours after injury and is performed by keratinocytes that move across the granulation tissue. Transforming growth factor beta 1 (TGF- $\beta$ 1) is one of the most important ligands for epithelial cell migration during re-epithelialisation (O'Kane & Ferguson, 1997). Furthermore, various hypoxia-inducible genes regulated through HIF-1, such as MMPs, play a crucial role in this process. These MMPs are upregulated by keratinocytes and macrophages in the wounded tissues under hypoxic conditions. Degradation of ECM components by MMPs is required to remove and reorganize provisional matrices and to allow migration of effector cells (Ravanti & Kahari, 2000).

It was found that NF- $\kappa$ B is constitutively activated in many diseased tissues including several types of tumours such as prostate cancer (Suh & Rabson, 2004; Rayet & Gelinas, 1999). Many studies have shown an important role for NF- $\kappa$ B in the regulation of cardiac myocyte survival through repression of apoptotic cell death triggered by hypoxia or ischaemic myocardial injury (Mustapha *et al*, 2000). In addition to hypoxia, NF- $\kappa$ B can be activated by bacterial products which, associated with chronic inflammation can lead to tumourigenesis. For example, in a mouse model of colitis-associated cancer (CAC), deletion of IKK- $\beta$  (leading to decreased NF- $\kappa$ B activity) in enterocytes showed that the tumour-promoting activity of NF- $\kappa$ B results from its ability to suppress the apoptosis of malignant cells. In this model, mice are injected with the procarcinogen azoxymethane (AOM), which undergoes metabolic activation in enterocytes, and this is followed by oral administration of dextran-sulphate sodium salt (DSS), which induces chronic colitis through disruption of the intestinal barrier and exposure of macrophages in the lamina propria to enteric bacteria. The exposure of macrophages to bacteria results in the activation of NF- $\kappa$ B in these cells through TLR signalling, leading to the production and secretion of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1, IL-6, and IL-8 that also activate NF- $\kappa$ B in intestinal epithelial cells (Greten *et al*, 2004).

### **1.13 Conclusion**

Macrophages are highly versatile cells residing in all tissues of the body. They change their functions depending on their location and activation status, and exhibit tissue-specific phenotypes. They are major players in the inflammatory response and secrete pro-inflammatory and antimicrobial mediators. These cells are recruited and retained

in hypoxic sites including tumours, atherosclerotic plaques, wounds, and arthritic joints. When macrophages are recruited to pathologic sites, they rapidly respond by activation of a diverse array of transcription factors including HIF-1/2 and NF- $\kappa$ B and their downstream target genes.

Hypoxia and bacterial pathogens can be present together in some pathological sites. For example, deep injuries are characterised by hypoxia (Rodero & Khosrotehrani, 2010) due to the damage to blood vessels (Lewis *et al*, 1999), and are also likely to be contaminated with microorganisms. Bacteria also promote hypoxic conditions via their high consumption of oxygen during rapid cell division. Therefore, revealing the transcriptomic picture in macrophages exposed to hypoxia and LPS is important in order to expand our understanding of macrophage behavior.

Several studies have shown that LPS treated macrophages up-regulate known hypoxia-inducible genes such as VEGF, Glut-1, iNOS and EPO through regulation of HIF-1 (Blouin *et al*, 2004; Frede *et al*, 2006). In contrast to hypoxia, which is believed not to up-regulate HIF-1 $\alpha$  mRNA, LPS has been found to stimulate HIF-1 $\alpha$  mRNA expression at the transcriptional level under normoxia in macrophages through a NF- $\kappa$ B site in the promoter of the HIF-1 $\alpha$  gene (Blouin *et al*, 2004).

### **1.14 Aim of this study**

In deep injuries, two stimuli, hypoxia and lipopolysaccharide, could be present. This hypoxic microenvironment could be due in part to altered blood supply owing to blood vessel damage (Lewis & Murdoch, 2005). The accumulation of leukocytes including macrophages also contributes to lower oxygen level due to oxygen consumption by these high numbers of cells (Murdoch *et al*, 2004). Deep injuries are usually

contaminated with bacteria because these injuries are open. Microorganisms can rapidly multiply and proliferate in such sites, which also enhances the hypoxic conditions due to their high oxygen consumption in these infected tissues. Given that hypoxia and bacterial contamination often co-exist, my hypothesis is that there is likely to be synergy between hypoxia and LPS in controlling macrophage gene expression.

As mentioned above in section (1.11), several studies have investigated the effect of hypoxia on gene expression of monocyte/macrophage cells for a short period of time using microarray technology (Bosco *et al*, 2006; Fang *et al*, 2009; Burke *et al*, 2003; White *et al*, 2004). However, no study to date has tried to determine the transcriptomic profile of primary human macrophages when exposed to acute hypoxia in the presence of lipopolysaccharide (LPS). Therefore, the aim of this project was to determine the gene expression profile of primary human macrophages exposed to hypoxia in the presence or absence of LPS. Particular focus was on the potential synergistic effects of combined treatment by hypoxia and LPS on macrophage gene expression.

## CHAPTER 2: Materials

### 2.1 Reagents

#### 2.1.1 Cell culture

Reagent	Catalogue number	Company
Cobalt chloride (CoCl <sub>2</sub> )	C-8661	Sigma-Aldrich, Dorset, UK
Desferrioxamine (DFO)	D-9533	Sigma-Aldrich, Dorset, UK
Dimethyl sulphoxide (DMSO)	D-5879	Sigma-Aldrich, Dorset, UK
Foetal Bovine Serum (FBS)	S0115	BiochRom AG, Berlin, Germany
Ficoll-Paque™ Plus	17-1440-03	GE healthcare, Buckinghamshire, UK
Hank's Balanced Salt Solution	H-6648	Sigma-Aldrich, Dorset, UK
Heparin (5000 U/ml)	Kindly provided by Glenfield Hospital, Leicester, UK	Leo laboratories limited, UK
Human AB serum	S4190-100	BioSera, Ringmer, UK
Iscove's Modified Dulbecco's medium	13390	Sigma-Aldrich, Dorset, UK
L-glutamine 200 mM	G-7513	Sigma-Aldrich, Dorset, UK
LY294002 (PI3 kinase inhibitor)	9901	New England Biolabs, Hitchin, UK
Non-essential amino acids (NEAA) 100X	M-7145	Sigma-Aldrich, Dorset, UK
Penicillin-Streptomycin 100 mM	P-0781	Sigma-Aldrich, Dorset, UK

Salmonella abortus equii lipopolysaccharide (SAE LPS)	581-009-L002	Alexis, San Diego, USA
Trypan blue	6146	Sigma-Aldrich, Dorset, UK
VLE RPMI 1640 medium (Very Low Endotoxin)	F1415	BioChrom AG, Berlin, Germany
Wortmannin	681675	EMD Biosciences, Darmstadt, Germany

**Table 2.1:** List of reagents used in the cell culture.

### 2.1.2 Reverse transcription and Real-Time PCR

Reagent	Catalogue number	Company
AMV Reverse Transcriptase	M510A	Promega Corporation Southampton, UK
Chloroform	C-2432	Sigma-Aldrich, Dorset, UK
Deoxyadenosine triphosphate (dATP)	U120D	Promega Corporation, Southampton, UK
Deoxycytidine triphosphate (dCTP)	U122D	Promega Corporation Southampton, UK
Thymidine triphosphate (dTTP)	U123D	Promega Corporation Southampton, UK
Deoxyguanosine triphosphate (dGTP)	U121D	Promega Corporation Southampton, UK
Diethyl pyrocarbonate (DEPC)	D-5758	Sigma-Aldrich, Dorset, UK
Ethanol	N/A	Chemistry department, Leicester University, UK
Random Hexanucleotides	N/A	Eurofins MWG Operon, Ebersberg, Germany

Isopropanol	P/7500/15	Fisher, Loughborough, UK
RNasin® Ribonuclease Inhibitor	N251B	Promega Corporation Southampton, UK
SYBR® Green JumpStart™ Taq ReadyMix™	S-1816	Sigma-Aldrich, Dorset, UK
TRI reagent	T-9424	Sigma-Aldrich, Dorset, UK

**Table 2.2:** List of reagents used in RNA isolation, reverse transcription and RT-PCR.

### 2.1.3 Molecular cloning

Reagent	Catalogue number	Company
Agarose	A5093	Sigma-Aldrich, Dorset, UK
Ampicillin	A9518	Sigma-Aldrich, Dorset, UK
Boric acid	100160	Merck, Darmstadt, Germany
Bovine Serum Albumin (BSA)	A-2153	Sigma-Aldrich, Dorset, UK
Bovine Serum Albumin (BSA) 100X	R396D	Promega Corporation Southampton, UK
DNA molecular weight marker (100 bp ladder)	N0467S	New England Biolabs, Hitchin, UK
DNA molecular weight marker (1 Kb ladder)	N3232S	New England Biolabs, Hitchin, UK
DNA molecular weight marker (HyperLadder™ V)	33031	Bioline, London, UK
Ethidium Bromide (EtBr)	46065	Sigma-Aldrich, Dorset, UK
Pfu DNA polymerase	EP0571	Fermentas, York, UK
REDAccuTaq® LA DNA Polymerase	D-1313	Sigma-Aldrich, Dorset, UK

<i>Sfi</i> I Restriction Enzyme	R0123S	New England BioLabs, Hitchin, UK
T4 DNA ligase	600011	Stratagene, Stockport, UK
Xylene cyanol	X4126	Sigma-Aldrich, Dorset, UK

**Table 2.3:** List of reagents used in general molecular cloning.

### 2.1.4 Western blotting

Reagent	Catalogue number	Company
Acrylamide/bisacrylamide	161-0158	Bio-Rad Laboratories, Hertfordshire, UK
Ammonium persulphate (APS)	A-3678	Sigma-Aldrich, Dorset, UK
Bromophenol blue	8122	Merck, Darmstadt, Germany
ECL advance™ western blotting detection kit	RPN2135	GE healthcare, Buckinghamshire, UK
Glycine	G-8898	Sigma-Aldrich, Dorset, UK
Methanol	M/4000/17	Fisher, Loughborough, UK
Pre-Stained High Molecular Weight Protein Standard	LC5699	Invitrogen Life Technology, Paisley, UK
Sodium Dodecyl Sulphate (SDS)	L-5750	Sigma-Aldrich, Dorset, UK
Tetramethylethylenediamine (TEMED)	T-9281	Sigma-Aldrich, Dorset, UK
Trizma™ (Tris base)	T-1503	Sigma-Aldrich, Dorset, UK
Tween-20	P-5927	Sigma-Aldrich, Dorset, UK

**Table 2.4:** List of reagents used in Western blotting.

### 2.1.5 Reagent Kits

Reagent Kit	Catalogue number	Company
Agilent RNA kits	5067-1548	Agilent , Cheshire, UK
Dual luciferase <sup>®</sup> Reporter Assay System <sup>*</sup>	E1910	Promega Corporation Southampton, UK
EndoFree <sup>™</sup> Plasmid Maxi Kit <sup>**</sup>	12362	QIAGEN, West Sussex, UK
ENDOSAFE cartridge testing kit	PTS20F	Charles River Laboratories, USA
GeneChip Human Gene 1.0 ST Array	901086	Affymetrix, Cleveland, OH, USA
JetPEI <sup>™</sup> DNA transfection reagent	101-40N	Polyplus-transfection Inc, New York, USA
Library Efficiency <sup>®</sup> DH5 $\alpha$ <sup>™</sup> competent cells <sup>***</sup>	18263-012	Invitrogen Life Technology, Paisley, UK
Luciferase assay system <sup>****</sup>	E1501	Promega Corporation Southampton, UK
Pierce 660 nm Protein Assay	22660	Thermo Scientific, Rockford, USA
QIAquick Gel Extraction kit	28704	QIAGEN, West Sussex, UK
RNeasy Mini Kit	74104	QIAGEN, West Sussex, UK

**Table 2.5:** List of reagent kits used in the project.

**NOTE:**

\* The Promega Dual Luciferase<sup>®</sup> Reporter Assay System kit contains 5x Lysis buffer, Luciferase Assay Reagent II and Stop & Glo reagent.

\*\* The Qiagen EndoFree<sup>™</sup> Plasmid Maxi Kit contains TE buffer (10mM Tris-HCl [pH7.5] and 1mM EDTA).

\*\*\* Library Efficiency<sup>®</sup> DH5 $\alpha$ <sup>™</sup> competent cells Kits contains control pUC19 DNA and S.O.C medium.

\*\*\*\* The Promega Luciferase Assay System kit contains 5x Lysis buffer and Luciferase Assay Reagent.

### 2.1.6 Consumables

Material	Catalogue number	Company
0.5 ml Centrifuge tube	311-02-051	Axygen Bioscience, California , USA
1.5 ml Centrifuge tube	311-08-051	Axygen Bioscience, California , USA
15 ml Centrifuge tube	430791	Corning Incorporated, Tampaulipas, Mexico
50 ml Centrifuge tube	21008-178	VWR International, West Chester, USA
5 ml Falcon tube (polystyrene)	352008	Becton Dickinson, New Jersey, USA
14 ml Falcon tube	352059	Becton Dickinson, New Jersey, USA
Hybond-P PVDF membrane	RPN303F	GE healthcare, Buckinghamshire, UK
LightCycler <sup>®</sup> Capillaries (20 µl)	04929292001	Roche Diagnostics, Burgess Hill, UK
MACS MS column	130-042-201	Miltenyi Biotec, Bergisch Gladbach, Germany
Multidish 6-Well Nunclon plates (adherent plates)	S3127XC	NUNC A/S, Roskilde, Denmark
1 ml Syringe	SZR-205-110L	Fisher, Loughborough, UK
20 ml Syringe	SZR-205-110L	Fisher, Loughborough, UK
50 ml Syringe	SZR-205-160T	Fisher, Loughborough, UK
75 cm <sup>2</sup> Tissue culture flasks	658170	Greiner Bio-One, Stonehouse, UK
75 cm <sup>2</sup> Tissue culture flasks filter cap	658175	Greiner Bio-One, Stonehouse, UK
6-Well Costar ultra low	CC227	Appleton Woods,

attachment plates		Birmingham, UK
96-well cell culture cluster	3596	Corning Incorporated, New York, USA

**Table 2.6:** List of materials used in the project.

## 2.2 Media

### 2.2.1 Media for cell culture

Medium	Reagents	Recipe
COMPLETE Iscove's Dulbecco's Modified Medium	Iscove's Dulbecco's Modified Medium 2 mM L-glutamine 200 U/ml Penicillin 200 µg/ml Streptomycin 2.5 % Human AB serum	500 ml Iscove's Dulbecco's Modified Medium 5 ml 200 mM L-glutamine 10 ml 10000 U/ml Penicillin / 10000 µg/ml Streptomycin 12.5 ml Human AB serum store at 4°C
COMPLETE RPMI 1640 medium	VLE RPMI1640 medium 2 mM L-glutamine 200 U/ml Penicillin 200 µg/ml Streptomycin 10x Non-essential amino acids 10 % FBS	500 ml VLE RPMI1640 medium 5 ml 200 mM L-glutamine 10 ml 10000 U/ml Penicillin / 10000 µg/ml Streptomycin 5 ml 100x NEAA the medium was passed through a Gambro U-2000 ultrafiltration column to eliminate potential contaminants such as endotoxin and other

		bacterial components then add 50 ml FBS and store at 4°C
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**Table 2.7:** Media used for cell culture.**2.2.2 Media for bacteria**

Medium	Reagent	Recipe
LB-medium with Ampicillin*	10 g/l NaCl 5 g/l Yeast extract 10 g/l Tryptone 100 µg/ml Ampicillin	10 g Luria broth powder 400 ml dH <sub>2</sub> O autoclave and let cool to 55°C add 400 µl 100 mg/ml Ampicillin store at 4°C
LB-Agar medium with Ampicillin*	10 g/l NaCl 5 g/l Yeast extract 10 g/l Tryptone 15 g/l Agar 100 µg/ml Ampicillin	10 g Luria broth 6 g Agar 400 ml dH <sub>2</sub> O autoclave and let cool to 55°C add 400 µl 100 mg/ml Ampicillin pour into plates store at 4°C

**Table 2.8:** Media used for bacterial culture.

\*LB-medium and LB-agar were prepared by the medium kitchen, Department of Infection, Immunity and Inflammation, University of Leicester

## 2.3 Primers

### 2.3.1 Primers for real-time PCR

Primers	Direction	Sequences
B2m	5` forward	5'-GGCTATCCAGCGTACTCCAAAG-3'
B2m	3` reverse	5'-CAACTTCAATGTCTGGATGGATG-3'
CA12	5` forward	5'-GGAATGGCAGGTTCAAGTTCCAC-3'
CA12	3` reverse	5'-CTCCACTGACAGGAGGTTGGAT-3'
DDIT4	5` forward	5'-CACTGGCTTCCGAGTCATCA-3'
DDIT4	3` reverse	5'-TATTCCCCACCTCCACCTT-3'
Glut-1	5` forward	5'-CAACTGGACCTCAAATTTATTGTGGG-3'
Glut-1	3` reverse	5'-CGGGTGTCTTATCACTTTGGCTGG-3'
HIF1-a	5` forward	5'-AATGCCACCACTACCACTGC-3'
HIF1-a	3` reverse	5'-TAACACGTTAGGGCTTCTTGGA-3'
MMP-7	5` forward	5'-ATGGACTTCCAAAGTGGTCACCTACAG-3'
MMP-7	3` reverse	5'-GGATACATCACTGCATTAGGATCAGAG-3'
TRAIL	5` forward	5'-TCACAGTAGTAGCCTCCAGGTTTCC-3'
TRAIL	3` reverse	5'-AGAGATGGGGTTTCACTATGTTGGTC-3'
VEGF	5` forward	5'-CAGCGCAGCTACTGCCATCCAATCGAGA-3'
VEGF	3` reverse	5'-GCTTGTACATCTGCAAGTACGTTCTTTA-3'
VCAN	5` forward	5'-ACAAGCATCCTGTCTCACG-3'
VCAN	3` reverse	5'-TGAAACCATCTTTGCAGTGG-3'
Wnt5a	5` forward	5'-ATATTAAGCCCAGGAGTTGCTTTG-3'
Wnt5a	3` reverse	5'-GCAGAGAGGCTGTGCTCCTA-3'

MT1M	5` forward	5'-GCAGTCGCTCCATTTATCGC-3'
MT1M	3` reverse	5'-AAATCCAGGTTGTGCAGCTTGT-3'
NIACR2	5` forward	5'-CGCTCCATCGGATCACTAGC-3'
NIACR2	3` reverse	5'-GGCTGGATTTCCAGGACTTGAGG-3'

**Table 2.9:** List of primers used in this project for Real Time-PCR.

### 2.3.2 Primers for PCR cloning

Primers	Sequences
TRAIL_F	5'-AATT <b>GGCCTAACTGGCCGGG</b> GATGGAGATCTGAGAAGG-3'
TRAIL_R	5'-AATT <b>GGCCGCCGAGGCCG</b> CCATGATCCTGTCAGAGTC-3'

**Table 2.10:** List of primers used for PCR cloning.

**Note:**

TRAIL promoter sequences were generated by PCR from human genomic DNA with the appropriate sets of primers designed using Primer3 web-based software (available at: <http://frodo.wi.mit.edu/primer3/input.htm>). The sequence "AATT" is a random extra sequence added to both ends of the clones. The bold sequence is the *Sfi*I restriction site.

## 2.4 Recipes

All solutions and buffers were prepared using nanopure deionised water (dH<sub>2</sub>O).

Where stated, buffers and solutions were autoclaved (20 minutes, 121°C).

Reagents	Concentration	Recipe
X % Agarose gel	X % (w/v) Agarose 1x TBE 0.25 µg/ml EtBr	X g Agarose 100 ml 1x TBE 2.5 µl 10 mg/ml EtBr bring to the boil in a microwave oven, let cool and pour into the

		gel tray
Ampicillin stock solution	100 mg/ml Ampicillin	1 g Ampicillin add to 10 ml autoclaved dH <sub>2</sub> O, filter sterilise and store aliquots at -20°C
2 mg/ml Aprotinin	2 mg/ml Aprotinin	5 mg in 2.5 ml dH <sub>2</sub> O Store in -20°C
10% APS	10% (w/v) Ammonium persulphate	10 g APS in 100 ml dH <sub>2</sub> O
1 mg/ml BSA	1 mg/ml BSA 1x luciferase lysis buffer	10 mg BSA 10 ml 1x luciferase lysis buffer
50 µg/ml BSA	50 µg/ml BSA 1x luciferase lysis buffer	100 µl 1mg/ml BSA 1.9 ml 1x luciferase lysis buffer
100 µg/ml BSA	100 µg/ml BSA 1x luciferase lysis buffer	100 µl 1mg/ml BSA 900 µl 1x luciferase lysis buffer
250 µg/ml BSA	250 µg/ml BSA 1x luciferase lysis buffer	100 µl 1mg/ml BSA 300 µl 1x luciferase lysis buffer
500 µg/ml BSA	500 µg/ml BSA 1x luciferase lysis buffer	100 µl 1mg/ml BSA 100 µl 1x luciferase lysis buffer
750 µg/ml BSA	750 µg/ml BSA 1x luciferase lysis buffer	100 µl 1mg/ml BSA 30 µl 1x luciferase lysis buffer
DEPC-dH <sub>2</sub> O	0.1 % Diethyl pyrocarbonate	1 litre dH <sub>2</sub> O add 1 ml DEPC and incubate

		at RT overnight, then autoclave
75% DEPC-Ethanol	75% (v/v) Ethanol	37.5 ml 100% Ethanol 12.5 ml 0.1% DEPC-dH <sub>2</sub> O store at -20°C
10 mM dNTP mix	10 mM dATP 10 mM dTTP 10 mM dCTP 10 mM dGTP	100 µl 100 mM dATP 100 µl 100 mM dTTP 100 µl 100 mM dCTP 100 µl 100 mM dGTP 600 µl DEPC-dH <sub>2</sub> O store aliquots at -20°C
1 M DTT	1 M Dithiothreitol	0.54 g DTT in 3.5 ml dH <sub>2</sub> O filter sterilise and store at -20°C
DNA 6x gel loading buffer (10 ml)	0.25% Bromophenol blue 0.25% Xylene cyanol 30% Glycerol	7.0 ml dH <sub>2</sub> O 250 mg Bromophenol blue 250 mg Xylene cyanol 3.0 ml Glycerol store aliquot at 4°C
10 mg/ml EtBr	10 mg/ml EtBr	g EtBr adjust to 10 ml with dH <sub>2</sub> O and store in the dark at RT
0.1M EGTA pH 8.0	380 g/l EGTA	1.9 g Adjust PH to 8.0 adjust to 50 ml with dH <sub>2</sub> O and autoclave
1% Formalin	1x PBS/2% FBS	100 µl formalin

	1% Formalin	9.9 ml 1x PBS/2% FBS
1M Hepes pH7.5	238.3 g/l Hepes	11.915 g adjust pH to 7.5 adjust to 50 ml with dH <sub>2</sub> O and autoclave
1M KCl	74.56 g/l KCl	3.72 g in 50 ml dH <sub>2</sub> O and autoclave
1 M NaCl	58.44 g/l NaCl	2.92 g in 50 ml dH <sub>2</sub> O and autoclave
1x PBS (Phosphate buffered saline)*	137 mM NaCl 2.7 mM KCl 10 mM Na <sub>2</sub> HPO <sub>4</sub> 2 mM KH <sub>2</sub> PO <sub>4</sub>	40 g NaCl 1 g KCl 7.2 g Na <sub>2</sub> HPO <sub>4</sub> 1.2 g KH <sub>2</sub> PO <sub>4</sub> adjust to 5 litre with dH <sub>2</sub> O autoclave passed through a Gambro U-2000 ultrafiltration column to eliminate potential contaminants such as endotoxin and other bacterial components store at 4°C
1x PBS/2% FBS	1x PBS 2% FBS	98 ml 1x PBS 2 ml FBS
4% polyacrylamide gel (stacking gel)	0.13 M Tris/HCl 6.8 0.25% SDS 4% Acrylamide/bisacrylamide 0.125% APS	3 ml dH <sub>2</sub> O 340 µl ml 1.5 M Tris/HCl 6.8 100 µl 10% SDS 500 µl 30% Acrylamide/bisacrylamide

	0.25% TEMED	50 µl 10% APS 10 µl TEMED After addition of APS and TEMED the gel was poured immediately and left at least 30 min at RT to polymerise
8% Polyacrylamide gel (resolving gel)	0.4 M Tris/HCl 8.3 0.1% SDS 8% Acrylamide/bisacrylamide 0.1% APS 0.3% TEMED	5.8 ml dH <sub>2</sub> O 1.27 ml 3 M Tris/HCl 8.3 100 µl 10% SDS 2.7 ml 30% Acrylamide/bisacrylamide 100 µl 10% APS 30 µl TEMED After addition of APS and TEMED the gel was poured immediately and left at least 30 min at RT to polymerise
10% SDS	10% (w/v) sodium dodecyl sulfate	10 g SDS in 100 ml dH <sub>2</sub> O
0.1 M Sodium vanadate	121.93 g/l Sodium vanadate	0.12 g in 10 ml dH <sub>2</sub> O Store in 4°C
10x TBE (Tris-borate-EDTA)	0.9 M Tris base 0.9 M Boric acid 2 mM EDTA PH 8.0	108 g Tris base 55 g Boric acid 40 ml 0.5 M EDTA adjust to 1 litre with dH <sub>2</sub> O autoclave
1x TBE with EtBr	90 mM Tris-borate	100 ml 10x TBE

	0.5 mM EDTA PH 8.0 0.25 µg/ml EtBr	25 µl 10 mg/ml EtBr Adjust to 1 litre with dH <sub>2</sub> O
10x TBS (Tris buffered saline)	200 mM Tris base 1.368 M NaCl	24.2 g Tris base 80 g NaCl adjust Ph to 7.6 adjust to 1 litre with dH <sub>2</sub> O and autoclave
1x TBS/Tween-20	1x TBS 0.1% Tween-20	100 ml 10x TBS 1 ml Tween-20 900 ml dH <sub>2</sub> O
1.5 M Tris/HCl	121.14 g/l Tris base	90.855 g Tris base adjust to pH 6.8 with HCl adjust to 500 ml with dH <sub>2</sub> O and autoclave
3 M Tris/HCl	121.14 g/l Tris base	181.71 g Tris base adjust to pH 8.3 with HCl adjust to 500 ml with dH <sub>2</sub> O and autoclave
Western blot blocking buffer	5% Skimmed milk powder 1x TBS 0.1% Tween-20	5 g Skimmed milk powder 100 ml 1x TBS/Tween-20
Western blot gel loading buffer 5x	250 mM Tris/HCl 6.8 10% SDS 0.5% Bromophenol blue 50% glycerol 500 mM DTT	3.4 ml dH <sub>2</sub> O 1.6 ml 1.5 M Tris/HCl 6.8 1 g SDS 0.05 g Bromophenol blue 5 ml glycerol 25 µl of 1 M DTT added to

		25 $\mu$ l 5x gel loading buffer before use
Western blot running Buffer 10x	250 mM Tris 2 M Glycine 10% SDS	30.29 g Tris 144.13 g Glycine 10 g SDS adjust to 1 litre with dH <sub>2</sub> O and autoclave Dilute 1:10 with dH <sub>2</sub> O to use
Western blot transfer buffer	25 mM Tris 200 mM Glycine 20% Methanol	3.03 g Tris 14.41 g Glycine 200 ml Methanol 800 ml dH <sub>2</sub> O

**Table 2.11:** List of recipes for all buffers and solutions.

**NOTE:**

\* 1x PBS was prepared by the media kitchen of the Department of Infection, Immunity and Inflammation, University of Leicester.

## 2.5 Antibodies

Reagent	Catalogue number	Company
Monoclonal anti- mouse DDIT4 antibody	A500-001A	Bethyl Laboratories, Inc. Montgomery, USA
Rabbit anti-Actin polyclonal antibody	A-2066	Sigma-Aldrich, Dorset, UK
Polyclonal anti-Rabbit Immunoglobulins/HRP	P0448	Dako, Cambridgeshire, UK
Monoclonal anti- mouse HIF-1 $\alpha$ antibody	610959	BD Biosciences, Oxford, England

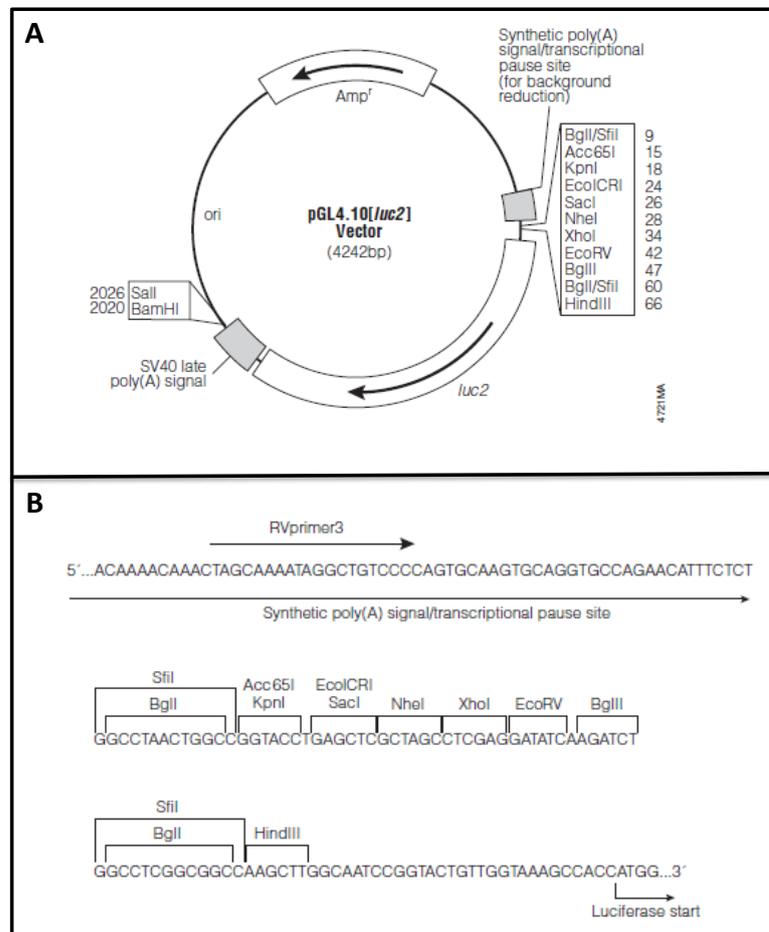
**Table 2.12:** List of antibodies used for immunoblotting experiments.

## 2.6 Reporter plasmid

### 2.6.1 pGL4.10 [luc2] luciferase reporter vector (#E6651, Promega, Southampton, UK)

The pGL4 luciferase reporter vectors are reporter vectors optimized for expression in mammalian cells (Fig 2.1). Numerous configurations of pGL4 vectors are available, including those with the synthetic firefly luc2 (*Photinus pyralis*) and Renilla hRluc (*Renilla reniformis*) luciferase genes, which have been optimized for more efficient expression in mammalian cells. Furthermore, both the reporter genes and the vector backbone such as Ampicillin (Amp<sup>r</sup>) gene and mammalian selectable marker genes have been engineered to reduce the number of consensus transcription factor binding sites, reducing background and the risk of anomalous transcription. The multiple cloning region of the pGL4 vectors has been synthetically constructed and is based on the multiple cloning region of the pGL3 vectors (Figure 2.1). However, differences between the two multiple cloning regions exist. The pGL4 vector multiple cloning region includes the following restriction sites: *BglII*, *SfiI*, *Acc65I*, *KpnI*, *EcoICRI*, *SacI*, *NheI*, *XhoI*, *EcoRV*, *BglII*, and *HindIII*. The *BglII*, *SfiI* and *EcoRV* restriction sites have been added to the pGL4 vector multiple cloning region and are not found in the pGL3 vector multiple cloning region. This increases the number of choices in choosing restriction sites during cloning. The *MluI* and *SmaI* restriction sites found in the pGL3 multiple cloning region have not been included in the pGL4 multiple cloning region. The two *BglII/SfiI* restriction sites in pGL4 facilitate the moving the DNA of interest such as response elements, enhancers and promoters between vectors. Additionally, transfers between pGL4 vectors using either the *BglII* or *SfiI* restriction enzymes retain the desired orientation of the DNA of interest due to the unique DNA recognition

properties of *BglI* and *SfiI*. Also pGL4 vectors contain a SV40 late poly (A) signal which increases the level of luciferase expression due to polyadenylation signals (Fig 2.1). In addition to the features listed, all pGL4 vectors contain a synthetic poly (A) signal/transcriptional pause site which is located upstream of the multiple cloning region. This site is present to reduce the effects of spurious transcription on luciferase reporter gene expression (Fig 2.1). In this project the *SfiI* restriction site of pGL4 luciferase reporter vector was chosen for TRAIL promoter cloning.



**Figure 2.1:** pGL4.10 plasmid vector and restriction sites. (A) Generic pGL4.10 [luc2] vector map. (B) The multiple cloning regions of the pGL4 vectors.

## 2.6.2 Use of the phRL-TK Renilla luciferase vector for normalisation of transfection data

The phRL-TK vector (#E2241, Promega, Southampton, UK) serves as an internal control reporter construct which can be used in combination with any experimental reporter vector (e.g. pGL4) to co-transfect mammalian cells. The phRL-TK vector contains a cDNA encoding Renilla luciferase, originally cloned from the marine organism *Renilla reniformis* (sea pansy) which can be used to normalize the firefly luciferase activity from the promoter reporter constructs. In this project, HMDM were transfected with TRAIL promoter pGL4.10 [luc2] reporter construct and co-transfected with a *Renilla* plasmid (phRL-TK) to normalise the transfection results.

## 2.7 Equipment

Equipment	Model	Company
Balance	BD202	Mettler Toledo, Greifensee, Switzerland
Bioanalyzer	2100	Agilent, Santa Clara, CA
Centrifuges	5717R	Eppendorf, Hamburg, germany
	Sorvall Legend	Thermo Scientific, Germany
Densitometer	GS-700	Bio-Rad Laboratories, U.S.A.
Incubators	Hera cell	Heraeus, Hanau, Germany
	Galaxy R	New Brunswick Scientific / RS Biotech
LightCycler <sup>®</sup> Real-Time PCR instrument	Version 3	Roche, Burgess Hill, UK
Luminometer	Sirius	Berthold detection system, Pforzheim, Germany
Micro plate reader (ELISA reader)	Model 680	Bio-Rad Laboratories, Hertfordshire, UK

Microscopes	H600	Hund, Wetzlar, Germany
	Wilovert S	Hund, Wetzlar, Germany
Media pressing apparatus pump	BP 742	FRESENIUS, Germany
NanoDrop spectrophotometer	1000	NanoDrop products, Wilmington, USA
Oxygen analyser	Mini O <sub>2</sub>	Analox sensor technology, North Yorkshire, UK
Portable test system, ENDOSAFE	1-843-766-7575	Charles River Laboratories, USA
pH-meter	MP225	Mettler Toledo, Greifensee, Germany
Shaker	Innova44	New Brunswick Scientific, Hertfordshire, UK
	Celloshaker	Glotec-Fischer, Reiskirchen, Germany
Thermocycler (PCR machine)	TC-3000	Techne, Cambridge, UK
	Progene 231-103	Techne, Cambridge, UK
Ultra steriset filter	09J-1048	GAMBRO, Darmstadt, Germany
UV- visible Spectrophotometer	Cary 50 Bio	Varian, Santa Clara, USA
Western blot apparatus	Xcell II Minicell	Novex San Diego, USA

**Table 2.13:** List of equipment.

## CHAPTER 3: Methods

### 3.1 Cell culture

#### 3.1.1 Filtration of cell culture medium to reduce endotoxin (LPS) contamination

Macrophages can be activated by very low concentrations of LPS. Since the purpose of the present study was to elucidate the effect of LPS and hypoxia on macrophage gene expression, it was important to minimise LPS contamination in culture media. Therefore, all culture media was filtered before seeding plates with macrophages. In the filtration (Schaefer *et. al.*, 2008), the medium was passed through a Gambro U-2000 ultrafiltration column (Gambro Medizintechnik GmbH, Planegg-Martinsried, Germany), which utilizes a unique synthetic membrane characterized by a very small pore size with high bacterial and endotoxin retention capabilities. After filtration 10% (v/v) low endotoxin foetal calf serum was added (BioSera; confirmed to contain <0.1EU/ $\mu$ l). Media filtration was carried out in a Class II flow cabinet. Filtered complete (apart from serum) Iscove`s media was stored in 200 ml aliquots at -20°C until needed.

#### 3.1.2 Endotoxin testing of filtered Iscove`s media

Three batches of Iscove`s filtered media were prepared and used during this project; 30% of the filtered media bottles were randomly selected to be tested for LPS contamination. The Limulus Amoebocyte Lysate (LAL) LPS test was used to detect endotoxin levels in the media. The LAL assay is highly sensitive and can detect down to

0.01 Endotoxin Units (EU)/ml (1pg/ml). In this test cartridges are pre-loaded by the manufacturer with all of the reagents required to perform an LAL test on the Endosafe®-PTS™ portable spectrophotometer. This integrated testing system eliminates preparation of multiple reagents and reduces the opportunity for technical error. The disposable cartridge contains four channels; two channels serve as the positive control channels, and two channels are for testing of samples. To perform the test, 25µL of sample was added into all four sample reservoirs. The reader's internal pump moves the samples to the reagent station for mixing and then into the optical cells of the cartridge to be read. One endotoxin unit (EU) is equal to 100 pg of LPS. The endotoxin levels in all tested filtered bottles ranged from 0.200 EU/ml to 0.5 EU/ml (20 pg/ml to 50pg/ml).

### **3.1.3 Isolation of peripheral blood mononuclear cells (PBMCs)**

Blood samples from healthy donors were collected into a standard laboratory syringe which contained sufficient heparin to give a final concentration of 10 U/ml, to prevent the blood clotting. The blood sample was diluted 1:1 with Hank's Balanced Salt Solution and then 30 ml of diluted blood was gently layered onto 15 ml of Ficoll-Paque™ Plus in a 50 ml centrifuge tube, followed by 30 minutes centrifugation (minimum acceleration and brake off) at 400g at room temperature (RT). Following centrifugation a thick concentrated PBMC layer, containing monocytes and lymphocytes, was visible at the interface between the original blood sample and the Ficoll-Paque Plus. The monocyte/lymphocyte layer was carefully transferred to a new 50 ml centrifuge tube and washed twice with Hank's Balanced Salt Solution (HBSS) and

spun down at 400 g for 10 minutes at RT. Finally the PBMC were washed once with Iscove's Modified Dulbecco's medium and spun down at 400 g for 10 minutes at RT. Isolated cells were re-suspended in 10ml of complete Iscove's Dulbecco's medium containing 2.5% human AB serum (See tables 2.1.1 and 2.1.6 for the reagents and materials) followed by cell counting using an improved Neubauer haemocytometer with a chamber depth of 0.1 mm (Staples *et al.*, 2007) (See section 3.1.4 and 3.1.5).

**Note:** Iscove's Dulbecco's medium containing 2.5% human AB serum, L-glutamine, penicillin and streptomycin is described as complete Iscove's medium in this project. Hank's Balanced Salt Solution and complete Iscove's medium were used at room temperature.

### 3.1.4 Determination of cell concentration

An aliquot of the cells was diluted 1:10 in HBSS. 10  $\mu$ l of the diluted cells was transferred to an improved Neubauer haemocytometer with a chamber depth of 0.1 mm. 25 central squares of the grid pattern bounded by triple lines (1mm x 1mm) were counted and the cell concentration (cells/ml) calculated as follows:

$$\text{Cells/ml} = \text{cells counted} \times 10^4 \times \text{dilution factor}$$

- $10^4$  = conversion factor of volume of counting chamber into ml.
- Dilution factor = 10

### **3.1.5 Generation of adherence-purified human monocyte-derived macrophages (HMDM)**

The PBMC concentration was adjusted to  $1 \times 10^6$  cell/ml in complete Iscove's medium; 6-well adherent Nunclon plates were seeded with 2ml of medium per well giving  $2 \times 10^6$  cells per well and incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  for 2 hours. After 2 hours, the medium containing the non-adherent cells (mainly lymphocytes) was removed and replaced with 2ml of fresh complete Iscove's medium. Adherent monocytes were incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  and 20.9%  $\text{O}_2$  for 5 days to allow differentiation into macrophages (Burke *et al.*, 2003; Staples *et al.*, 2011). Previous analysis of maturation markers (CD68) and morphology of monocyte-derived macrophages (MDMs) over a period of 3-5 days incubation concluded that this length was appropriate for the generation of macrophages (Staples *et al.*, 2007) (See table 2.1.1 and 2.1.6 for the reagents and materials).

### **3.1.6 Culture of U937 cells**

U937 cells are a non-adherent human monocytic cell line derived from a histiocytic lymphoma (Hosaya *et al.*, 1999). U937 cells were cultured in  $75\text{cm}^2$  tissue culture flasks using filtered RPMI 1640 medium containing 10% FCS, L-glutamine, penicillin and streptomycin and non-essential amino-acid (See table 2.1.1). Cells were split once every 3 days to keep the cells healthy; an aliquot of cells was diluted 1:1 with 1% filtered Trypan blue and then counted as described in section 3.1.4. Trypan blue stains the cytoplasm of apoptotic and dead cells blue but it is unable to penetrate the cell membrane of living cells. After determining the cell concentration, cells were diluted to  $2.0 \times 10^5$  cells/ml and 10 ml transferred to a  $75\text{cm}^2$  flask. The flask was labelled with

the date split and the cell concentration and returned to incubation at 37 °C with 5% CO<sub>2</sub> and 20.9% O<sub>2</sub> (See table 2.1.1 and 2.1.6 for the reagents and materials).

### **3.1.7 Mono Mac 6 cell line**

Mono Mac 6 cells are a monocytic cell line, obtained from the peripheral blood of a monoclastic leukaemia patient (Ziegler-Heitbrock *et al*, 1994). The cell line exhibits characteristics of mature blood monocytes including, CD14 expression, and production of the cytokines: interleukin-1 (IL-1), Interleukin-6 (IL-6), and tumour necrosis factor (Ziegler-Heitbrock *et al*, 1994). Cells are un-adherent and are seeded at a density of  $2 \times 10^5$  cells/ml in a volume of 10ml of filtered VLE RPMI medium , supplemented with: 200U/ml penicillin, 200µg/ml streptomycin, 2mM L-Glutamine (Life Technologies), 5ml Oxaloacetate Pyruvate Insulin, 10% foetal Calf Serum (Biowhittaker). Splitting, counting and culture were as for U937.

## **3.2 Cell treatments**

### **3.2.1 Normoxic & Hypoxic Incubation**

Depending on the experiment, cells were either cultured under normal oxygen concentrations (normoxia) in a humidified atmosphere of 95% air (20.9% O<sub>2</sub>), 5% CO<sub>2</sub>, or under hypoxia in a humidified multi-gas oxygen control Galaxy R incubator at 0.2% O<sub>2</sub>, 5% CO<sub>2</sub>, 94.8% N<sub>2</sub> (See table 2.15). Oxygen levels indicated on the incubator display screens were verified using a separate oxygen analyser (Analox). Cells were incubated under normoxia or hypoxia for 18 hours, 24 hours or 5 days depending on the experiment.

### **3.2.2 Lipopolysaccharide (LPS) stimulation of adherence-purified HMDM prior to RNA isolation for cDNA array and RT-PCR analysis**

The LPS used for cell stimulation was commercially isolated and purified (by Alexis Biochemicals Inc.) from *Salmonella abortus equii* (SAE) (See table 2.1.1). The reason for using this particular LPS was that at the time these experiments were carried out, this was the purest (i.e. lacking detectable contamination with other TLR ligands) available. SAE LPS was diluted with RPMI 1640 medium to make 10 ng/ $\mu$ l working stock solutions. After 5 days incubation under normal oxygen tensions,  $2 \times 10^6$  adherent HMDM were treated with SAE LPS at final a concentration of 100 ng/ml and incubated under normoxia or hypoxia for a further 18 hours prior to RNA isolation.

### **3.2.3 Cobalt chloride (CoCl<sub>2</sub>) stimulation of adherence-purified HMDM**

CoCl<sub>2</sub> increases HIF-1 $\alpha$  level by reducing ubiquitination and hence proteolytic degradation of this protein (Maxwell *et al.* 1999). CoCl<sub>2</sub> (See table 2.1.1) was dissolved in dH<sub>2</sub>O to prepare a laboratory stock solution with a concentration of 30 mM. CoCl<sub>2</sub> was added to cells at final concentration of 300  $\mu$ M, and incubated for 18 hours in the presence or absence of 100 ng/ml of LPS, prior to RNA isolation (See section 3.4.2).

### **3.2.4 Desferrioxamine (DFO) stimulation of adherence-purified HMDM**

DFO is an iron chelating agent which is often used for HIF-1 $\alpha$  stabilisation (Maxwell *et al.*, 1999). DFO (See table 2.1.1) was dissolved in dH<sub>2</sub>O to prepare a laboratory stock solution with a concentration of 20 mM. DFO was added to cells at final concentration of 200  $\mu$ M, for 18 hours in the presence or absence of 100 ng/ml of LPS, prior to RNA isolation (See section 3.4.2).

### **3.2.5 PI3-Kinase inhibitor treatment of adherence-purified HMDM**

LY294002 and wortmannin have been shown to act as highly selective inhibitors of PI3-Kinase (Brunn *et al*, 1996). They were dissolved in sterile DMSO to generate laboratory stock solutions with a concentration of 10 mM and 0.2 mM respectively (See table 2.1.1). After 5 days incubation under normal oxygen tensions,  $2 \times 10^6$  adherent HMDM were treated with LY290042 at final concentrations of 2  $\mu$ M and 5  $\mu$ M for 18 hours in normoxia or hypoxia in the presence or absence of 100 ng/ml of LPS (See section 3.2.1), prior to RNA isolation (See section 3.4). Wortmannin at a final concentration of 200 nM was added to  $2 \times 10^6$  adherent HMDM after incubation for 5 days in normoxia. Dilutions were prepared such that all wells received 2  $\mu$ l of DMSO/inhibitor solution. The same volume of DMSO (2  $\mu$ l) was added to cells as a carrier control. Cells were then incubated under normoxia or hypoxia (See section 3.2.1) for a further 18 hours prior to RNA isolation (See section 3.4.2).

## **3.3 General molecular cloning**

### **3.3.1 Amplification of the TRAIL promoter by polymerase chain reaction**

The polymerase chain reaction (PCR) is a powerful method developed by Kary Mullis in the 1980s (Mullis & Faloona, 1987) (Mullis *et al*, 1992) and occurs in three stages; denaturation of the template DNA, primer annealing, and primer extension. PCR is based on the ability of DNA polymerase to synthesize new strands of DNA complementary to the offered template strand by using two primers which are short pieces (usually around 20 base pairs) of synthetic DNA. Amplification of the targeting sequence on the template is achieved by cycles of reactions that elongate the primers

according to the targeting sequence. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify.

Forward and reverse primers which were used to amplify regions of the TRAIL promoter for PCR cloning are shown in table 2.3.2. Below is the reaction mix and PCR set up to amplify the region of the TRAIL promoter using a thermocycler (See table 2.1.1 and 2.1.6 for the reagents and materials).

Reagent	Volume	Concentration
10x Pfu buffer with MgSO <sub>4</sub>	5 µl	1 x
dNTP (10 mM)	1 µl	0.2 mM
Genomic DNA (50 pg/µl)	1 µl	50 pg
Forward primer (20 mM)	1 µl	0.4 mM
Reverse primer (20 mM)	1 µl	0.4 mM
Sterile dH <sub>2</sub> O	50.5 µl	-
Pfu DNA polymerase	0.5 µl	0.025 U/µl
Final Volume	50 µl	-

**Table 3.1:** PCR reaction mix for 685-bp (-585bp/+100bp) constructs of TRAIL promoter.

Segment	Temperature	Time	cycles
Initial Denaturation	96 <sup>o</sup> C	30 sec	1
Denature	94 <sup>o</sup> C	15 sec	40
Annealing	68 <sup>o</sup> C	30 sec	
Extension	68 <sup>o</sup> C	15 sec	
Final Extension	68 <sup>o</sup> C	20 min	1
Final Extension	4 <sup>o</sup> C	-	-

**Table 3.2:** PCR cycle for amplification of 685-bp (-585bp/+100bp) of TRAIL promoter.

### **3.3.2 Agarose gel electrophoresis of the PCR product DNA**

Electrophoresis is used to separate DNA fragments by applying an electric field to a gel. DNA is negatively charged, independent of the pH of the solution, and migrates towards the positive pole of an electric field. The rate of migration of DNA in a gel depends on the concentration of the agarose, the pore size of the gel, the applied voltage, and the conformation and length of the DNA molecules. In general bigger fragments migrate slower through the gel.

The sizes of PCR amplified fragments of the TRAIL promoter were confirmed by running on an agarose gel and compared with a DNA marker (See table 2.1.3 and 2.4 for all the reagents and buffers). For agarose gel electrophoresis, a solution of agarose in 100ml of 1x TBE containing 0.25 µg/ml ethidium bromide (See table 2.4) was heated in a microwave oven until the agarose was dissolved. The solution was left to cool for a few minutes and then poured into a gel tray with a 1.0 mm comb in it and the gel allowed to set. Ethidium bromide (EtBr) is a fluorescent dye which intercalates between stacked bases of double stranded DNA and allows visualisation of nucleic acids by exposure to UV light. Once the agarose solution was solidified and complete wells were formed, the comb was removed and the gel was placed in the electrophoresis tank which was filled with sufficient 1x TBE buffer (containing EtBr) to cover the gel (See table 2.4). The DNA samples of amplified fragments of TRAIL promoter were diluted with 1:6 with 6x gel loading buffer and slowly loaded into the complete wells. The size of a fragment is estimated by comparison with a DNA molecular weight marker ladder which contains fragments of known length. 250 ng of DNA molecular weight ladder was loaded at the same time as the samples into a

different well. A voltage of 100 V was applied for 2 hours. The gel run was terminated when the bromophenol blue migrated about 3/4 of the length of the gel. The gel was then examined under UV light and photographed.

### 3.3.3 Restriction digestion for cloning of the TRAIL promoter

Restriction endonucleases can be used for cleaving double-stranded DNA molecules at specific positions which are called restriction sites. The cleavage site is located within the recognition sequence and depending on the enzyme, DNA fragments with blunt ends or cohesive (sticky) ends are generated.

In this project, for TRAIL promoter cloning, the *SfiI* restriction site was chosen, which is cleaved by the *SfiI* restriction enzyme (isolated from an *E. coli* strain that carries the *SfiI* gene from *Streptomyces fimbriatus*). The *SfiI* endonuclease cleaves DNA at the sequence 5'-GGCCNNNN↓NGGCC-3', (where N is any base and ↓ is the point of cleavage) and creates sticky ends. The *SfiI* restriction enzyme was used for the digestion of PCR amplified fragments of the TRAIL promoter which carry *SfiI* restriction sites and also the pGL4.10 luciferase reporter plasmid (See section 2.6.1). Restriction digestion was carried according to the manufacturer's instructions (See table 3.3). The reaction was incubated at 50°C for 2 hours to allow digestion. After 2 hours, digested DNAs were separated on agarose gels as described in section 3.3.5.

**Note:** Concentrations of the amplified TRAIL promoter PCR products were determined by comparing to known concentrations of DNA molecular weight marker separated on agarose gels (See section 3.3.2).

Reagents	Volume	Final concentration
<i>Sfi</i> I Restriction Enzyme ( 20 U/ $\mu$ l)	1 $\mu$ l	1 U/ $\mu$ l
DNA	11 $\mu$ l	1-5 $\mu$ g / 20 $\mu$ l
10x <i>Sfi</i> I Buffer	2.0 $\mu$ l	1 x
10x BSA	2.0 $\mu$ l	1 x
Sterile dH <sub>2</sub> O	4.0 $\mu$ l	-
Final volume	20 $\mu$ l	-

**Table 3.3:** *Sfi*I digestion reaction mixture for PCR amplified fragments of TRAIL promoter.

### 3.3.4 QIAquick DNA Agarose Gel Extraction

The digested pGL4.10 vector and PCR amplified fragments of TRAIL promoter sequences were extracted and purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions (See table 2.1.5).

First, 3 volumes of QG Buffer was added to 1 volume of extracted gel slice (e.g. 300  $\mu$ l of QG Buffer to 100 mg of extracted gel) in a 1.5 ml sterile centrifuge tube and then incubated at 50°C for 10 minutes. When the gel slice was dissolved 1 volume of isopropanol was added to 1 volume of extracted gel slice (e.g. 100  $\mu$ l of isopropanol to 100 mg of extracted gel) to increase the yield of DNA fragments. Then the resulting mixture was transferred to a QIAquick spin column and centrifuged at 13000 g for 1 minute to bind DNA to the column. 750  $\mu$ l of PE Buffer containing ethanol was then added to the column and spun down at 13000 g for 1 minute to wash the DNA. Then, the column was placed over a new sterile 1.5 ml centrifuge tube and finally, to elute DNA, 30  $\mu$ l of EB Buffer was added to the centre of column and after 1 min at RT it was

centrifuged at 13000 g for 1 minute. The purified DNA fragments were analysed by agarose gel electrophoresis (See section 3.3.2).

### 3.3.5 Ligation of DNA into plasmid vector

DNA ligation is the process of joining together two DNA molecule ends, most commonly to ligate an insert DNA molecule into a plasmid vector, ready for bacterial transformation. It involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. This reaction is usually catalyzed by a DNA ligase enzyme such as T4 DNA ligase which is the most commonly used DNA ligase to ligate sticky or blunt ends. Typically, insert and plasmid vector DNA are individually cut to yield complementary ends, then both are added to a ligation reaction to be circularised by DNA ligase (See table 2.1.1 and 2.1.6 for the reagents and materials).

In this project the digested pGL4.10 [luc2] plasmid vector and digested fragments of TRAIL promoter sequence were ligated at 1:6 ratio according to the manufacturer's instructions (See table 3.5, Fig 2.1 for the cloning region of the pGL4.10 vector). To prepare the ligation mixture, first PGL4.10 plasmid vector, TRAIL DNA fragments and sterile dH<sub>2</sub>O were transferred into a 0.5 ml sterile centrifuge tube and incubated in the Thermocycler (PCR machine) at 42°C for 2 minutes and then allowed to cool down in RT. The rest of the reagents were then added and incubated at 4°C for 16 hours to complete the ligation and followed by storage at -20°C (See table 3.6).

**Note:** Concentrations of the digested fragment of TRAIL promoter products and digested pGL4.10 plasmid vector were estimated by comparing to known

concentrations of DNA molecular weight marker separated on agarose gels (See section 3.3.2).

Digested fragment of TRAIL promoter	pGL4 vector ratio	Insert DNA ratio	pGL4 vector (ng)	Insert DNA (ng)	pGL4 vector ( $\mu$ l)	Insert DNA ( $\mu$ l)
685-bp (-585bp/+100bp) (5ng/ $\mu$ l)	1	6	100	100	15	20

**Table 3.4:** Ratio of vector DNA (pGL4.10 vector): insert DNA (digested TRAIL promoter product). pGL4.10 vector was used at 6.6 ng/ $\mu$ l.

Reagents	Volume	Final concentration
Digested pGL4.10 [luc2]	See table above	2.5-5ng
Digested TRAIL promoter	See table above	0.5-10ng
Sterile dH <sub>2</sub> O	Variable	-
Incubate in a PCR machine at 42°C for 2 minutes		
T4 DNA Ligase (4U/ $\mu$ l)	2	0.4 U/ $\mu$ l
10x Reaction buffer	2	1 x
ATP (10mM)	2	1mM
Final volume	20	-
incubate at 4°C for 16 hours to complete ligation		

**Table 3.5:** Reaction mixture for ligation of the digested TRAIL promoter sequences into the pGL4.10 vector.

### 3.3.6 Transformation of plasmid DNA into DH5- $\alpha$ competent cells

Bacterial transformation is a technique to introduce a foreign plasmid into bacteria and to use those bacteria to amplify the plasmid in order to make large quantities of it. This is based on the natural function of a plasmid.

For this project, Library Efficiency DH5- $\alpha$  Competent Cells were used as these cells are suitable for cloning experiments in which limiting amounts of plasmid DNA will be used (See table 2.1.1 and 2.1.6 for the reagents and materials). For the transformation, first the ligation product (pGL4.10 plasmid-TRAIL promoter sequence) was diluted 1:5 with TE buffer (10mM Tris-HCl [pH7.5] and 1mM EDTA). A 200  $\mu$ l of aliquot of DH5- $\alpha$  competent cells was thawed on ice and 100  $\mu$ l of cells were transferred into 14 ml Falcon tube; 1  $\mu$ L (1~10 ng) of the diluted ligation product and 5  $\mu$ l (50 pg) of the control pUC19 DNA were added into each tube and gently shaken to mix. The bacteria were incubated on ice for 30 minutes, then heat-shocked at 42°C in a water bath for 45 seconds followed by 2 minutes incubation on ice. Then, 900  $\mu$ l of room temperature S.O.C medium was added to each tube, and the competent cells were incubated in 37°C with shaking at 225 rpm for 1 hour. Then, 100  $\mu$ l, 300  $\mu$ l, and 500  $\mu$ l of competent cells were plated out onto LB-agar plates containing 100  $\mu$ g/ml ampicillin for selection (See table 2.2.2) and the plates incubated overnight at 37°C.

Transformation efficiency of competent cells was determined by transforming 50 pg of the control pUC19 DNA into competent cells, with 100  $\mu$ l of cells being plated out.

$$\text{Transformation efficiency (CFU/}\mu\text{g)} = \frac{\text{no. of colonies} \times 10 \times 10^6}{\text{used pUC19 DNA in pg}}$$

- Factor 10: 100  $\mu\text{l}$  transformed bacteria streaking into LB-agar
- Factor  $10^6$ : converting factor of pg to  $\mu\text{g}$

### 3.3.7 Purification of plasmid-DNA

Plasmid DNA was prepared using the Qiagen EndoFree™ Plasmid Maxi Kit according to the manufacturer's instructions. The plasmid purification protocol is based on a modified alkaline lysis procedure. Insoluble complexes containing chromosomal DNA, salt, detergent and proteins are removed by passing the lysate through a filter. The filtered lysate is incubated on ice with a specific removal buffer which prevents the binding of LPS molecules to an anion-exchange column in the subsequent step allowing purification of DNA containing less than 0.1 endotoxin units per  $\mu\text{g}$  of DNA. Plasmid DNA is bound to an anion-exchange column under low salt and pH conditions and RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid-DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. A final wash step with 70% ethanol removes residual salt. The purified DNA is briefly air-dried and dissolved in a small volume of TE buffer (See table 2.1.1 and 2.1.6 for the reagents and materials).

The procedure is as follows. A starter culture of 3 ml LB medium containing 100  $\mu\text{g/ml}$  ampicillin (see table 2.2.2) was inoculated with a single bacterial colony picked from a

selective plate and incubated ~8 hours at 37°C shaking at 224 rpm. The starter culture was diluted 1:1000 into 100 ml of selective LB medium containing 100 µg/ml ampicillin and grown 12-16 hours at 37°C shaking at 224 rpm. 50 ml of over-night grown bacterial culture were centrifuged at 6000 g for 30 minutes at 4°C. The bacterial pellet were lysed by re-suspending in 10 ml of buffer P1 followed by adding 10 ml of buffer P2. The buffer and bacteria were mixed gently by inverting the tube 5 times and then incubated 5 minutes at RT. During the incubation time, the QIAfilter cartridge was prepared by screwing the cap onto the outlet nozzle of it and placing into a convenient tube. Then, 10 ml of chilled buffer P3 was added to the lysate which was mixed gently by inverting the tube 5 times followed by transferring lysate into the QIAfilter cartridge and incubated in RT for 10 minutes. After incubation, the QIAfilter cartridge was placed over a sterile 50 ml centrifuge tube and the cap was removed and then the lysate was filtered into the tube by applying a plunger. The filtered lysate was mixed gently with 2.5 ml of buffer ER and incubated on ice for 30 minutes. A QIAGEN-tip column was equilibrated by applying 10 ml of QBT buffer and allowed to empty by gravity flow. Then, the filtered lysate was transferred into the QIAGEN-tip column and allowed to enter the resin by gravity flow. The QIAGEN-tip column was washed twice with 30 ml buffer QC and the DNA was eluted with 15 ml buffer QN into a sterile 50 ml centrifuge tube. Addition of 10.5 ml isopropanol and centrifugation at 15000 g for 30 minutes at 4°C precipitated the DNA. The supernatant was decanted and the DNA pellet was washed with 5 ml of RT endotoxin-free 70% ethanol and centrifuged at 10000 g for 10 minutes at 4°C. The DNA-pellet was air dried for 10-15 minutes and re-suspended in 300-450 µl of endotoxin-free TE buffer and stored at -20°C.

A small volume of purified plasmid DNA was used to determine DNA concentration (See section 3.3.8). DNA sequencing to confirm the sequence of cloned fragments was done by the Protein Nucleic Acid Chemistry Laboratory (PNAACL), University of Leicester.

### **3.3.8 Quantification of nucleic acids**

Absorption spectroscopy is a non-destructive method of measuring the concentration of deoxyribonucleic acid (DNA). Nucleic acids absorb light maximally at a wavelength of 260nm, and this physical property can be used as a basis to determine the concentration of the nucleic acids in a given solution. DNA samples are sometimes contaminated with trace of protein which has a maximum absorption at 280nm. So the purity of the nucleic acids can be estimated by calculating the ratio of readings taking at 260nm and 280nm ( $OD_{260}:OD_{280}$ ). Pure preparations of DNA have  $OD_{260}/OD_{280}$  values of ~1.8 whereas contamination with protein results in lower values.

The measurement of purified DNA (pGL4.10 plasmid-TRAIL promoter) was carried out in a special quartz cuvette since glass or plastic cuvettes show a high absorption at 260 nm. In general, to save the samples, they were diluted 1:100 with TE buffer. Then the DNA concentration was determined by spectrophotometer at  $OD_{260}$  and calculated using the following formula:

$$\text{Concentration of nucleic acid } (\mu\text{g/ml}) = \text{OD}_{260} \times \epsilon \times d$$

- $\epsilon$ : extinction coefficient (50  $\mu\text{g/ml}$  for dsDNA).
- $d$ : dilution factor.

## 3.4 Quantification of mRNA

### 3.4.1 Isolation of total RNA for microarray experiments

The RNeasy Mini Kit allows efficient purification of total RNA from small amounts of starting material. Cell-culture media in all conditions were aspirated and quickly 500  $\mu\text{l}$  of lysis buffer (RLT) was added into each flask. Then cells were scraped off the flask and the lysates were collect into a microcentrifuge tube and then vortexed to mix to ensure no clumps are visible. To shear the DNA, lysates were passed at least 8 times through a 20-gauge needle (0.9 mm diameter) fitted to a 2 ml syringe; 700  $\mu\text{l}$  of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. Then, 700  $\mu\text{l}$  of the sample was transfer to an RNeasy spin column and placed in a 2 ml collection tube and centrifugated for 15 seconds at  $\geq 8000 \times g$  and the flow-through discarded. The last step was repeated for the rest of the lysate; 700  $\mu\text{l}$  Buffer RW1 was added to the RNeasy spin column and centrifugated for 15 seconds at  $\geq 8000 \times g$  to wash the spin column membrane. Then 500  $\mu\text{l}$  Buffer RPE was added to the RNeasy spin column and centrifugated for 15 seconds at  $\geq 8000 \times g$  to wash the spin column membrane. For a second time, 500  $\mu\text{l}$  Buffer RPE was added to the RNeasy and it was centrifuged as before. To eliminate any possible carryover of Buffer RPE, the RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute; 20

$\mu\text{l}$  of RNase-free water directly was added to the spin column membrane and centrifuged for 1 minute at  $\geq 8000 \times g$  to elute the RNA. Using the same column, the eluted product was added into the column and centrifuged for 1 minute at  $\geq 8000 \times g$  and the RNA was stored at  $-20^{\circ}\text{C}$ .

### **3.4.2 Isolation of total RNA using Tri reagent**

Total RNA was isolated from either normoxic or hypoxic adherent HMDM (See section 3.1.3) using TRI reagent lysis and isopropanol precipitation according to the manufacturer's instructions as described below (Staples *et al.*, 2000 & 2007 & 2011) (See table 2.1.1 and 2.1.6 for the reagents and materials).

Cells were lysed in 500  $\mu\text{l}$  TRI reagent. The lysate, containing RNA, DNA and protein was transferred into a new sterile 1.5 ml centrifuge tube and allowed to stand for 5 minutes at RT to permit complete dissociation of nucleoprotein complexes. At this stage the lysate was either stored at  $-80^{\circ}\text{C}$  or used for mRNA isolation. 100  $\mu\text{l}$  of chloroform was added to the lysate and vortexed vigorously for 15 seconds to form a white emulsion which was allowed to stand for 10 minutes at RT. The resulting mixture was centrifuged at 12000 g for 15 minutes at  $4^{\circ}\text{C}$ . This separates the mixture into 3 phases; a colourless upper aqueous phase containing RNA, an interphase containing DNA and a red organic phase containing protein. The top aqueous layer was transferred into a new autoclaved 1.5 ml centrifuge tube and 500  $\mu\text{l}$  of isopropanol was added for RNA precipitation. The sample was incubated at RT for 10 minutes and then centrifuged at 12000 g for 10 minutes at  $4^{\circ}\text{C}$ . The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol and spun down at 12000 g for 5

minutes at 4°C. The supernatant was discarded and the pellet air-dried for 10 minutes at RT under a *laboratory fume hood*. The RNA pellet was re-suspended in 20 µl of DEPC-dH<sub>2</sub>O and either immediately reverse transcribed (See section 3.4.2) or stored at -80°C.

### 3.4.3 Reverse transcription

In this enzymatic reaction, isolated total RNA is reverse transcribed into cDNA. For this reaction, 9 µl of total RNA (See section 3.4.1 and 3.4.2) was transferred into an autoclaved 0.5 ml centrifuge tube and incubated in a thermocycler (PCR machine) for 5 minutes at 70°C to denature RNA secondary structure. An arbitrary volume (9 µl) of total RNA was used for each sample because when this work was carried out there was no spectrophotometer available in this Department which was capable of accurately quantifying the low concentrations of RNA typically found in these samples. Rather than standardising the input RNA quantity, data was normalized against the RT-PCR data for the housekeeping gene  $\beta$ -2 microglobulin ( $\beta$ -2m) for each sample. This approach has been validated in detail previously (Staples et al., 2010). The sample was cooled to 42°C and then 11 µl of reaction mix (See table 3.9) was added and the reaction was incubated at 42°C for 1 hour to allow reverse transcription of RNA into cDNA. The reaction was terminated by heating to 90°C for 4 minutes to denature the reverse transcriptase enzyme. The cDNA was stored at -20°C or used immediately for Real-Time-PCR (See table 2.1.1 and 2.1.6 for the reagents and materials).

Reagent	volume	Final concentration
Hexanucleotides (0.2 µg/µl)	1 µl	10 ng/µl
10 mM dNTP mix	2 µl	1 mM
DEPC-dH <sub>2</sub> O	2.4 µl	-
5x AMV Buffer	4.0 µl	1 x
AMV Reverse Transcriptase (10 U/µl)	0.8 µl	0.4 U/µl
RNasin® Ribonuclease Inhibitor (40 U/µl)	0.8 µl	1.6 U/µl
RNA	9 µl	-
Total volume	20 µl	-

**Table 3.6:** Reaction mix for reverse transcription. Reagents and reaction mix were kept on ice.

### 3.4.4 Real-Time PCR

Semi-quantitative PCR was performed using a Roche LightCycler, an integrated thermal cycler and fluorimeter that amplifies the target nucleic acid and monitors the development of the amplification products via measurement of fluorescence. Amplification of target DNA is carried out in the presence of the fluorescent dye SYBR Green which binds to the minor groove of the DNA double helix. The fluorescent intensity is directly proportional to the amount of the amplified product. Subsequent to an amplification run, a melting curve analysis of the PCR product is performed to allow discrimination between specific products and non-specific products. The melting cycle comprises three steps; rapid heating to 94°C to denature all DNA, cooling the reaction below the annealing temperature of the DNA fragments, and finally slowly heating to monitor the fluorescence of the double-stranded DNA as it melts at a characteristic temperature.

For each PCR assay, four 1:5 serially diluted standard samples from a pool of the cDNAs to be tested were prepared. Then 3 µl of samples (standard samples and tested

cDNA) were transferred into a Roche LightCycler capillary and 17  $\mu$ l of PCR master mix (See table 3.10) was added to each reaction capillary. The PCR master mix contains SYBR Green JumpStart with Taq DNA polymerase which is inactive at RT, due to the presence of heat-labile blocking groups on some of the amino acid residues of the enzyme and subsequently minimizes non-specific amplification products as no elongation takes place during the period when primers can bind non-specifically. The polymerase is activated by removing the blocking groups at high temperatures in the initial denaturation step of the Real-Time PCR. One extra capillary was prepared using 3  $\mu$ l of autoclaved dH<sub>2</sub>O with 17  $\mu$ l of PCR master mixture as a negative control to confirm that the PCR master mix is not contaminated with DNA. Then all the capillaries were capped and spun down at 400 g for 10 seconds at 4°C. LightCycler capillaries were transferred to a Roche PCR LightCycler real-time PCR instrument for amplification (See table 2.1.1 and 2.1.6 for the reagents and materials). The reaction mix and set up was prepared according to the following tables.

Reagent	Volume	Final concentration
2x SYBR® Green JumpStart™ Taq ReadyMix	10 $\mu$ l	1 x
5' Primer (5 $\mu$ M)	2 $\mu$ l	0.5 $\mu$ M
3' Primer (5 $\mu$ M)	2 $\mu$ l	0.5 $\mu$ M
Sterile dH <sub>2</sub> O	3 $\mu$ l	-
Total volume	17 $\mu$ l	-
DNA template	3 $\mu$ l	-

**Table 3.7:** Reaction master mix for Real-Time PCR. Primers have been listed in table 2.4.1.

Segment	Temperature	Time	Cycles
Initial denaturation	95°C	10 minutes	1
Denature	96°C	10 sec	45
Annealing	60°C	10 sec	
Extension	72°C	25 sec	

**Table 3.8:** PCR cycle used for amplification and detection mRNA of  $\beta$ 2-M and all other genes.

### 3.4.5 Quantification of mRNA

Real-Time PCR was initially performed using primers for the house keeping gene  $\beta$ -2 microglobulin ( $\beta$ -2M) which is expressed in all nucleated cells (Gussow *et al.*, 1987; Lee *et al.*, 2007). Different studies have shown that  $\beta$ -2M expression does not alter under low oxygen conditions (Jogi *et al.*, 2002; Foldager *et al.*, 2009; Watson *et al.*, 2009) and it has been used previously as an internal control in hypoxia experiments carried out at 0.2% O<sub>2</sub> (Staples *et al.*, 2011). The amount of cDNA of the gene of interest was then determined in a second Real-Time PCR. In each run, four internal standards were used consisting of serial 1:5 dilutions of a cDNA pool containing cDNA from each sample (See section 3.4.4). These four standard samples were plotted as a standard curve by LightCycler software to enable it to calculate the relative concentration of the cDNA of interest in the test samples. Then each sample was normalised by dividing the calculated cDNA value for the gene of interest by the calculated amount of  $\beta$ -2M in the same sample. The following formulae were used to calculate the relative level and hypoxic induction of the gene of interest (TRAIL in this example);

$$\frac{\text{TRAIL calculated value for normoxic cDNA}}{\beta 2\text{m calculated value for normoxic cDNA}} = \text{Relative TRAIL abundance in normoxia}$$

$$\frac{\text{TRAIL calculated value for hypoxia cDNA}}{\beta 2\text{m calculated value for hypoxia cDNA}} = \text{Relative TRAIL abundance in hypoxia}$$

$$\frac{\text{Relative TRAIL calculated value in hypoxia}}{\text{Relative TRAIL calculated value in normoxia}} = \text{TRAIL hypoxic fold induction}$$

## 3.5 Transfection

### 3.5.1 JetPEI™ DNA transfection reagent

HMDM were transfected using JetPEI™ transfection reagent to assess the hypoxic inducibility of TRAIL and DDIT4 promoter constructs. JetPEI™ is a useful transfection reagent and ensures effective and reproducible DNA and oligonucleotide transfection into mammalian cells with low toxicity. JetPEI™ is a linear polyethylenimine (PEI), synthesized and purified by PolyPlus LTD which condenses DNA into positively charged particles. The particles then interact with anionic cell surface residues such as proteoglycans and phospholipids and are taken into the cell via endocytosis. Once inside the lysosomes, PEI works as a proton sponge, resulting in an influx of counterions by accepting protons leading to endosomal pH increasing. Raising the endosomal pH has been suggested to alter protein folding and inactivate endosomal enzymes,

resulting in protection of DNA from degradation. This mechanism also leads to endosome swelling and rupture, permitting the release of the JetPEI/DNA complexes into the cytoplasm, thereby allowing nuclear transport for subsequent transcription.

The overall charge of the JetPEI™/DNA complexes is therefore crucial for efficient transfection. It is determined by the DNA to reagent ratio. This ratio, which represents the ionic balance within the complexes, is defined as the N/P ratio which refers to the number of nitrogen residues (N) in the JetPEI™ per phosphate (P) of DNA. To obtain positively charged complexes, an N/P ratio of >3 is required. The following formula, recommended by the manufacturer PolyPlus, was used in order to calculate the N/P ratio which is taking into account the volume of JetPEI™ reagent used for a given amount of DNA;

$$\text{N/P ratio} = \frac{7.5 \times \mu\text{l of JetPEI}^{\text{TM}}}{3 \times \mu\text{g of DNA}}$$

- Factor 7.5 : concentration of nitrogen residues in JetPEI™
- 3: 1 µg of oligonucleotide contains 3 nmoles of anionic phosphate

In preliminary investigations to optimise the transfection procedure, HMDM generated from PBMC were transfected by JetPEI™ (See table 2.5) according to the manufacturer's instruction. PBMC at  $2 \times 10^6$  were cultured in 6-well adherent plates with complete Iscove's Dulbecco's medium and after 5 days incubation under normal

oxygen conditions (See section 3.2.1), HMDM were transfected with plasmid DNA as described below. In order to normalise the transfection results, HMDM were co-transfected with a Renilla plasmid (pRL-TK) which contains Renilla luciferase and serves as an internal control for normalization (See section 2.6.2). Cells were then incubated under normoxia or hypoxia for 24 hours or 5 days (See section 3.2.1) prior to luciferase assay (See section 3.5.3).

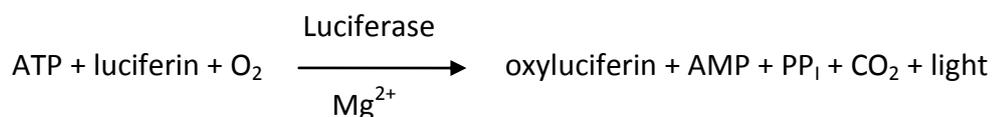
In this project, my preliminary experiments established that the best PBMC transfection results were obtained using an N/P ratio of 8 (1 µg of DNA, 3.2 µl of JetPEI™). PBMC were isolated (see section 3.1.3) and seeded in 4ml complete Iscove's medium at a density of  $4 \times 10^6$  per well in 6-Well Costar ultra low attachment plates and incubated at 37°C with 5% CO<sub>2</sub> and 20.9% O<sub>2</sub> for 5 days to allow differentiation of monocytes to macrophages. On the day of transfection, cells were de-adhered by repetitive pipetting using a 1 ml Gilson and transferred into a sterile 50 ml centrifuge tube. Cells were returned to 37°C prior to transfection. For each transfection (using two wells per transfection) 2 µg of DNA was diluted with 200 µl of 150mM NaCl into a sterile 0.5 ml centrifuge tube. In a new sterile 0.5 ml centrifuge tube, 6.4 µl of JetPEI™ reagent was diluted with 200 µl of 150 mM NaCl. Both tubes were gently mixed and spun down briefly. The 200 µl of diluted JetPEI™ solution were then added all at once to the 200 µl of diluted DNA solution and immediately mixed by vortexing and spun down briefly to bring the drops to the bottom of the tube. The JetPEI™ / DNA mixture was incubated for 30 minutes at RT and then 400 µl of mixture was added to 4 ml of complete Iscove's medium containing  $4 \times 10^6$  PBMC which had been previously de-adhered as described above. Transfected cells were divided into 2 equal parts and

plated in 2 ml at a density of  $2 \times 10^6$  per well in separate 6-Well Nunclon plates (adherent plates) and returned to 37°C with 5% CO<sub>2</sub> and 20.9% O<sub>2</sub> for 1 hour. Cells were then incubated under either normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 24 hours or 5 days (See section 3.2.1) prior to luciferase assay (See section 3.5.3).

### 3.5.2 Luciferase reporter gene assays

In order to study the activity of a specific promoter, the promoter can be cloned into a vector containing a reporter gene (e.g. luciferase). Transfection of the vector into cells results in expression of luciferase using the host cell's transcription factors as well as its transcription and translation machinery.

Luciferase catalyses the formation of light from ATP and luciferin (Reaction is shown below). The emitted light intensity is linearly related to the amount of luciferase expressed and is measured using a luminometer (See table 2.7). The measurements are expressed in relative light units (RLU) per second.



### 3.5.3 Luciferase assay system

PBMC were transfected with TRAIL or DDIT4 promoter regions which were cloned into the pGL4.10 [luc2] luciferase reporter vector (see sections 3.3 and 3.5.1) and cultured

under normoxia or hypoxia (see section 3.2.1) prior to luciferase assay. The assay was performed according to the manufacturer's instructions (see table 2.1.1 and 2.1.6 for the reagents and materials).

Medium was first removed from the wells and the cells were washed twice with 1 ml of 1x PBS. To each well 250  $\mu$ l of 1x lysis buffer was added. The cell lysates were incubated at -80°C for 10 minutes followed by thawing at RT for 30 minutes. Then, 100  $\mu$ l of luciferase assay reagent (LAR) was added to a 5 ml polystyrene Falcon tube and which was measured as background in the luminometer (See table 2.7). The luminometer measured the emitted light units for 30 seconds and converted the detected light units into Relative Light Units per second, RLU/s. Then 20  $\mu$ l of the cell lysate were added to the tube containing 100  $\mu$ l LAR and mixed gently and measured in the luminometer. All transfection experiments were performed triplicate.

In addition, a dual luciferase assay system (See table 2.7) was used according to the manufacturer's instructions to measure the luciferase signals expressed by HMDM which were transfected with DNA-plasmid (e.g. TRAIL promoter pGL4.10 plasmid) and co-transfected with pRL-TK (See section 2.6.2). Medium was first removed from the wells and to each well 250  $\mu$ l of 1x lysis buffer were added. The cell lysates were incubated at -80°C for 10 minutes followed by thawing at RT for 30 minutes. The luminometer measured the emitted light units for 30 seconds and converted the collected light units into RLU/s. To quantify the firefly luciferase expressed by the pGL4.10 plasmid, 20  $\mu$ l of the room temperature cell lysate were added to the Falcon tube containing 100  $\mu$ l luciferase assay reagent II (LAR II) and mixed gently and measured in the luminometer. Then by adding 100  $\mu$ l of Stop & Glo reagent to the

same tube, the firefly luciferase activity is quenched and the Renilla luciferase expressed by pRL-TK, will be activated and measured in the luminometer. For each experiment, 100  $\mu$ l of LAR II and then 100  $\mu$ l of Stop & Glo reagent were sequentially added to the 5 ml polystyrene Falcon tube and the RLU quantified, and the values used as firefly and Renilla background values.

### 3.6 Microarray experiments and hybridization

The microarray experiments for gene expression were performed as a service in the Nottingham Arabidopsis Stock Centre (NASC) as follows: Total RNA was reverse transcribed to generate cDNA libraries by two-cycles of cDNA synthesis. For first strand cDNA synthesis, 1 $\mu$ g of total RNA was incubated with (concentration of primer) T7-oligo dT primer at 70°C for 6 minutes, cooled down to 4°C for 2 minutes and then incubated at 42°C for 1h with first-cycle first-strand master mix (Invitrogen, Paisley, UK). The RT enzyme was then inactivated by heating at 70°C for 10 minutes, and the sample then cooled to 4°C for 2 minutes. Ten microliters of first-cycle second-strand cDNA master mix (4.8 $\mu$ l RNase-free water, 4  $\mu$ l 17.5mM freshly diluted MgCl<sub>2</sub>, 0.4  $\mu$ l 10mM dNTP, 0.6  $\mu$ l *E.coli* DNA polymerase I and 0.2  $\mu$ l RNase H; Invitrogen) was added and incubated at 16°C for 2h then at 75°C for 10 minutes and cooled to 4°C for 2 minutes. First-cycle IVT (*in vitro* transcription) amplification of cRNA was then carried out at 37°C for 16h. The cRNA was then purified following the manufacturer's instructions (Affymetrix). The quality and quantity of the resulting cRNA was estimated using an Agilent

2100 Bioanalyzer. The resulting amplified RNA (600ng) was used for second-cycle, first-strand cDNA synthesis following the manufacturer's instructions (Affymetrix). Next, second-cycle first-strand master mix (5X 1<sup>st</sup> strand reaction mix, 0.1M DTT, RNase inhibitor, 10mM dNTP and superscript II: Invitrogen) was added and the reactions incubated at 42°C for 1h followed by incubation at 4°C for 2 minutes. Then RNase H (Invitrogen) was added to the mixture and incubated at 37°C for 20 minutes, followed by incubation at 95°C for 5 minutes then at 4°C for 2 minutes. For second-cycle, second-strand cDNA synthesis, 5µM T7-oligo (dT) primer was added and the reaction incubated at 70°C for 6 minutes then cooled to 4°C for 2 minutes. Second-cycle, second-strand master mix was incubated at 16°C for 2h then T4 polymerase was added and incubated at 16°C for 10 minutes and cooled to 4°C for 2 minutes. cDNA was then purified following the manufacturer's instructions (Invitrogen). Biotin-labelled cRNA was synthesised using the GeneChip IVT labelling kit (Affymetrix) at 37°C for 16h. Biotin-labelled cRNA was cleaned up following the manufacturer's instructions. The cRNA quality and quantity for microarray hybridization was estimated using an Agilent 2100 Bioanalyzer. Human Genome ST 1.0 gene chips arrays were equilibrated to room temperature and wetted by using a pre-hybridization mix at 45°C for 10 minutes with rotation. The hybridization cocktail was prepared according to Affymetrix manufacturer's instructions and heated to 99°C for 5 minutes then incubated at 45°C for 5 minutes. Arrays were hybridized with a hybridization cocktail containing biotin-labelled cRNA at 45°C for 16h on rotater at 60 rpm. The washing and staining were

performed according to the Affymetrix manufacturer's instructions. Gene chips then were scanned using an Affymetrix GenChip 3000 scanner.

## **3.7 Protein analysis**

### **3.7.1 Total protein extraction**

The PBMC concentration was adjusted to  $1 \times 10^6$  cell/ml in complete Iscove's medium; 6-well adherent Nunclon plates were then seeded with 2ml of medium per well giving  $2 \times 10^6$  cells per well and incubated at 37°C, 5% CO<sub>2</sub> for 2 hours. Adherent monocytes were then generated as described in section (3.1.5) (Burke *et al.*, 2003; Staples *et al.*, 2011). Cells were either cultured under normoxia or hypoxia in the presence or absence of 100 ng/ml LPS for 18 hours. Cells then were taken quickly from incubators and the media was removed. Cells were washed with 5 ml of ice-cold PBS. Then, the PBS was poured off and 1ml of fresh ice cold PBS was added. Using a plastic scraper, the plate was scraped and cells were removed and transferred into a 1.5 ml sterile centrifuge tube followed by 30 seconds centrifugation at 4°C for 10,000 g. The supernatant was removed and the cells were lysed 50 µl of SDS lysis buffer (see table 2.11). Cells were then incubated for 5 minutes at 100°C, and then chilled briefly on ice. The lysate was passed 10 times up and down through a 26 gauge needle using a 1ml syringe. Samples were centrifuged at 10,000g for 10 minutes at 4°C. The supernatant, containing the protein, was transferred into a 1.5 ml sterile centrifuge tube and stored at -20°C for future use.

### 3.7.2 Quantification of protein concentration

The Thermo Scientific Pierce 660 nm protein assay is a quick, ready-to-use colorimetric method for total protein quantification (See table 2.1.5). The assay is reproducible, rapid and more linear than Coomassie-based Bradford assays and compatible with higher concentrations of most detergents, reducing agents and other commonly used reagents. The Pierce 660nm protein assay is based on the binding of a proprietary dye-metal complex to protein in acidic conditions that causes a shift in the dye's absorption maximum, which is measured at 660nm. The dye-metal complex is reddish-brown and changes to green upon protein binding. The colour change is produced by deprotonation of the dye at low pH facilitated by interactions with positively charged amino acid groups in proteins. Therefore, the dye interacts mainly with basic residues in proteins, such as histidine, arginine and lysine and to a lesser extent tyrosine, tryptophan and phenylalanine.

Protein concentrations are estimated by reference to absorbance obtained for a series of standard protein dilutions assayed alongside the unknown samples. In this project, bovine serum albumin (BSA) was used as standard protein and the assay was performed according to the manufacturer's instructions (See table 2.1.5). First a serial dilution of BSA in 1x luciferase Lysis buffer was prepared as follows: 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml and 750 µg/ml (See table 2.11). For the assay, 10 µl of standards, luciferase cell lysate and 1x Lysis buffer as blank were pipetted into a 96-well plate. Then, 150 µl of protein assay reagent was then added to each well and the plate was covered and mixed on a plate shaker at 200 rpm for 1 minute following 5 minutes incubation at RT. After incubation the protein concentration was measured in

an ELISA reader at 660 nm (See table 2.7). To calculate the protein expression, a standard curve was prepared by plotting the average blank-corrected 660 nm measurement for each BSA standard versus its concentration in  $\mu\text{g/ml}$ . Then the standard curve was used to determine the protein concentration of each cell lysate sample.

### **3.7.3 Western blotting**

Western blotting is a technique used to identify and locate proteins based on their ability to bind to specific antibodies. Western blot analysis can detect a protein of interest from a mixture of a great number of proteins. In western blotting, prior to protein immobilization on the PVDF or nitrocellulose membrane, sample proteins are separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) which can give information about the size of the protein with comparison to a protein size marker or ladder, and also give information on protein expression with comparison to a control such as untreated sample or another cell type or tissue.

The Xcell II Minicell electrophoresis and blotting system was used according to the manufacturer's instruction (See table 2.13). Please see table 2.11 and 2.12 for the reagents which were used for western blotting. For electrophoresis, total protein extract samples (100  $\mu\text{g}$ ) (See section 3.6) were diluted 1:6 with 6x loading buffer to a maximum of 18  $\mu\text{l}$  (kept on ice at all times) following 5 minutes incubation at 95°C in the thermocycler and then loaded to the gel. Samples were then size fractionated on SDS-PAGE gel containing 8% (resolving gel) and 4% polyacrylamide (stacking gel) in 1x western blot running buffer for 60 minutes at 40 mA. A pre-stained high molecular

weight protein ladder was also used to estimate test sample protein sizes. Following electrophoresis, proteins were electro-blotted onto Hybond-P PVDF membrane in western blot transfer buffer for 90 minutes at 20 V.

#### **3.7.4 Immunodetection of DDIT4 and HIF-1 $\alpha$ proteins**

Blotted membranes were washed three times in 1x TBS/Tween 20 buffer for 5 minutes at RT and then were blocked overnight at 4°C in Western blot blocking buffer. Next day, the membranes were washed twice for 10 minutes in 1x TBS/Tween 20 buffer followed by incubation for 1 hour at RT in a 1:1000 dilution of rabbit polyclonal anti-DDIT4 antibody in Western blot blocking buffer. For HIF-1 $\alpha$  detection, the membrane was also incubated for 1 hour at RT in a 1:1000 dilution of monoclonal anti-HIF-1 $\alpha$ . The membranes then were washed twice for 20 minutes and twice for 10 minutes in 1x TBS/Tween 20 buffer. Then the membranes were probed with secondary antibody for 1 hour at RT in a 1:2500 dilution of polyclonal anti-rabbit immunoglobulins/HRP in western blot blocking buffer. For HIF-1  $\alpha$ , membranes was probed with secondary antibody for 1 hour at RT in a 1:1000 dilution of polyclonal anti-rabbit immunoglobulins/HRP in western blot blocking buffer. Again, the membranes were washed twice for 20 minutes and twice for 10 minutes in 1x TBS/Tween 20 buffer and the antibody-labelled proteins were detected using ECL Advance western blotting detection reagents (Amersham) according to the manufacturer's instructions and visualised on film (See table 2.5).

### 3.7.5 Re-probing the membranes

Membranes were re-probed for  $\beta$ -actin protein as an internal control for western blotting analysis. For re-probing, the membranes were washed three times for 10 minutes in 1x TBS/Tween 20 buffer following incubation overnight at 4°C in western blot blocking buffer. Rabbit polyclonal anti-actin antibody was used at a 1:2500 dilution in western blot blocking buffer for 1 hour at RT. The membranes were probed with secondary antibody for 1 hour at RT in a 1:2500 dilution of polyclonal anti-rabbit immunoglobulins/HRP in western blot blocking buffer. The immunodetection procedure was performed as described in section 3.6.4.

**Note:** Please see table 2.11 and 2.12 for the buffers and antibodies which used in Immunodetection of  $\beta$ -actin protein.

### 3.7.6 Densitometric analysis

The intensity of bands on the membrane of Western blot assays are dependent on the protein concentration. A densitometer scanner was used to translate the X-ray films of the Western blots into values that subsequently are presented as graphs with accompanying statistics. This gives accurate assessment of the data obtained by immunoblotting experiments. Densitometric analyses in this project were performed by using Molecular Analyst®/PC image analysis software installed on a GS-700 scanner from Bio-Rad Laboratories.

### 3.8 Database analysis

The TRAIL and DDIT4 proximal promoters sequence was searched using MatInspector (Genomatix, [http://www.genomatix.de/online\\_help/help\\_matinspector/matinspector\\_help.html](http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html)) and transcription element search system (TESS) software (<http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME>) for putative transcription factor binding sites.

### 3.9 Microarray data analysis software

The microarray raw data have analyzed using the high-throughput software from Bioconductor version 2.9. (<http://bioinf.wehi.edu.au/limma/>). The heat map for synergy expressed genes was analyzed and generated by using DNA-Chip Analyzer (d-Chip) software downloaded from (<http://biosun1.harvard.edu/complab/dchip/>).

### 3.10 Statistics

The statistical tests used in this project for all data were parametric. Statistical analysis was done using the statistical software package GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA). P-values were calculated for assessment of significance level. The results were considered statistically significant if \*\*\*,  $p \leq 0.001$ ; \*\*,  $p \leq 0.01$ ; and \*,  $p \leq 0.05$ .

## CHAPTER 4: Results

### **Hypoxia-induced gene regulation in the presence or absence of LPS in primary human macrophages *in vitro*: microarray and quantitative PCR analysis**

#### **4.1 Introduction**

Hypoxia is a common feature of several pathological conditions, such as inflammatory lesions, deep injuries (Eltzschig & Carmeliet, 2011), solid tumours (Brahimi-Horn *et al*, 2007), atherosclerotic plaques (Bjornheden *et al*, 1999), and arthritic joints (Stevens *et al*, 1991). Blood monocytes respond and migrate to these sites and differentiate into macrophages (Bosco *et al*, 2008). Macrophages are versatile cells which can adapt their phenotypes according to the environmental conditions of their surroundings. They are able to function under such conditions by altering gene expression by up- or down-regulation of certain genes needed to maintain their viability and proper function (Lewis *et al*, 1999). Recruitment and adaptation of monocytes to hypoxic tissues is a very complex process involving expression of many receptors and chemokines (Murdoch *et al*, 2004).

In the last few years, microarray technology has become an invaluable tool for characterization of gene expression in response to a particular stimulus. This technology enables researchers to monitor thousands of genes at the same time, and it is an excellent tool to understand the molecular mechanisms of many biological phenomena (Zarrinkar *et al*, 2001). Several previous studies have investigated the gene expression changes induced by hypoxia in macrophages using microarray technology. Some of them investigated a limited number of genes (Burke *et al*, 2003; Fong *et al*,

2007), whereas the others covered the whole genome (White *et al*, 2004; Bosco *et al*, 2006; Fang *et al*, 2009), However, none of these studies have investigated the effect of combined treatment with microbial immunostimulatory molecules plus hypoxia on macrophage gene expression. This is relevant to pathological conditions such as wounds, and chronic infections such as tuberculosis in which macrophages encounter both hypoxia and microbial stimuli simultaneously. Therefore, in this study we aimed to fill the gap in knowledge in this area by characterising the combined effect of hypoxia and the best characterised microbial immunostimulatory molecule, lipopolysaccharide (LPS) on human macrophage gene expression.

Affymetrix arrays are a well known commercial platform used in molecular biology. In the present study, the analysis of the whole transcriptome was performed using Affymetrix GeneChip Human Gene 1.0 ST Arrays. These can interrogate 28,869 genes, with approximately 26 probes for each gene, spread across the full length of the gene. This provides accurate and robust human gene expression profiling. The principle of array technology is that RNA extracted from the treated cells (Total RNA) is converted into cDNA, labelled and then hybridized to the oligonucleotide probes in the gene chip that represent all known genes in the human genome.

## **4.2 Aims and Hypothesis**

The aim of the work described in this chapter was to elucidate the full range of genes up- or down-regulated when macrophages are exposed to hypoxia (0.2% O<sub>2</sub>), or LPS (100 ng/ml), or both together. In other words, I wanted to ask whether the treatment of human macrophages with hypoxia plus LPS would give a different gene profile than either hypoxia or LPS alone, or both these sets of genes added together, ie, would

some genes be regulated synergistically, only when both stimuli were present. Synergy is defined as two factors interacting together, so that their combined effect is greater than the sum of their individual effects. Secondly, would some genes which were regulated by each of the two stimuli alone show further additive or synergistic up- or down-regulation in response to both stimuli together. My working hypothesis was that there would be synergy between LPS and hypoxia in regulating gene expression in primary human macrophages.

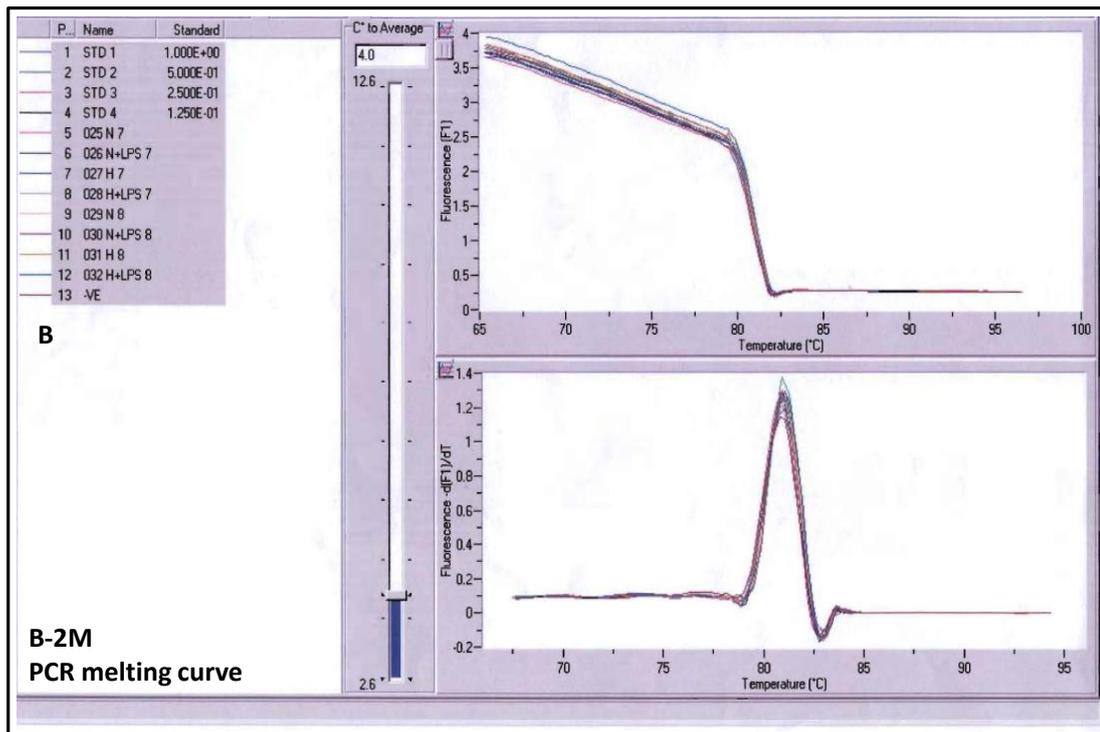
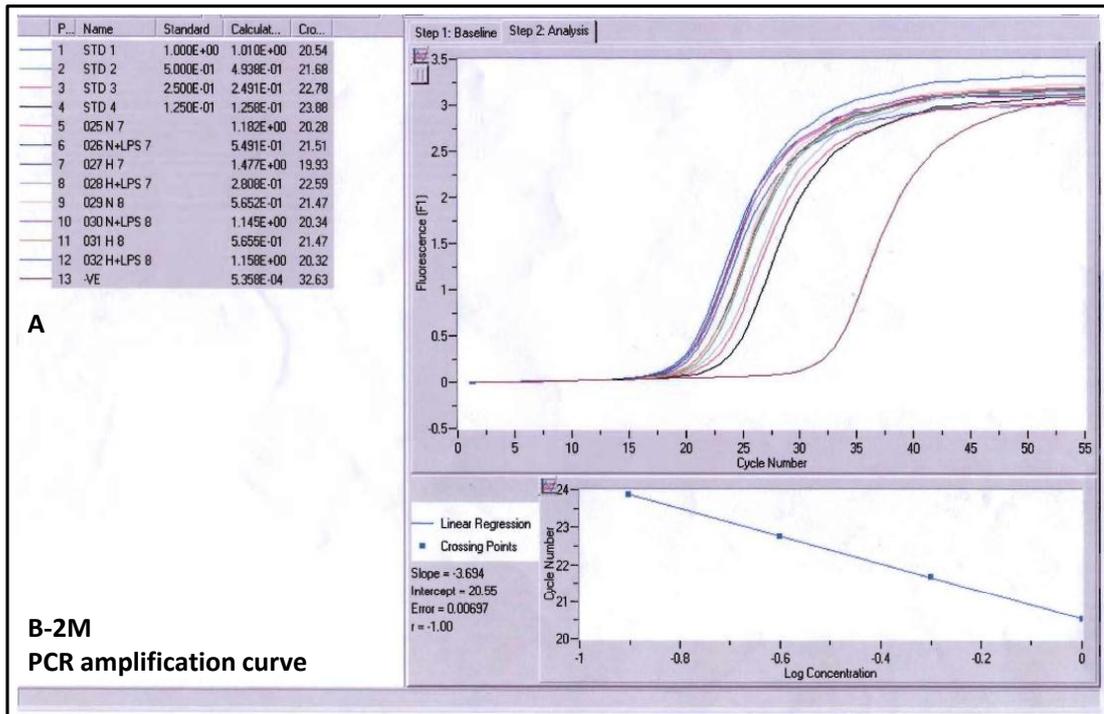
## 4.3 Results

### 4.3.1 Confirmation of hypoxic induction of selected hypoxia-inducible genes in human macrophages by quantitative real-time RT-PCR

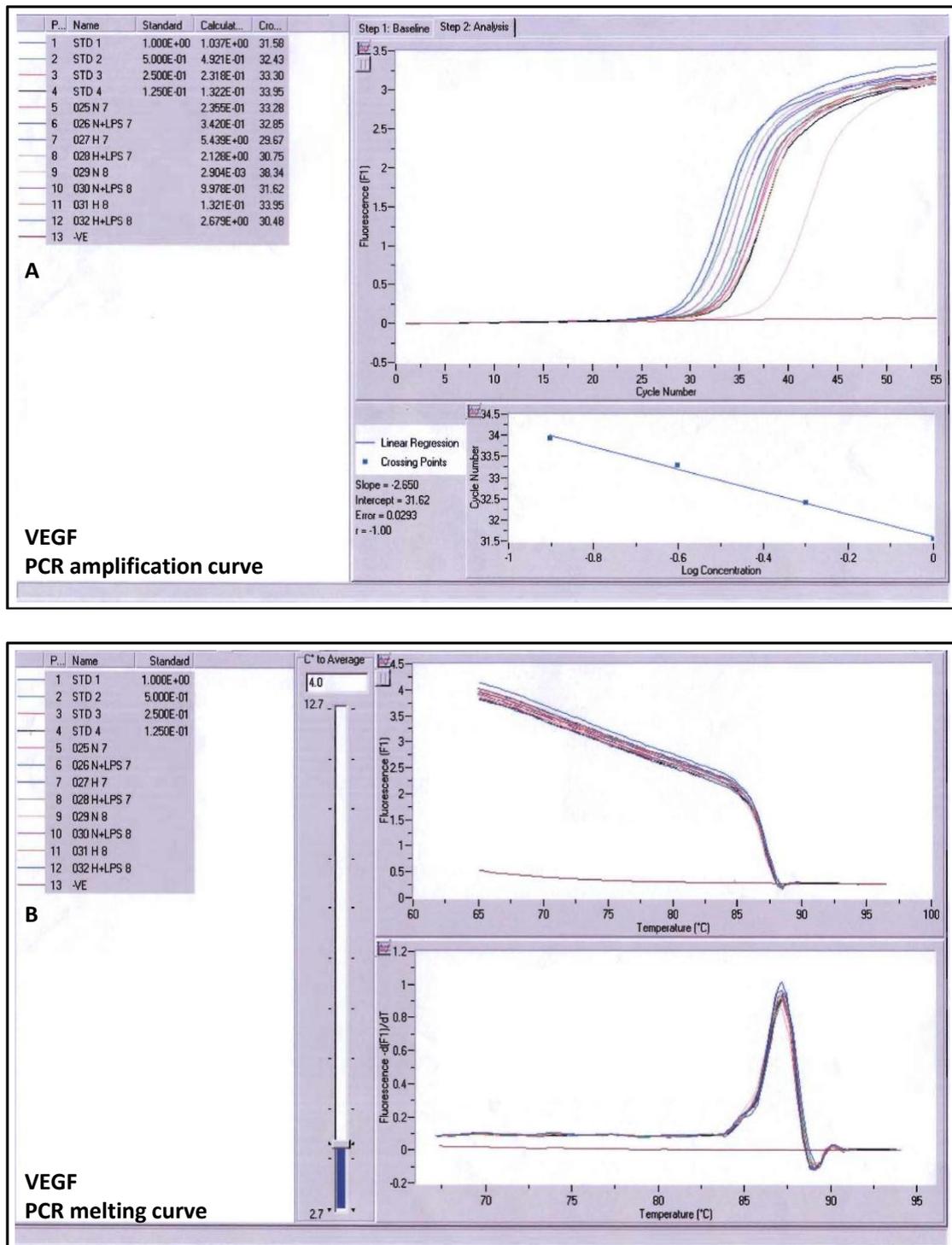
Prior to running microarray experiments, the effect of 18 hours hypoxia (0.2% O<sub>2</sub>) on selected known hypoxia-inducible genes in primary human MDMs was investigated, to confirm that the RNA isolated was suitable for microarray experiments. Primary human MDMs were either cultured under normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) in the presence or absence of 100ng/ml of LPS for 18 hours. RNAs were isolated and cDNA for each sample was generated by reverse transcription, and then real-time PCR was performed.

The reliability of any RT-PCR gene expression analysis can be improved by including an invariant (stable) endogenous control (reference gene) in the assay to correct for sample to sample variation (Bustin *et al*, 2005). Work in this lab has shown that the beta 2-microglobulin ( $\beta$ -2M) gene has a constant level of expression in macrophages under normoxia and hypoxia (Staples *et al*, 2010). Therefore, it was used in this project

as an internal control for quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR was carried out for the  $\beta$ -2M gene by using four 2-fold serial-diluted standards (STD1-4; 1, 0.5, 0.25, and 0.125) run in parallel with the samples and one negative control (PCR water; PCR-ve control). A standard curve was plotted by LightCycler (Roche) software to calculate the relative concentration of  $\beta$ -2M cDNA for each sample (See figure 4.1 A). The specificity and purity of the quantified samples was confirmed by examining the melting curve as seen in figure 4.1 B which shows that all PCR products melt at the same temperature. Then quantitative real-time RT-PCR was carried out for selected genes of interest. Figures 4.2 A and B show an example of amplification and melting curves of one selected gene, vascular endothelial growth factor (VEGF).

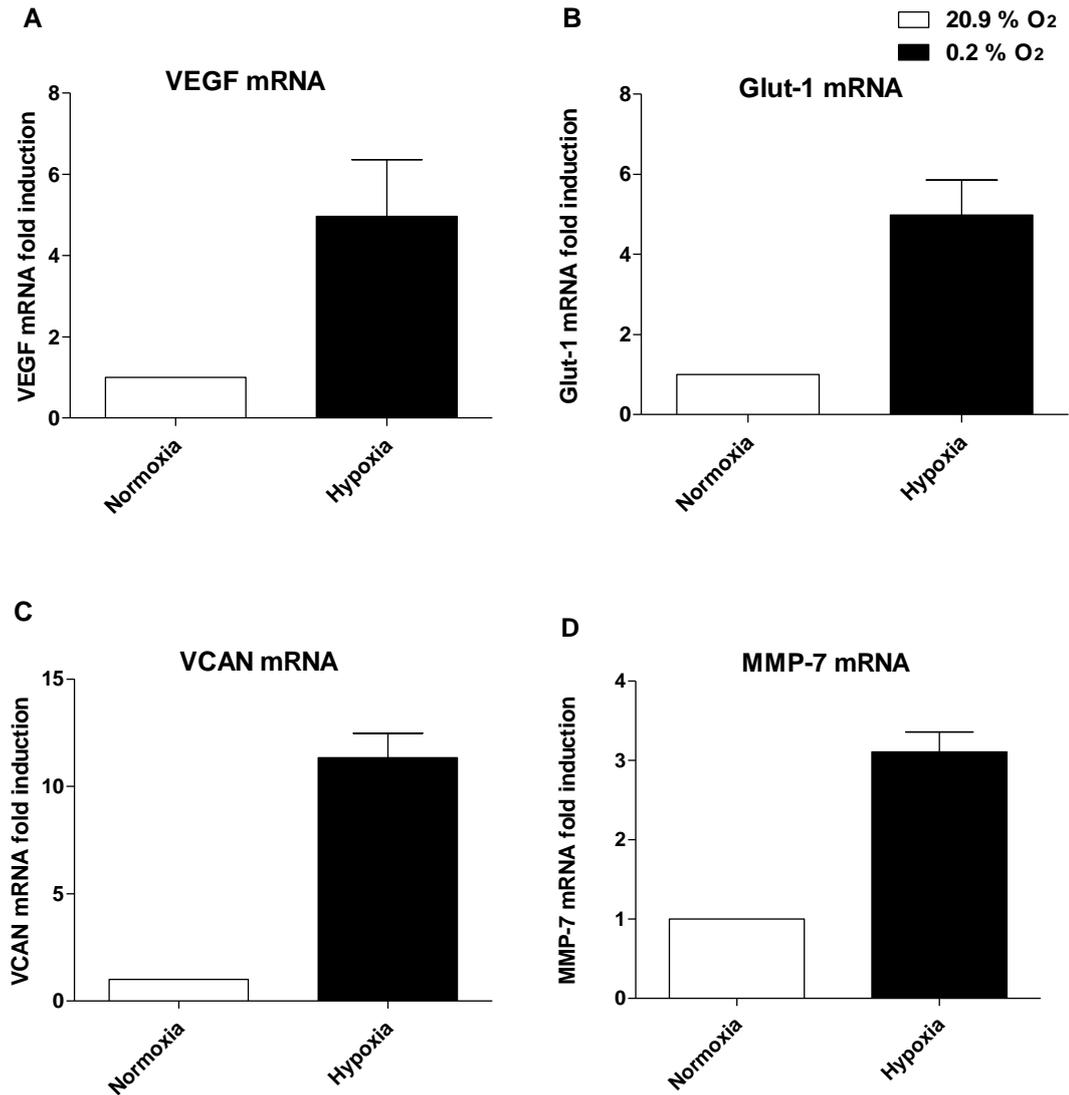


**Figure 4.1: LightCycler PCR amplification (A) and melting (B) curves of  $\beta$ -2M in adherence-purified human MDMs:** The figures show four standards (STD1-4) produced by two fold serial dilution (1, 0.5, 0.25, and 0.125) which were prepared and run in parallel with the samples (N, N+LPS, H, and H+LPS) and negative control (-ve). The standard curve was plotted by the LightCycler software to calculate the relative concentration of  $\beta$ -2M cDNA for each sample. These two figures show: (A) amplification and (B) melting curves of two sets of treated samples obtained from two different donors.



**Figure 4.2: LightCycler PCR amplification (A) and melting (B) curves of VEGF cDNA of adherence-purified human MDMs:** The figures show four standards (STD1-4) produced by two folds serial dilution (1, 0.5, 0.25, and 0.125) which were prepared and run in parallel with the samples (N, N+LPS, H, and H+LPS) and negative control (-ve). The standard curve was plotted by the LightCycler software to calculate the relative concentration of VEGF cDNA for each sample. These two figures show: (A) amplification and (B) melting curves of two sets of treated samples obtained from two different donors.

Up-regulation of selected previously-reported hypoxia-inducible genes, vascular endothelial growth factor (VEGF) and glucose transporter-1 (Glut-1) (Harmey *et al*, 1998; Cramer *et al*, 2003) was confirmed by quantitative real-time PCR. In addition to that, up-regulation of two other hypoxia-inducible genes, matrix metalloproteinase 7 (MMP-7) (Burke *et al*, 2003) and versican (VCAN) (Asplund *et al*, 2010) were also confirmed. Quantitative RT-PCR analysis showed that up regulation of VEGF, Glut-1, VCAN, and MMP-7 by hypoxia is substantial (figure 4.3). These RT-PCR data were obtained from the RNA of the two donors used in microarray experiments. As shown in figure 4.3, data are consistent with previous studies (Burke *et al*, 2003; Bosco *et al*, 2006; Asplund *et al*, 2010; Fang *et al*, 2009) indicating that the macrophages used were cultured and exposed to hypoxia properly, and therefore the RNA isolated from these cells was suitable for microarray experiments.



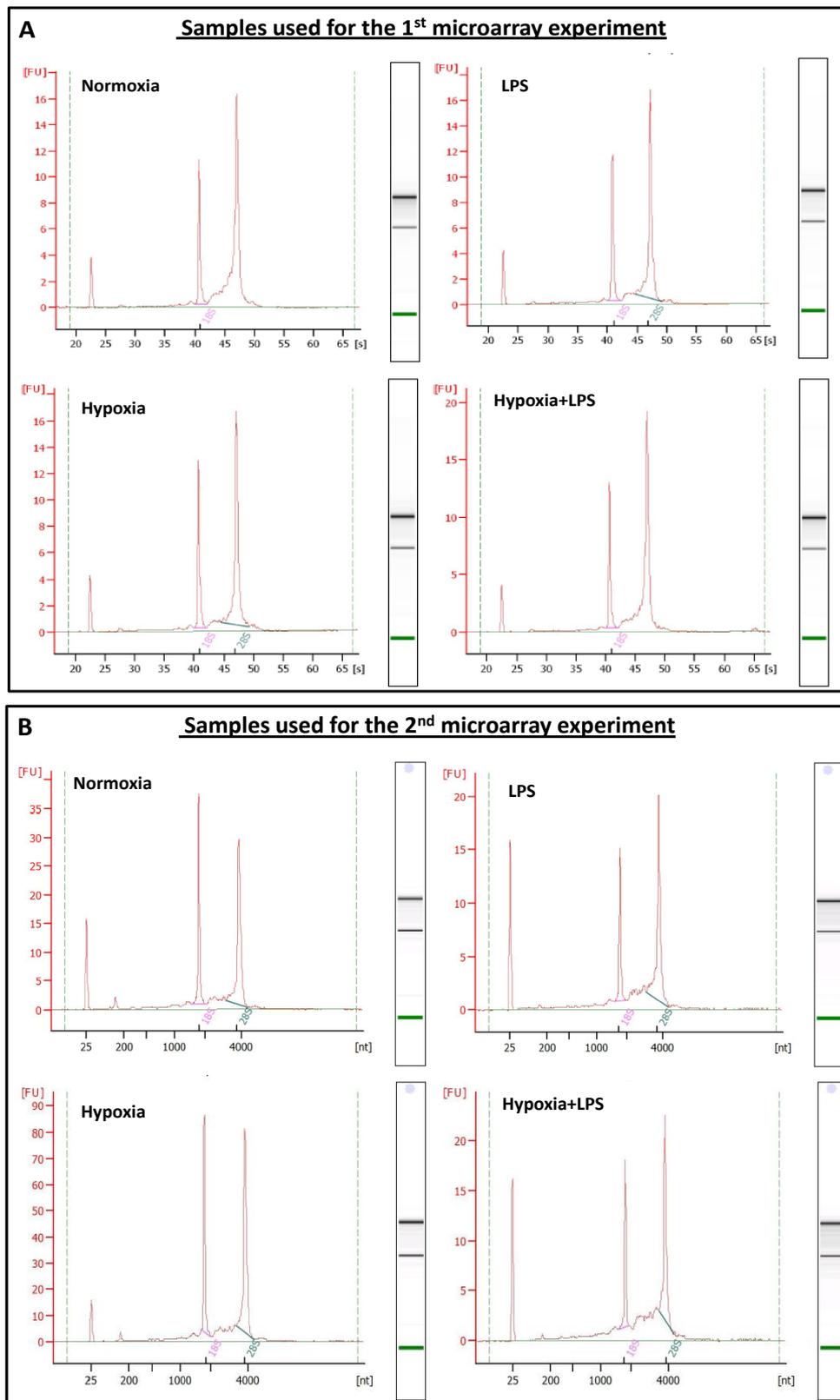
**Figure 4.3: Up-regulation of selected hypoxia-inducible genes in MDM by real-time RT-PCR:** mRNA levels of hypoxia-inducible genes relative to normoxia for (A) VEGF (vascular endothelial growth factor), (B) Glut-1 (glucose transporter type 1), (C) VCAN (versican), and (D) MMP-7 matrix metalloproteinase-7. mRNA expression level was evaluated by quantitative RT-PCR, and fold induction of each gene was calculated after normalization to the mRNA level of the  $\beta$ -2M housekeeping gene. Data from 2 independent experiments using different donors expressed as means  $\pm$  SEM. These samples were from the two donors used in microarray experiments.

### 4.3.2 Total RNA quality assessment

The quality of the total RNA to be used in microarray experiments was checked using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The Bioanalyzer produces electropherogram and gel images for each sample (figure 4.4) that serve the same purpose as an RNA gel. The X axis measures transfer time, which translates to fragment size. The shorter the time, the smaller the RNA fragment. The Y-axis measures the fluorescence intensity which represents the amount of RNA at that size, and this would be the equivalent to more intense bands in the gel. A 25bp RNA fragment is always spiked in to every sample; this appears at the 23 second mark in the graph. The two ribosomal RNA subunits, 18S and 28S, are used to indicate the integrity level of total RNA in the sample. The 28S/18S ratio is an indicator of RNA quality, and this ratio is automatically calculated by the Bioanalyzer instrument. The ratio in a sample with good intact RNA should be between 1.8 and 2.0, whereas with degraded samples, the two major rRNA subunits (18S and 28S) are shorter and produce a decrease in fluorescence signal as dye intercalation sites are destroyed in degraded subunits. The large 28S rRNA subunit usually tends to be degraded more easily than 18S subunit during RNA isolation, therefore, the 28S/18S ratio would become lower, and this ratio has been adopted as an indicator for RNA integrity.

Figure 4.4 shows electropherograms of normoxia, LPS, hypoxia, and hypoxia+LPS RNA samples, for samples sets used in the 1<sup>st</sup> (Panel A) and 2<sup>nd</sup> (Panel B) microarray experiments, respectively. Agilent Technologies has developed a new numbering system tool for RNA quality assessment called RNA Integrity Number (RIN) which also generated automatically by the Bioanalyzer. The RIN for each sample was determined

based on the shape of the curve in the electropherogram allowing classification of total RNA from 1 to 10, where 1 is the most degraded and the 10 is the most intact RNA. The RIN number of all microarray samples used in this project ranged from 9.0 to 9.7 (data not shown).



**Figure 4.4: Electropherogram image of RNA samples used in microarray experiments:** Electropherogram of high-quality RNA showing clearly visible peaks of two ribosomal RNA subunits (18S and 28S). (A) Normoxia, LPS, hypoxia, and hypoxia+LPS treated samples used in 1<sup>st</sup> microarray experiment. (B) Normoxia, LPS, hypoxia, and hypoxia+LPS treated samples used in 2<sup>nd</sup> microarray experiment.

### 4.3.3 Microarray experiments

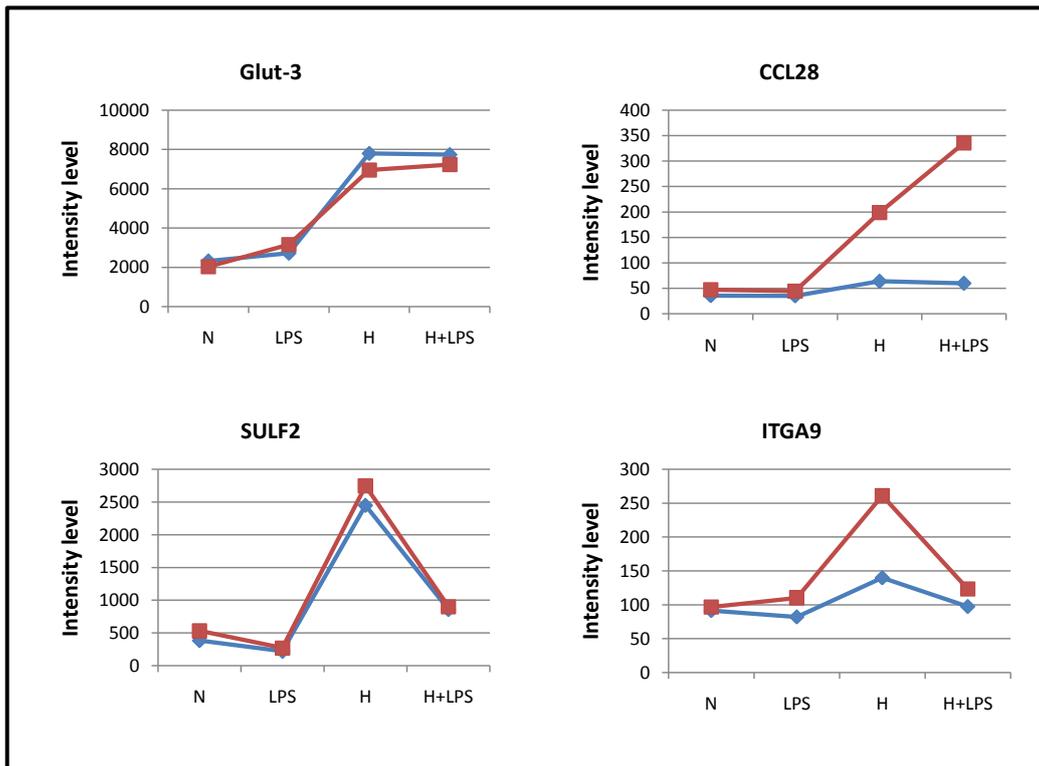
The microarray experiments were performed as a service at the Nottingham Arabidopsis Stock Centre (NASCC). NASCC (<http://affymetrix.arabidopsis.info/>) is a well respected centre and is certified by Affymetrix, the gene chip provider for its reliable and accurate microarray experiment processing. The experiment was done twice using independently generated RNA samples isolated from two different donors for array 1 and 2. Both donors were male, aged 24 and 27 years old, from Chinese and Indian ethnicity respectively, and were healthy non smokers.

### 4.3.4 General microarray data analysis

The initial microarray raw data analysis was performed at the University of Birmingham by our collaborator Dr. Wenbin Wei. Further analysis, for example to calculate the fold change for each gene in each condition using the fluorescence intensity values obtained from raw data after removing the replicate and un-annotated transcripts from the microarray raw data, was carried out by me. Specific criteria were applied for selection of genes of interest from the microarray data. Genes were defined as being differentially regulated by hypoxia, LPS, or hypoxia plus LPS if they exhibited  $\geq 2$  fold increase, or a  $\leq 0.5$  fold decrease (which equals  $\leq 2$  fold down regulation) compared to untreated normoxic cultures.

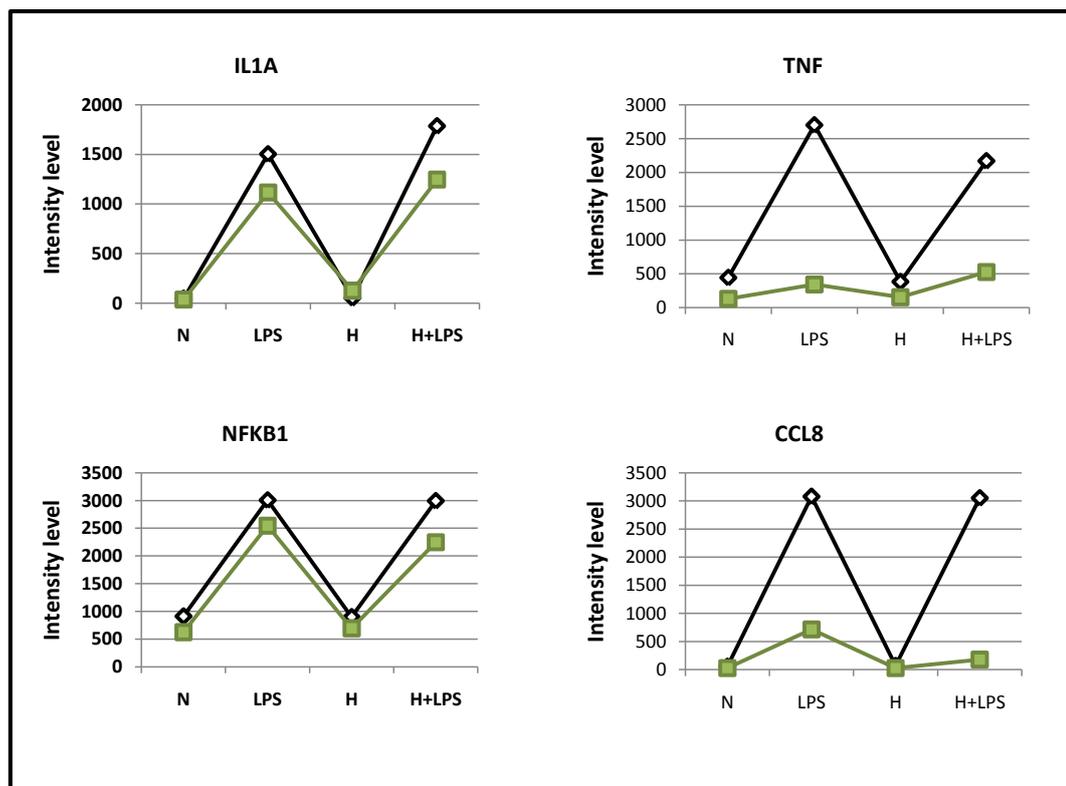
In order to minimize the effects of variation in the data, two microarray experiments were performed. To attain realistic and accurate analysis of the microarray data, the raw data of the two microarray experiments were analyzed separately and then genes were selected in each array according to the  $\geq 2$  fold change criteria mentioned above.

Intensity values of microarray data represent the expression level of each gene. In this study, some genes had consistent, similar intensity values between the two microarray experiments performed, but the others did not. I did not want to focus my work on genes with very low intensity values, ie very low mRNA expression, for three reasons: 1) Quantification by PCR might be problematic, 2) the apparent fold induction values (from one of the arrays, for example) might not be real if the intensity values were so low that they were close to the background, 3) if the mRNA levels of a particular gene were very low, it might be that the gene is not a very significant mediator of macrophage activity and phenotype. Intensity values of microarray data of selected genes induced under hypoxia in primary human MDMs are shown in figure 4.5. Glucose transporter (Glut-3) and sulfatase 2 (SULF2) have very similar values in both arrays for each condition. However, CCL28 and ITGA9 genes have very different intensity values between the 1<sup>st</sup> and 2<sup>nd</sup> arrays, (figure 4.5).



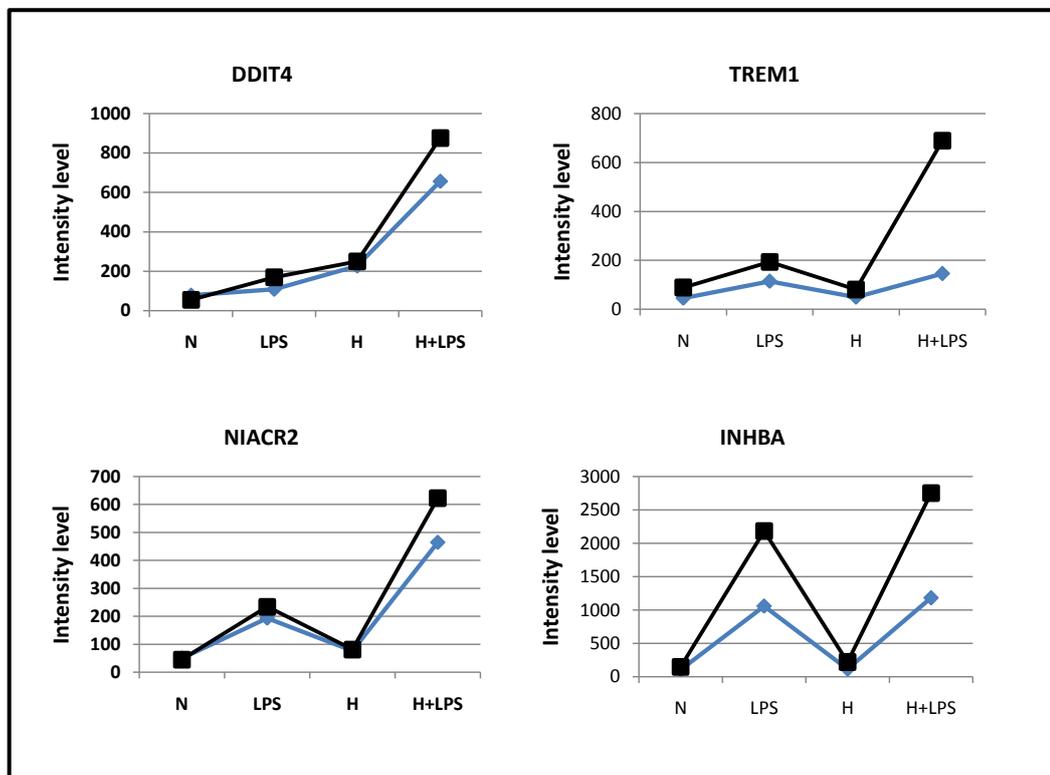
**Figure 4.5: Intensity expression level of selected hypoxia-inducible genes:** Four genes induced by hypoxia were selected from microarray data, glucose transporter 3 (Glut-3), sulfatase 2 (SULF2), Chemokine (C-C motif) ligand 28 (CCL28), and Integrin, alpha 9 (ITGA9). The four different conditions of spots (N, LPS, H, H plus LPS) are taken on X-axis and the intensity level is taken on Y-axis. This graph shows levels of mRNA expression of each particular gene when macrophages exposed to hypoxia for 18 hours. The red and blue lines are the 1<sup>st</sup> and 2<sup>nd</sup> array data, respectively.

Similar data were also found for some LPS-inducible genes. As shown in figure 4.6, Interleukin 1 alpha (IL-1 $\alpha$ ) and nuclear factor of kappa B (NF $\kappa$ B1) show similarity in both arrays data sets, but in contrast tumour necrosis factor (TNF) and CCL8 have different expression pattern in LPS and H+LPS treated samples (figure 4.6).



**Figure 4.6: Intensity expression level of selected genes induced by LPS:** Selected genes induced by LPS from microarray data, Interleukin 1, alpha (IL-1A), Nuclear factor of kappa B1 (NF $\kappa$ B1), tumour necrosis factor (TNF), and chemokine (C-C motif) ligand 8 (CCL8). The four different conditions of spots (N, LPS, H, H plus LPS) are taken on X-axis and the intensity level is taken on Y-axis. This graph shows levels of mRNA expression of each gene when macrophages exposed to LPS for 18 hours. The black and green lines with markers are 1<sup>st</sup> and 2<sup>nd</sup> array respectively.

Figure 4.7 shows the intensities of 4 selected synergy genes induced by combined hypoxia plus LPS. These transcripts produced by macrophages under hypoxia plus LPS were highly increased compared with hypoxia or LPS alone which indicates the synergistic effect of both conditions in macrophages. Two of them, DNA-damage-inducible transcript 4 (DDIT4) and Niacin receptor 2 (NIACR2) showed consistency in both arrays in each point; therefore, these two were included in the synergy gene list (Table 4.3).

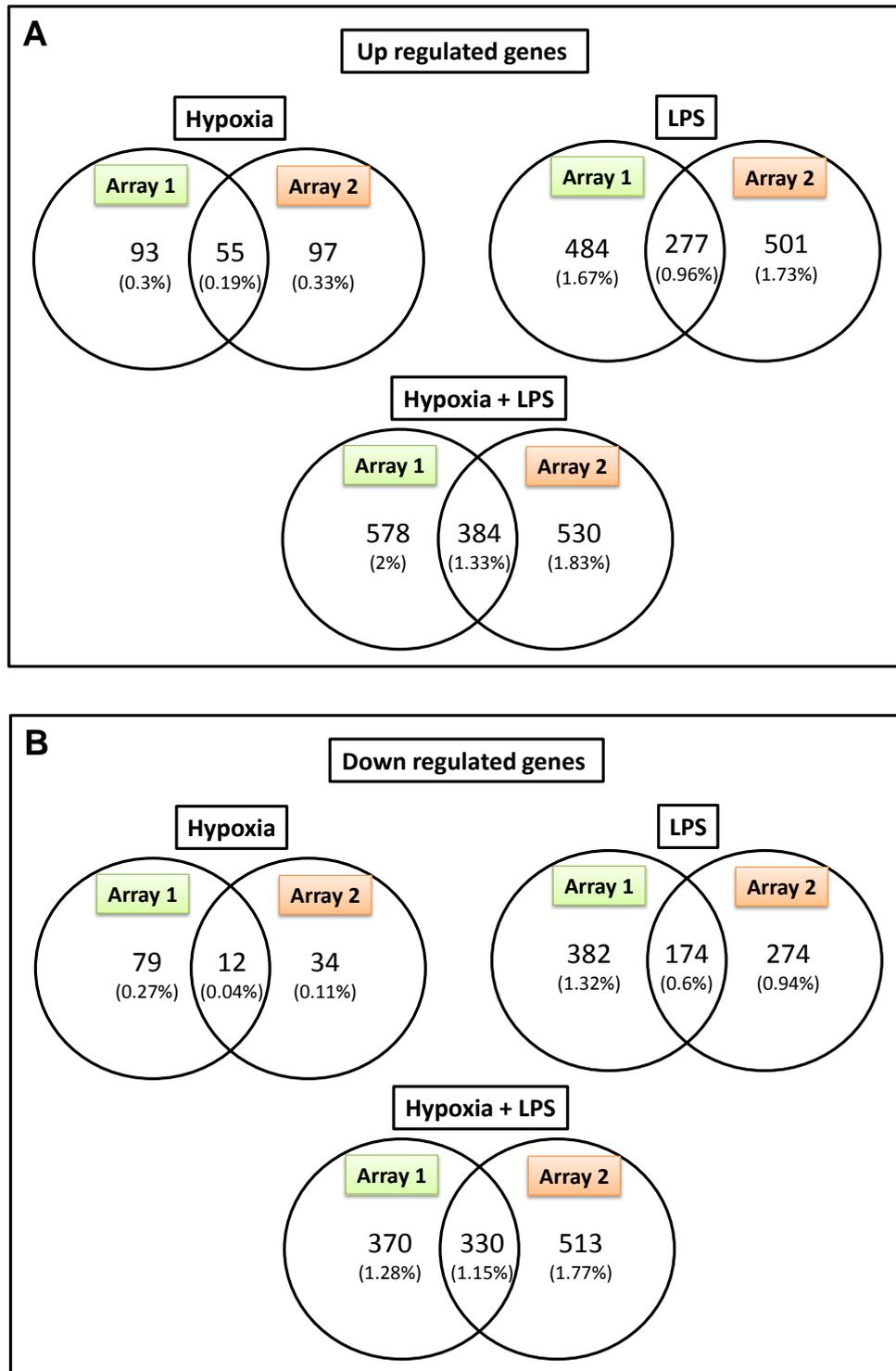


**Figure 4.7: Intensity expression level of selective synergy genes induced by combined treatment of hypoxia plus LPS:** Genes induced by hypoxia plus LPS were selected from microarray data, DNA-damage-inducible transcript 4 (DDIT4), Niacin receptor 2 (NIACR2), Triggering receptor expressed on myeloid cells 1 (TREM1), and Inhibin, beta A (INHBA). The four different conditions of spots (N, LPS, H, H+LPS) are taken on X-axis and the intensity level is taken on Y-axis. This graph shows levels of mRNA expression of each gene when macrophages exposed to combined treatment of hypoxia plus LPS for 18 hours. The black and blue lines represent the 1<sup>st</sup> and 2<sup>nd</sup> arrays, respectively.

Looking at figure 4.5, 4.6 and 4.7, it is clear that intensity values vary markedly between genes, and also between different experiments using cells from different donors. With this in mind it was decided to calculate fold induction values within each array data set, rather than by pooling and averaging across the two data sets. This made sure that high intensity values in one array did not dominate over lower intensity values in the other array when the two sets of microarray data were merged in order to calculate the average. For example; the hypoxic intensity value of PSAP, APLP2, FLNA, and PDIA3P genes in array 1 were 11788, 8561, 7505, and 3755 respectively, whereas, the hypoxic intensity of the same genes in array 2 are 8879, 5764, 5665, and 1609. As shown with these values, the intensity values in array 1 were higher than produced by array 2. In contrast, some genes have higher intensity values in array 2 than in array 1, for example the intensity of genes such as LRRC33, ERGIC1, PSENEN, and SCAMP2 in array 1 under hypoxia were 1177, 1708, 522, and 3475, but in array 2 the intensities for each gene are 2372, 2360, 2357, and 5582 respectively. Therefore, if the intensities from array 1 and 2 of a specific gene were merged together in order to calculate the average fold change of both, the differences between these two arrays disappear. This way of calculation may lead to false fold changes due to low intensity of either array. Therefore, in this project, fold induction of genes in each array were calculated separately using the intensity values and then the average of both was calculated.

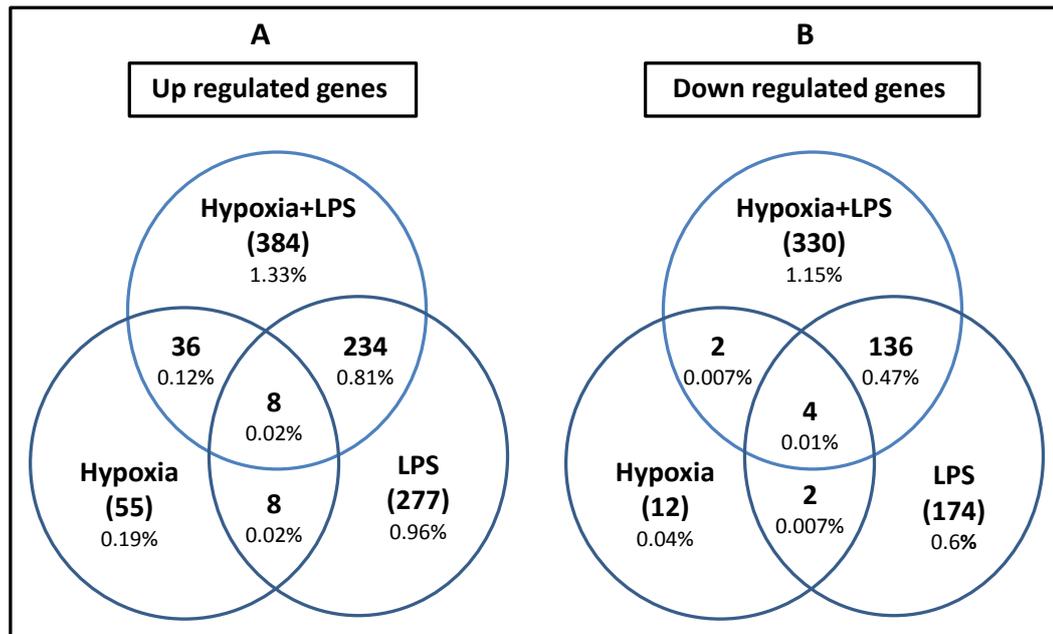
I observed in my data that most genes transcripts remained unchanged by the different treatments. As seen in figure 4.8 A, approximately 0.3%, 1.67%, and 2% of the genes were up-regulated by hypoxia, LPS, or by hypoxia plus LPS respectively. This percentage is in respect to the total number of interrogated transcripts in gene chip

which around (28,869 genes). Similar findings were obtained looking at down-regulated genes. As indicated in figure 4.8 B, only 0.27% and 0.11% of genes were down-regulated by hypoxia in array 1 and 2 respectively. LPS suppressed 1.32% in array 1 whereas only 0.94% in array 2 of genes in macrophages. In addition, the data show that 1.28% and 1.77% of total number of genes were down-regulated by hypoxia plus LPS in arrays 1 and 2 respectively.



**Figure 4.8: Two circle Venn diagrams showing the number of regulated genes found in common between arrays 1 and 2:** Primary human macrophages were exposed to hypoxia (0.2 % O<sub>2</sub>) in the presence or absence of 100ng/ml LPS for 18 hours. After removing replicate and un-annotated transcripts, data represent the total numbers of genes up- or down-regulated (A and B respectively) by 2 fold or more in each treatment. The percentage of modulated genes was calculated in respect to the total number of interrogated transcripts in the gene chip (in brackets).

Only genes that were significantly differentially up- or down-regulated in both duplicate array experiments (as found in the overlapping areas of the two-circle Venn diagrams in Figure 4.9) were selected for further analysis in this project. The data from the two arrays were combined to obtain the average fold changes for genes found to be regulated by at least 2 fold in both arrays. As seen in figure 4.9, the data showed that 55 genes were up-regulated by hypoxia in both arrays, whereas only 12 genes were down-regulated by the same condition. Substantially more genes, 277, were up-regulated by LPS, and 174 genes were down-regulated. Moreover, the array data shows that 384 genes were found to be up-regulated by combined treatment by hypoxia plus LPS, and 330 genes were down-regulated. These numbers are higher than for the single treatments. This suggests synergy is taking place. In addition, some genes were found to be regulated by more than one treatment; these genes are enumerated (numbers without parentheses) in the overlapping segments of the Venn diagram (Fig 4.9).



**Figure 4.9: Venn diagrams of genes up/down-regulated in all conditions:** Primary human macrophages were exposed to hypoxia (0.2 % O<sub>2</sub>) in the presence or absence of 100ng/ml LPS for 18 hours. After removing replicate and un-annotated transcripts, data represent the total numbers of genes down- or up-regulated by 2 fold or more in each treatment (in brackets). The numbers of genes regulated by more than one treatment are shown in the relevant overlapping segments.

The Venn diagram in figure 4.9 shows that macrophages responded to the treatments by regulating gene expression in both directions, up- and down. There are 55 genes up-regulated by hypoxia, and 277 genes up-regulated by LPS and 384 up-regulated by combined hypoxia plus LPS. The total number of genes up-regulated by the combination of the two stimuli (384) is higher than the sum of those regulated by both separately (55+277=332), a difference of 52 extra up-regulated genes. This suggests that there is indeed interplay, ie synergy, between hypoxia and LPS which caused these additional 52 genes to be up-regulated is worthy of further investigation. This idea is further supported by the fact that only 8 genes were up-regulated independently by both hypoxia alone and by LPS alone, and yet the combination of the two caused up-regulation of 52 extra genes. As would be expected, we observed that the same 8 genes (MT1M, MT1G, MT1F, MT1JP, CP, ADM, CA12, and VEGF) were also found to be

up-regulated in all three conditions (figure 4.9 A). Interestingly, half of these 8 genes are metallothionein family members.

The array data showed that 330 genes were down-regulated by hypoxia plus LPS. Of these 330 genes, 136 were also down-regulated by LPS alone, but only 2 genes; GDF15 (growth differentiation factor 15) and TAF9B (TATA box binding protein (TAF)-associated factor 9B) were also down-regulated by hypoxia alone. Only 2 down-regulated genes were suppressed in common between hypoxia and LPS, SNORD79 (small nuclear RNA, C/D 79) and ASRGL1 (asparaginase like 1). In addition, 4 genes were found to be decreased in common by all three treatments,: EPAS1 (endothelial PAS domain protein 1, or HIF-2 $\alpha$ ), SLOC4C1 (solute carrier organic anion transporter member, 4C1), CDC42EP3 (CDC42 effector protein), and JAKMIP2 (Janus kinase and microtubule interacting protein 2) (figure 4.9 B).

Overall, the analysis of the array data showed that the number of genes up-regulated is higher than the number down-regulated, especially for treatment with hypoxia alone.

In this study I was especially interested in determining whether some genes were synergistically regulated by hypoxia and LPS. Therefore, tables 4.1 and 4.2 list synergistically regulated genes selected from the first and second microarray experiments, respectively. Then the average values of both tables were calculated as shown in table 4.3. The up-regulated genes synergistically regulated by hypoxia plus LPS listed in table 4.3 are genes which show low or moderate up-regulation under hypoxia or LPS, but with further up-regulation of at least  $\geq 2$  fold when combined treatment was applied. I have attempted to divide the genes listed in table 4.3 into

those in which there seems to be true synergy (“multiple” fold up-regulation by H plus LPS), and those in which there is an additive rather than truly synergistic effect.

Mucin 1 (MUC1) is one of the selected synergy genes which shows no change with either hypoxia nor LPS treatment, however when the hypoxia was combined with LPS, this synergistically up-regulated mucin 1 by 6.41 mean fold change relative to normoxia. The expression under combined treatment in the 2<sup>nd</sup> array is much more than in the 1<sup>st</sup> array, although both sets of data met the criteria. NOD2 and P4HA2 showed slight up-regulation by hypoxia or LPS. However, when hypoxia plus LPS were combined, both genes were up-regulated by 4.04 and 3.62 fold, respectively. Ceruloplasmin (CP), Metallothionein 1M (MT1M) and carbonic anhydrase XII (CA12) genes were up-regulated by hypoxia or LPS; the level of expression by either hypoxia or LPS is similar except for MT1M which showed induction by hypoxia more than by LPS. However, when these two factors (hypoxia plus LPS) were combined, synergy was observed in CP, MT1M and CA12.

My data also has shown some synergy genes were induced mainly by hypoxia such as BNIP3 (BCL2/adenovirus E1B 19kDa interacting protein 3), DDIT4 (DNA-damage-inducible transcript 4), PLOD2 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2), MT1M (metallothionein 1M), and VEGF (vascular endothelial growth factor). However, when LPS was added, their induction dramatically increased which indicates synergistic cooperation between hypoxia and LPS. In contrast, other synergy genes in table 4.3 were induced mainly by LPS such as NIACR2 (Niacin receptor 2), TRAIL (TNF-related apoptosis-inducing ligand), PLAC8 (Placenta-specific 8) and wnt5a (wingless-type MMTV integration site family, member 5A). Other genes such as CA12 (carbonic

anhydrase XII), INSIG2 (insulin induced gene 2), MUC1 (mucin 1, cell surface associated), NOD2 (nucleotide-binding oligomerization domain containing 2), and P4HA2 (prolyl 4-hydroxylase, alpha polypeptide II) have the same level of up-regulation by hypoxia and LPS, but again when these two factor present together, the up-regulation is more than with either treatment alone, which indicates synergy (table 4.3).

**Table 4.1: Fold change of selected synergy genes of the 1<sup>st</sup> array in human primary macrophages *in vitro*:** Selection of synergy genes in this table based on increases found in hypoxia plus LPS compared with hypoxia or LPS alone. The up-regulated genes synergistically regulated by hypoxia plus LPS listed in this table are genes which show low or moderate up-regulation under hypoxia or LPS, but with further up-regulation of at least  $\geq 2$  fold when combined treatment was applied. In addition, intensity values of both microarray experiments for these particular genes were considered (data not shown).

Gene	Gene description	Accession no.	Hypoxia	LPS	H+ LPS
CP	Ceruloplasmin (ferroxidase)	NM_000096	5.01	4.16	12.585
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	NM_004052	4.63	0.85	10.396
DDIT4	DNA-damage-inducible transcript 4	NM_019058	2.91	1.39	8.4437
CA12	Carbonic anhydrase XII	NM_001218	3.37	3.21	9.5778
NIACR2	Niacin receptor 2	NM_006018	1.53	3.88	9.3277
TRAIL	TNF-related apoptosis-inducing ligand	NM_003810	1.54	3.93	21.519
PLAC8	Placenta-specific 8	NM_016619	1.09	1.81	5.0473
INSIG2	Insulin induced gene 2	NM_016133	1.57	2.34	4.8958
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	NM_182943	2.34	0.85	11.748
WNT5A	Wingless-type MMTV integration site family, member 5A	NM_003392	1.02	1.89	12.995
MUC1	Mucin 1, cell surface associated	NM_001018016	1.12	1	2.9598
NOD2	Nucleotide-binding oligomerization domain containing 2	NM_022162	1.4	1.39	3.6248
MT1M	Metallothionein 1M	NM_176870	5.74	2.9	23.795
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	NM_004199	1.5	1.52	3.5839
VEGFA	Vascular endothelial growth factor A	NM_001025366	3.17	3.03	7.9031

**Table 4.2: Fold change of selected synergy genes of the 2<sup>nd</sup> array in human primary macrophages *in vitro*:** Selection of synergy genes based on marked increases in mRNA level in hypoxia plus LPS compared with hypoxia or LPS alone. The up-regulated genes synergistically regulated by hypoxia plus LPS listed are genes which show low or moderate up-regulation under hypoxia or LPS, but with further up-regulation of at least  $\geq 2$  fold when combined treatment was applied. In addition, intensity values of both microarray experiments for these particular genes were considered (data not shown).

Gene	Gene description	Accession no.	Hypoxia	LPS	H+ LPS
CP	Ceruloplasmin (ferroxidase)	NM_000096	3.7	4.86	21.652
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	NM_004052	7.2	1.77	17.2
DDIT4	DNA-damage-inducible transcript 4	NM_019058	4.6	3.09	15.995
CA12	Carbonic anhydrase XII	NM_001218	4.6	3.64	15.156
NIACR2	Niacin receptor 2	NM_006018	1.8	5.19	13.838
TRAIL	TNF-related apoptosis-inducing ligand	NM_003810	1.8	5.09	11.7848
PLAC8	Placenta-specific 8	NM_016619	1	5.46	12.952
INSIG2	Insulin induced gene 2	NM_016133	1.1	1.56	3.3295
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	NM_182943	4.6	1.13	12.872
WNT5A	Wingless-type MMTV integration site family, member 5A	NM_003392	0.9	2.84	6.1167
MUC1	Mucin 1, cell surface associated	NM_001018016	1.3	1.44	9.8656
NOD2	Nucleotide-binding oligomerization domain containing 2	NM_022162	1.8	1.60	4.4716
MT1M	Metallothionein 1M	NM_176870	6.7	4.89	14.26
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	NM_004199	1.8	1.76	3.6758
VEGFA	Vascular endothelial growth factor A	NM_001025366	4.6	2.62	9.3163

**Table 4.3: Average fold change (array 1 + array 2) of selected synergy genes up-regulated by combined Hypoxia plus LPS treatment in human primary macrophages *in vitro*:** Genes showing marked (> 2 fold) increase in expression in response to Hypoxia+LPS compared with Hypoxia or LPS treatment alone. In addition, intensity values of both microarray experiments for these particular genes were considered (data not shown). Genes with high and consistent intensity values in both microarray data sets were selected. All values in this table represent the average of two experiments in each condition.

Gene	Gene description	Accession no.	Hypoxia	LPS	H+ LPS	Type of synergy
CP	Ceruloplasmin (ferroxidase)	NM_000096	4.35	4.51	17.11	Multiplying
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	NM_004052	5.91	1.31	13.79	Multiplying
DDIT4	DNA-damage-inducible transcript 4	NM_019058	3.75	2.24	12.21	Multiplying
CA12	Carbonic anhydrase XII	NM_001218	3.98	3.42	12.36	Multiplying
NIACR2	Niacin receptor 2	NM_006018	1.66	4.53	11.58	Multiplying
TRAIL	TNF-related apoptosis-inducing ligand	NM_003810	1.67	4.51	16.65	Multiplying
PLAC8	Placenta-specific 8	NM_016619	1.04	3.63	8.99	Multiplying
INSIG2	Insulin induced gene 2	NM_016133	1.33	1.95	4.11	Additive
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	NM_182943	3.47	0.99	12.31	Multiplying
WNT5A	Wingless-type MMTV integration site family, member 5A	NM_003392	0.96	2.36	9.55	Multiplying
MUC1	Mucin 1, cell surface associated	NM_001018016	1.21	1.22	6.41	Multiplying
NOD2	Nucleotide-binding oligomerization domain containing 2	NM_022162	1.60	1.49	4.04	Multiplying
MT1M	Metallothionein 1M	NM_176870	6.22	3.89	19.02	Multiplying
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	NM_004199	1.65	1.64	3.62	Additive
VEGFA	Vascular endothelial growth factor A	NM_001025366	3.88	2.82	8.60	Additive

My microarray experiments generated a lot of data which I have divided into six further tables according to the type of treatment and data. Tables 4.4 and 4.5 list genes up- and down-regulated under acute hypoxia respectively, tables 4.6 and 4.7 contain genes up- or down-regulated by LPS, whereas tables 4.8 and 4.9 include genes up- or down-regulated by combined treatment.

Macrophages are versatile cells which respond rapidly to hypoxia by altering gene expression and adapting their metabolic activity. Hypoxia increased the activity of a wide array of enzymes involved in energy production and intracellular glucose transport to enhance survival under hypoxia. As seen in table 4.4, aldolase C (ALDOC) and members of the glucose transporter family (Glut-1, 3, and 5) were up-regulated in human MDMs by acute hypoxia. Furthermore, table 4.4 showed up-regulation of receptors such as FCGR2A (Fc fragment of IgG, low affinity IIa, receptor (CD32)), CD180, and CD300A molecules, all membrane proteins involved in macrophage immune responses. Macrophages represent the first line of defence against bacteria and other microorganisms, so it is not surprising to find so many genes related to the immune response. In addition to that, genes involved in cell signalling and transcription such as SULF2, COLEC12 and TMEM51 were also up-regulated in primary human MDMs under hypoxia, presumably to facilitate a rapid response to the new stimulus. In infection, reduced oxygen tension is expected due to blood vessel damage and high consumption of oxygen by bacteria, and macrophages exposed to hypoxia up-regulated genes facilitating recruitment of leukocytes to these hypoxic sites, such as versican (VCAN), tensin (TNS1), and integrin (ITGA9), table 4.4.

**Table 4.4: Fold change of genes up-regulated by hypoxia in primary human macrophages *in vitro*:** The average fold changes of genes up-regulated by hypoxia in both arrays were calculated. Then genes with an average of  $\geq 2$  fold change relative to normoxia were selected for this table.

Function	Gene symbol	Gene description	Accession no.	Fold change		
				1 <sup>st</sup> array	2 <sup>nd</sup> array	Average
<b>Glucose/amino acids transport</b>						
	Glut-1	Glut-1 (glucose transporter), member 1	NM_006516	4.27	4.98	4.62
	Glut-3	Glut-3 (facilitated glucose transporter), member 3	NM_006931	3.35	3.41	3.38
	Glut-5	Glut-5 (glucose/fructose transporter), member 5	NM_003039	3.80	4.92	4.36
	GATM	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	NM_001482	4.28	3.16	3.72
<b>Glycolytic metabolism</b>						
	ALDOC	Aldolase C	NM_005165	4.49	7.45	5.97
	GYS1	Glycogen synthase 1 (muscle)	NM_002103	2.52	2.38	2.45
	PGM1	Phosphoglucomutase 1	NM_002633	2.19	2.30	2.24
	ARRDC3	Arrestin domain containing 3	NM_020801	2.65	2.80	2.72
	PDK1	Pyruvate dehydrogenase kinase, isozyme 1	NM_002610	2.98	2.49	2.73
	GBE1	Glucan (1,4-alpha-), branching enzyme 1	NM_000158	2.26	2.89	2.57
	LDHA	Lactate dehydrogenase A	NM_005566	2.18	2.09	2.13
	SLC25A19	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	NM_001126121	2.06	2.04	2.05
<b>Nonglycolytic metabolism</b>						
	CA12	Carbonic anhydrase XII	NM_001218	3.37	4.63	4.0
	MT1M	Metallothionein 1M	NM_176870	5.74	9.74	7.74
	P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I	NM_000917	3.31	3.16	3.23
<b>Immune responses</b>						
	C5orf20	Chromosome 5 open reading frame 20	NM_130848	4.43	4.19	4.31
	CD180	CD180 molecule	NM_005582	4.04	2.02	3.03
	CCL28	Chemokine (C-C motif) ligand 28	NM_148672	2.79	4.20	3.49
	SLAMF9	SLAM family member 9	NM_033438	2.49	2.99	2.74
	CD300A	CD300a molecule	NM_007261	2.43	2.42	2.42
	FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	NM_001136219	2.95	2.65	2.80

	IL18BP	Interleukin 18 binding protein	NM_173042	2.81	2.41	2.61
<b>Cell signalling/transcription</b>						
	SULF2	Sulfatase 2	NM_018837	6.35	5.14	5.74
	TMEM51	Transmembrane protein 51	NM_001136218	2.52	2.05	2.28
	COLEC12	Collectin sub-family member 12	NM_130386	2.38	2.10	2.24
	ZNF395	Zinc finger protein 395	NM_018660	3.37	3.30	3.33
	KDM3A	Lysine (K)-specific demethylase 3A	NM_018433	3.10	2.19	2.64
<b>Adhesion/stress</b>						
	VCAN	Versican	NM_004385	5.96	2.60	4.28
	TNS1	Tensin 1	NM_022648	2.61	2.94	2.77
	ITGA9	Integrin, alpha 9	NM_002207	2.53	2.69	2.61
	DDIT4	DNA-damage-inducible transcript 4	NM_019058	2.90	4.56	3.73
	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063	2.78	2.24	2.51
<b>Angiogenesis</b>						
	ADM	Adrenomedullin	NM_001124	4.07	4.94	4.50
	VEGF	Vascular endothelial growth factor A	NM_001025366	3.16	4.64	3.90

**Table 4.5: Fold change of genes down-regulated by hypoxia in primary human macrophages *in vitro*:** Average fold changes of genes down-regulated by hypoxia in both arrays were calculated. Then genes with an average of  $\leq 0.5$  fold decrease relative to normoxia were selected for this table.

Function	Gene symbol	Gene description	Accession no.	Fold change		
				1 <sup>st</sup> array	2 <sup>nd</sup> array	Average
<b>Apoptosis</b>						
	AIFM1	Apoptosis-inducing factor, mitochondrion-associated, 1	NM_001130847	0.23	0.48	0.35
<b>Cell activity/viability</b>						
	TOMM22	Translocase of outer mitochondrial membrane 22 homolog (yeast)	NM_020243	0.37	0.45	0.41
	TAF9B	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa	NM_015975	0.47	0.45	0.46
<b>Tissue differentiation/proliferation</b>						
	GDF15	Growth differentiation factor 15	NM_004864	0.33	0.46	0.39
	HBEGF	Heparin-binding EGF-like growth factor	NM_001945	0.38	0.48	0.43
<b>Transcription</b>						
	SNORD79	Small nucleolar RNA, C/D box 79	NR_003939	0.46	0.39	0.42
<b>Stress</b>						
	EPAS1	Endothelial PAS domain protein 1	NM_001430	0.38	0.46	0.42
	HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	NM_001530	0.42	0.50	0.46
<b>Transporters</b>						
	SLCO4C1	Solute carrier organic anion transporter family, member 4C1	NM_180991	0.31	0.45	0.38
	TFRC	Transferrin receptor (p90, CD71)	NM_003234	0.44	0.30	0.37

LPS is a potent activator of immune system cells including macrophages. Indoleamine 2,3-dioxygenase 1 (IDO1), Interleukin 1, beta (IL-1 $\beta$ ), and Interferon-induced protein 44-like (IFI44L) were markedly up-regulated by LPS in primary human MDMs, and these three genes have the highest up-regulation levels observed in the whole of my microarray data, (Table 4.6).

The intracellular activation of macrophages in response to LPS results in the release of a huge array of inflammatory mediators such as tumour necrosis factor (TNF) and interleukin 6 (IL-6). IL-6 is highly induced by LPS as seen in table 4.6, and is a potent inducer of the acute phase response and plays an essential role in the final differentiation of B-cells into Ig-secreting cells which are needed to produce antibodies to neutralize foreign antigens. Microarray data also showed up-regulation of many different chemoattractants induced by LPS such as CCL1, CCL8, CCL5, and CCL20 which are chemotactic factors that attract monocytes, lymphocytes, basophils, T cells, eosinophils, and neutrophils to the inflamed tissues (table 4.6).

The microarray data also elucidated genes up-regulated by LPS involved in controlling cell growth and proliferation such as IFITM1 (Interferon induced transmembrane protein 1) and epiregulin (EREG) respectively (table 4.6). My data showed that exposure of primary human macrophages to LPS induces a distinct set of transcription factors involved in regulation of many genes. These transcription factors include signal transducer and activator of transcription 4 (STAT4), NF $\kappa$ B1 and NF $\kappa$ BIZ. NF $\kappa$ B1 and NF $\kappa$ BIZ are two subunits involved in regulation of NF-kappa-B transcription factor complexes. Furthermore, the data revealed that LPS can up-regulate some genes that facilitate invasion of cells: integrin beta 8 (ITGB8), CD38, matrix metalloproteinase 14

(MMP14), and integrin beta 3 (ITGB3); (table 4.6). Both hypoxia and LPS can also suppress expression of some genes in primary hMDM (tables 4.5 and 4.7).

**Table 4.6: Fold change of genes up-regulated by LPS in primary human macrophages *in vitro*:** Average fold changes of up-regulated genes by LPS in both arrays were calculated. Then the average fold changes of genes with  $\geq 2$  fold change relative to normoxia were selected for this table.

Function	Gene symbol	Gene description	Accession no.	Fold change		
				1 <sup>st</sup> array	2 <sup>nd</sup> array	Average
<b>Immune response</b>						
	IDO1	Indoleamine 2,3-dioxygenase 1	NM_002164	88.99	175.78	132.38
	IL1B	Interleukin 1, beta	NM_000576	82.76	91.79	87.27
	IFI44L	Interferon-induced protein 44-like	NM_006820	65.92	49.03	57.47
	IL6	Interleukin 6 (interferon, beta 2)	NM_000600	13.35	77.28	45.31
	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	NM_007115	32.58	42.34	37.46
	IL1A	Interleukin 1, alpha	NM_000575	32.13	31.48	31.80
	PTGS2	Prostaglandin-endoperoxide synthase 2	NM_000963	13.40	27.92	20.66
	CFB	Complement factor B	NM_001710	16.80	22.24	19.52
	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	NM_001548	24.65	9.45	17.05
	CLEC4E	C-type lectin domain family 4, member E	NM_014358	7.87	14.17	11.02
<b>Chemoattraction</b>						
	CCL1	Chemokine (C-C motif) ligand 1	NM_002981	40.04	63.42	51.73
	CCL8	Chemokine (C-C motif) ligand 8	NM_005623	55.71	26.14	40.92
	CCL5	Chemokine (C-C motif) ligand 5	NM_002985	45.63	29.69	37.66
	CCL20	Chemokine (C-C motif) ligand 20	NM_004591	5.52	48.21	26.86
	CXCL1	Chemokine (C-X-C motif) ligand 1	NM_001511	10.06	8.32	9.19
	IL8	Interleukin 8	NM_000584	28.50	17.65	23.05
	CCR7	Chemokine (C-C motif) receptor 7	NM_001838	5.16	20.58	12.86
<b>Signaling/cell-cell interaction</b>						
	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	NM_007115	32.58	42.34	37.46
	SLAMF1	Signaling lymphocytic activation molecule family member 1	NM_003037	13.14	11.44	12.29
	THBS1	Thrombospondin 1	NM_003246	5.58	12.47	9.02
	RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	NM_005739	6.90	8.19	7.54
	EMR1	egf-like module containing, mucin-like, hormone receptor-like 1	NM_001974	4.90	7.39	6.14
<b>Cell growth/proliferation</b>						

	IFITM1	Interferon induced transmembrane protein 1	NM_003641	5.37	6.30	5.83
	LHFP	Lipoma HMGIC fusion partner	NM_005780	2.18	10.45	6.31
	EREG	Epiregulin	NM_001432	3.46	6.30	4.88
	MET	met proto-oncogene (hepatocyte growth factor receptor)	NM_001127500	3.08	5.69	4.38
	TNF	Tumor necrosis factor (TNF superfamily, member 2)	NM_000594	2.64	6.09	4.36
<b>Transcription</b>						
	STAT4	Signal transducer and activator of transcription 4	NM_003151	11.47	24.21	17.84
	NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	NM_031419	5.42	4.68	5.05
	IRF1	Interferon regulatory factor 1	NM_002198	4.31	3.65	3.98
	PARP14	Poly (ADP-ribose) polymerase family, member 14	NM_017554	3.74	3.77	3.75
	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_003998	3.30	4.08	3.69
<b>Cell viability</b>						
	SERPINB2	Serpine peptidase inhibitor, (ovalbumin) member 2	NM_001143818	10.60	16.28	13.43
<b>Cell adhesion/migration</b>						
	ITGB8	Integrin, beta 8	NM_002214	37.63	40.2	38.91
	CD38	CD38 molecule	NM_001775	13.66	40.5	27.08
	SIGLEC1	Sialic acid binding Ig-like lectin 1, sialoadhesin	NM_023068	7.31	11	9.15
	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	NM_000212	4.18	3.29	3.73
	MMP14	Matrix metalloproteinase 14 (membrane-inserted)	NM_004995	2.66	3.74	3.20
<b>Apoptosis</b>						
	XAF1	XIAP associated factor 1	NM_017523	10.20	7.58	8.89
	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	NM_006187	6.62	5.69	6.15
	GPR132	G protein-coupled receptor 132	NM_013345	3.13	7.12	5.12
	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	NM_003810	3.93	5.09	4.51
	CASP5	Caspase 5, apoptosis-related cysteine peptidase	NM_004347	2.86	6.01	4.43
	OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	NM_016816	3.97	3.25	3.61

**Table 4.7: Fold change of genes down-regulated by LPS in primary human macrophages *in vitro*:** Average fold changes of genes up-regulated by LPS in both arrays were calculated. Then genes with  $\leq 0.5$  average fold decrease relative to normoxia were selected for this table.

Function	Gene symbol	Gene description	Accession no.	Fold change		
				1 <sup>st</sup> array	2 <sup>nd</sup> array	Average
<b>Cell metabolism</b>						
	FABP4	Fatty acid binding protein 4, adipocyte	NM_001442	0.17	0.16	0.16
	PDK4	Pyruvate dehydrogenase kinase, isozyme 4	NM_002612	0.40	0.29	0.34
	GGTA1	Glycoprotein, alpha-galactosyltransferase 1	NR_003191	0.42	0.30	0.36
	GPR120	G protein-coupled receptor 120	NM_181745	0.45	0.17	0.31
	Glut-5	Glut-5 (glucose/fructose transporter), member 5	NM_003039	0.36	0.38	0.37
<b>Cell proliferation/migration</b>						
	PRKACB	Protein kinase, cAMP-dependent, catalytic, beta	NM_182948	0.26	0.21	0.23
	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	NM_002204	0.31	0.16	0.23
	CCND2	Cyclin D2	NM_001759	0.31	0.37	0.34
	NCAPH	Non-SMC condensin I complex, subunit H	NM_015341	0.43	0.32	0.37
	ZNF589	Zinc finger protein 589	NM_016089	0.45	0.31	0.38
	SGMS2	Sphingomyelin synthase 2	NM_001136258	0.42	0.38	0.40
	DOK2	Docking protein 2, 56kDa	NM_003974	0.37	0.44	0.40
<b>Immune response</b>						
	CD180	CD180 molecule	NM_005582	0.32	0.23	0.27
	CD300LB	CD300 molecule-like family member b	NM_174892	0.30	0.20	0.25
	TREM2	Triggering receptor expressed on myeloid cells 2	NM_018965	0.42	0.28	0.35
	SCARB1	Scavenger receptor class B, member 1	NM_005505	0.31	0.42	0.36
	HLA-DMB	Major histocompatibility complex, class II, DM beta	NM_002118	0.37	0.44	0.40
	FAIM3	Fas apoptotic inhibitory molecule 3	NM_005449	0.38	0.44	0.41
	CD22	CD22 molecule	NM_001771	0.40	0.43	0.41
	CD163	CD163 molecule	NM_004244	0.21	0.42	0.32
	CR1	Complement component (3b/4b) receptor 1	NM_000573	0.48	0.36	0.42
<b>Adhesion</b>						
	ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	NM_000885	0.39	0.34	0.36

**Table 4.8: Fold change of genes up-regulated by combined treatment of hypoxia plus LPS in primary human macrophages *in vitro*:** Average fold changes of genes up-regulated by hypoxia plus LPS in both arrays were calculated. Then genes with  $\geq 2$  fold average change relative to normoxia were selected for this table.

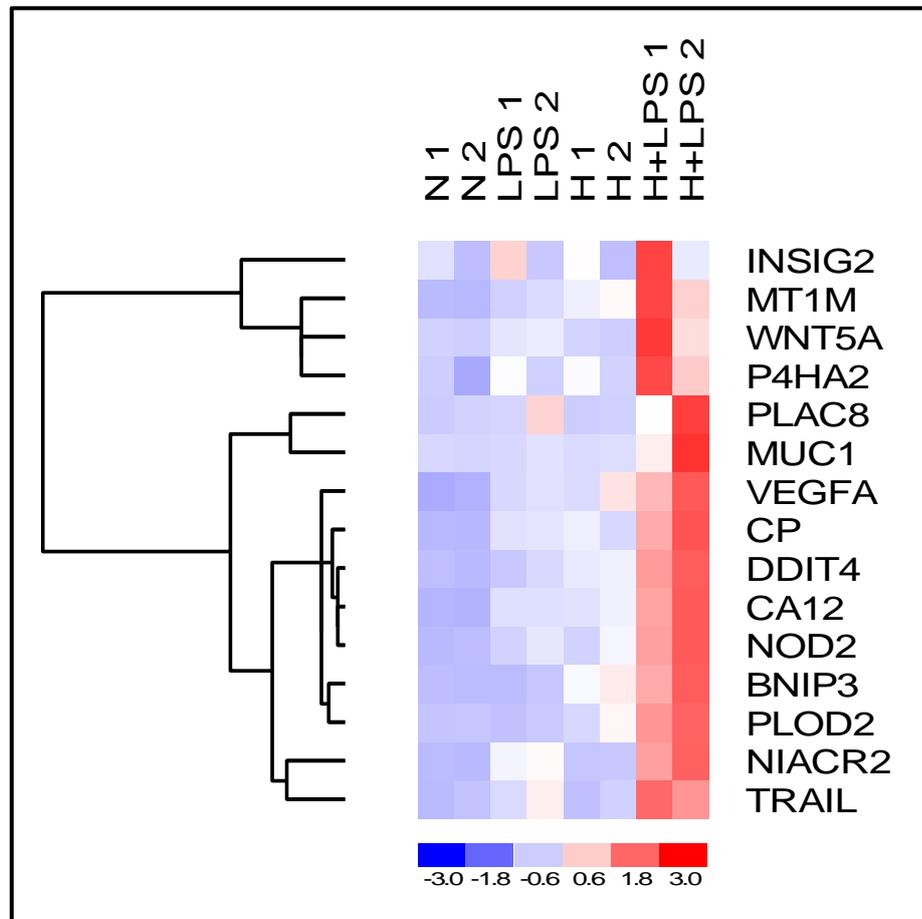
Function	Gene symbol	Gene description	Accession no.	Fold change		
				1 <sup>st</sup> array	2 <sup>nd</sup> array	Average
<b>Immune response</b>						
	IRG1	Immunoresponsive 1 homolog (mouse)	ENST00000377462	68.79	22.16	45.47
	PTGS2	Prostaglandin-endoperoxide synthase 2	NM_000963	46.24	44.45	45.34
	IL1A	Interleukin 1, alpha	NM_000575	38.17	35.14	36.65
	NCF1	Neutrophil cytosolic factor 1	NM_000265	12.10	5.69	8.89
	OLR1	Oxidized low density lipoprotein (lectin-like) receptor 1	NM_002543	7.63	4.16	5.89
	TREM1	Triggering receptor expressed on myeloid cells 1	NM_018643	7.74	3.21	5.47
	NOD2	Nucleotide-binding oligomerization domain containing 2	NM_022162	3.62	4.47	4.04
<b>Apoptosis</b>						
	TRAIL	TNF-related apoptosis-inducing ligand	NM_003810	21.51	6.78	14.14
	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	NM_004052	10.39	17.19	13.79
	NLRP3	NLR family, pyrin domain containing 3	NM_004895	4.010	2.47	3.24
	DDIT4	DNA-damage-inducible transcript 4	NM_019058	8.44	15.99	12.21
<b>Angiogenesis/growth</b>						
	INHBA	Inhibin, beta A	NM_002192	11.56	18.83	15.19
	VEGFA	Vascular endothelial growth factor A	NM_001025366	7.90	9.31	8.60
	ANGPTL4	Angiopoietin-like 4	NM_139314	2.07	7.06	4.56
	PLAC8	Placenta-specific 8	NM_016619	5.04	12.95	8.99
<b>Adhesion/migration</b>						
	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	NM_001547	22.65	3.66	13.15
	CERCAM	Cerebral endothelial cell adhesion molecule	NM_016174	2.56	5.07	3.81
	WNT5A	Wingless-type MMTV integration site family, member 5A	NM_003392	12.99	6.11	9.55
	MUC1	Mucin 1, cell surface associated	NM_001018016	2.95	9.86	6.40
<b>Metabolism/cell integrity</b>						
	PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	NM_182943	11.74	12.87	12.30
	CP	Ceruloplasmin (ferroxidase)	NM_000096	12.58	21.65	17.11
	CA12	Carbonic anhydrase XII	NM_001218	9.57	15.15	12.36

**Table 4.9: Fold change of genes down-regulated by combined treatment of hypoxia plus LPS in primary human macrophages *in vitro*:** Average fold changes of genes down-regulated by hypoxia plus LPS in both arrays were calculated. Then genes with  $\leq 0.5$  average fold decrease relative to normoxia were selected for this table.

Function	Gene symbol	Gene description	Accession no.	Fold change		
				1 <sup>st</sup> array	2 <sup>nd</sup> array	Average
<b>Intracellular transporter</b>						
	SLC26A11	Solute carrier family 26, member 11	NM_173626	0.20	0.18	0.19
	SLCO2B1	Solute carrier organic anion transporter family, member 2B1	NM_001145211	0.23	0.28	0.25
<b>Immune response/stress</b>						
	TLR5	Toll-like receptor 5	NM_003268	0.26	0.17	0.21
	HLA-DMB	Major histocompatibility complex, class II, DM beta	NM_002118	0.21	0.25	0.23
	ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	NM_000689	0.31	0.23	0.27
	FGD6	FYVE, RhoGEF and PH domain containing 6	NM_018351	0.31	0.28	0.29
<b>Migration/proliferation</b>						
	PLAU	Plasminogen activator, urokinase	NM_002658	0.22	0.41	0.31
	SUCNR1	Succinate receptor 1	NM_033050	0.36	0.29	0.32
	PRKACB	Protein kinase, cAMP-dependent, catalytic, beta	NM_182948	0.13	0.10	0.11
	ITGA6	Integrin, alpha 6	NM_000210	0.15	0.21	0.18
	GDF15	Growth differentiation factor 15	NM_004864	0.20	0.21	0.20
<b>Transcription</b>						
	EPAS1	Endothelial PAS domain protein 1	NM_001430	0.10	0.12	0.11
<b>Cell viability</b>						
	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	NM_032717	0.17	0.14	0.15
	DNASE2B	Deoxyribonuclease II beta	NM_021233	0.17	0.07	0.12
	TAF9B	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa	NM_015975	0.36	0.15	0.25

The heat map is a way to visualize microarray data which uses colours to represent data values in a two-dimensional image. Microarray heat maps are a special type of heat map used to highlight gene expression in molecular biology and it is a very useful way to compact a large amount of information into a small space to bring out easy ways to analyse the data from different angles (Weinstein, 2008). In the case of gene expression data, the colour assigned to a point in the heat map grid indicates how much of a particular RNA is expressed in a given sample, and this is represented by a given intensity number. The heat maps were generated using D-chip software which was downloaded from the following web site (<http://biosun1.harvard.edu/complab/dchip/download.htm>). As seen in Figure 4.10 the gene expression level is generally indicated by red for high expression and blue for low expression. This coloured grid is linked by a dendrogram (a tree diagram) to hierarchically cluster genes and it helps find groups of genes which behave similarly across a set of different treatments. Generally, the rows of a microarray heat map represent genes with each column of that row representing a different sample. Figure 4.10 shows a heat map of synergy genes (Taken from table 4.3). The data in the heat map can be viewed in two dimensional profiles; the first is gene expression profile which examines the expression of particular gene in a different treatment (Row), and the second is test expression profile which examines the expression of a specific treatment in a different gene set (Column). The two columns of test expression labelled with hypoxia+LPS 1 and 2 show similarity in most genes listed except for some genes such as the PLAC8 and MUC1 genes show less expression in array 1 than array 2 in treated samples by hypoxia+LPS. Also INSIG shows higher expression in array 1 than array 2 in the same treatment. Most genes listed in figure 4.10 panel A show no

expression by neither hypoxia nor LPS which mean no significant effect of hypoxia or LPS in gene induction, however when combined treatment was applied, the expression of these genes are markedly increased.



**Figure 4.10: Heat map images of synergy genes up-regulated by hypoxia plus LPS in microarray data:** Genes highly up-regulated by combined treatment of hypoxia plus LPS selected from table 4.3. The intensity value of each treated sample was converted to a heat map image represented by two colours. The intensity of colour refers to the intensity of gene expression level: *Red* refers to the up-regulated, and *Blue* refers to the down-regulated genes. This image shows distinct difference between hypoxia or LPS alone and combined hypoxia plus LPS treatment in human macrophages in synergy genes.

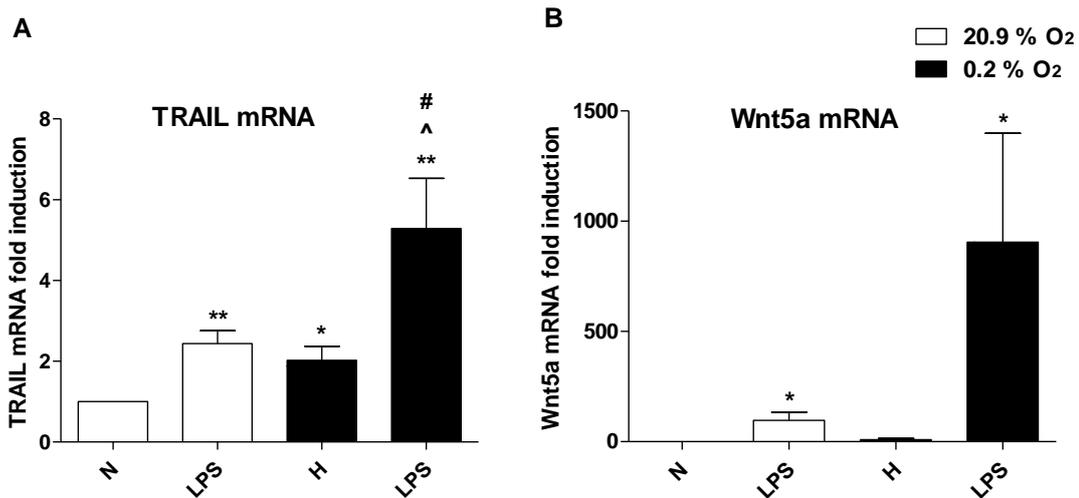
### **4.3.5 Verification of microarray data of selected synergy genes by quantitative real-time PCR**

It is important to verify microarray data using a second technique such as RT-PCR, because with so many genes on an array, and given that the array experiment was only done twice, it is likely that some spurious values will be obtained. The effect of hypoxia (0.2% O<sub>2</sub>) on selected synergy genes was elucidated by microarray analysis in primary human MDMs as seen in Table 4.3. Six genes were chosen to be confirmed by real time RT-PCR: DDIT4, CA12, NIACR2, TRAIL, Wnt5a, and MT1M. I chose these genes because they showed similar trends for each treatment between the 1<sup>st</sup> and 2<sup>nd</sup> array. As previously described in section 4.3.1, primary human MDMs purified from adherent cells of PBMC obtained from 12 different healthy donors, in 12 independent experiments conducted in different weeks, were cultured in 10 ml complete filtered Iscove's medium. Cells were either cultured under normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) in the presence or absence of 100 ng/ml of LPS for a further 18 hours. RNA was isolated and separated into two aliquots; one used in hybridization for microarray experiments and the other one was used for reverse transcription to generate cDNA for quantitative real-time RT-PCR. Two lots of samples were used for the two microarray experiments (1<sup>st</sup> and 2<sup>nd</sup> lot), and these were also used to confirm the up-regulation of selected synergy genes by real-time RT-PCR (Figs 4.11, 4.12, and 4.13). In addition to the two donors used in microarray experiments (1<sup>st</sup> and 2<sup>nd</sup> lot) 1 and 2, another six lots of samples from different healthy donors were used for synergy gene confirmation by RT-PCR (3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, and 9<sup>th</sup> lots), so samples from eight donors were used in real-time PCR (see table 4.10).

Quantitative real-time RT-PCR was carried out for selected synergy genes (TRAIL, wnt5a, DDIT4, CA12, MT1M, and NIACR2), which were found to be markedly up-regulated by combined treatment (Hypoxia plus LPS) in the microarray data (Table 4.3).

As seen in figure 4.11 panel A, the expression of TRAIL is significantly increased by combined treatment of hypoxia plus LPS compared to either treatment alone (P value  $\leq 0.05$ ), indicating the synergistic effect of hypoxia plus LPS when combined together, in accordance with the microarray data.

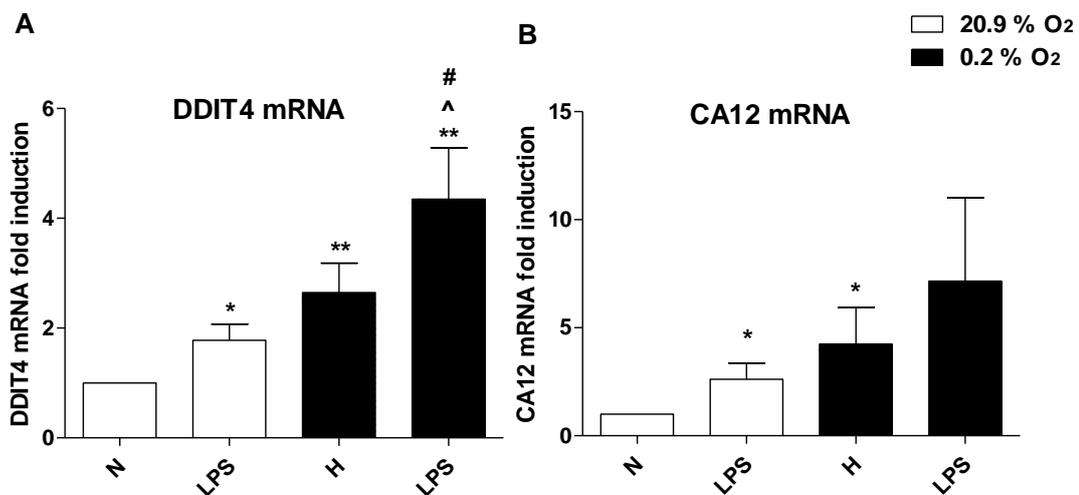
The mRNA expression of the wnt5a gene was also investigated in primary HMDMs. As seen in figure 4.11 panel B, wnt5a mRNA was markedly increased by hypoxia plus LPS and this data was statistically significant when compared with normoxia (P value  $\leq 0.05$ ). However, this up-regulation is not statistically significant when compared with treatment with hypoxia or LPS alone due to the high variability among the different donors.



**Figure 4.11: RT-PCR confirmation of up-regulated synergy genes TRAIL and wnt5a:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells then were treated with 100 ng/ml of LPS and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior to RNA isolation. (A) TNF-related apoptosis-inducing ligand (TRAIL) and (B) wingless-type MMTV integration site family, member 5A (wnt5a) mRNA expression were quantified by quantitative real-time RT-PCR then data were normalized with  $\beta$ -2M mRNA levels in order to calculate fold induction. Data from 8 independent experiments using different donors expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-tests. \* = P value  $\leq$  0.05 considered as significant when compared with normoxia, \*\* = p  $\leq$  0.01 when compared with normoxia, ^ = P value  $\leq$  0.05 when compared with hypoxia, # = P value  $\leq$  0.05 when compared with LPS sample.

Another two synergy genes, DDIT4 and CA12, were also verified by real time RT-PCR. As shown in table 4.3, both DDIT4 and CA12 were induced moderately by either hypoxia or LPS, however when both treatments (hypoxia plus LPS) were applied together, hypoxia and LPS synergistically up-regulated the mRNA of both genes. Quantitative RT-PCR analysis confirmed this synergy in DDIT4 (figure 4.12 A). The statistical analysis showed significant up-regulation by hypoxia plus LPS in comparison with hypoxia alone or LPS alone (P value  $\leq$  0.05). DDIT4 has been found to be hypoxia-inducible in previous studies (Shoshani *et al*, 2002) through the HIF-1 pathway. My real-time RT-PCR data has shown that up-regulation of DDIT4 by LPS is statistically significant (P value  $\leq$  0.05) when compared with normoxic sample (figure 4.12 A),

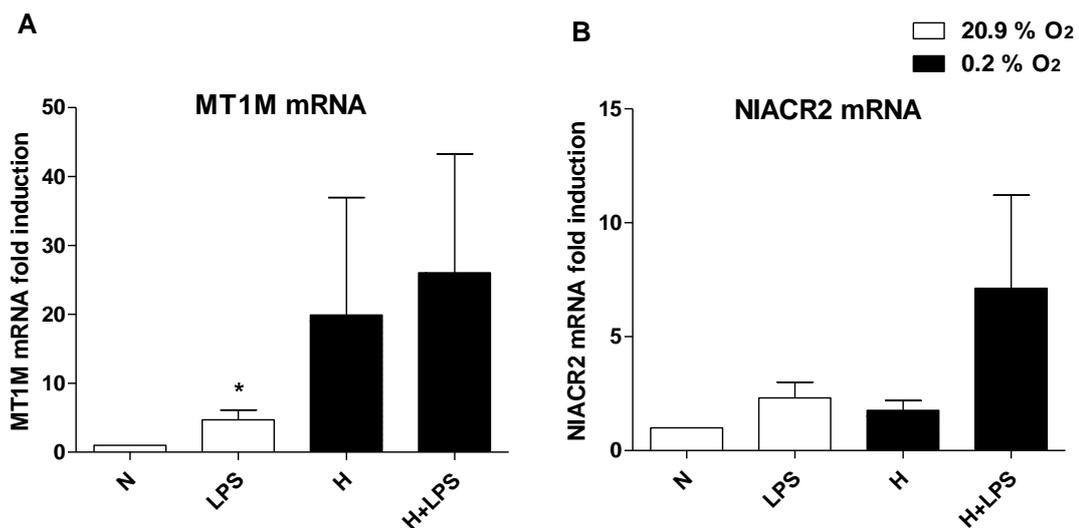
concordant with my array data (Table 4.3). To my knowledge this is the first study to show up-regulation of DDIT4 by LPS alone and hypoxia plus LPS in primary human macrophages. In addition, RT-PCR analysis showed up-regulation of CA12 by combined treatment with hypoxia plus LPS (figure 4.12 B). However, this up-regulation is not significant when hypoxia plus LPS was compared with hypoxia or LPS alone. This may be due to the huge variation in between the donors in this particular gene. RT-PCR showed significant up-regulation of CA12 by either hypoxia or LPS samples when compared to normoxia ( $P$  value  $\leq 0.05$ ), and this up-regulation was consistent with array data as shown in table 4.3 shows (4.0 and 3.4 fold change for hypoxia and LPS respectively).



**Figure 4.12: RT-PCR confirmation of up regulated synergy genes by combined treatment with hypoxia and LPS.** Primary human macrophages were incubated under normoxic condition for 5 days. Cells then were treated with 100 ng/ml and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior the RNA isolation. (A) DNA-damage-inducible transcript 4 (DDIT4), and (B) Carbonic anhydrase XII (CA12) mRNA expression were quantified by quantitative real-time RT-PCR then data were normalized with  $\beta$ -2M mRNA levels in order to calculate fold induction. Data from 8 independent experiments using different donors expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-tests. \* =  $P$  value  $\leq 0.05$  considered as significant when compared with normoxia, \*\* =  $p \leq 0.01$  when compared with normoxia, ^ =  $P$  value  $\leq 0.05$  when compared with hypoxia, # =  $P$  value  $\leq 0.05$  when compared with LPS sample.

The confirmation by real-time RT-PCR also has extended to another two putative synergy genes, metallothionein 1M (MT1M) and Niacin receptor 2 (NIACR2). As

indicated in figure 4.13, treatment by hypoxia plus LPS markedly induced mRNA expression of MT1M and NIACR2 in human MDMs. However, this up-regulation of both genes is not statistically significant. Significant up-regulation of MT1M by only LPS was found when compared with the normoxia sample (P value  $\leq 0.05$ ), (figure 4.13 A). It was noted that mRNA induction of MT1M in all treatment conditions is much higher than found with NIACR2. The data indicate that the up-regulation of MT1M mRNA by hypoxia plus LPS is not synergistic, because it is not different from the induction observed with hypoxia alone, or in fact from the untreated normoxic sample, because of the large error bars (figure 4.13).



**Figure 4.13: RT-PCR confirmation of up regulated synergy genes by combined treatment hypoxia and LPS.** Primary human macrophages were incubated under normoxic condition for 5 days. Cells then were treated with 100 ng/ml and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior the RNA isolation. (A) Metallothionein 1M (MT1M) and (B) Niacin receptor 2 (NIACR2) mRNA expression were quantified by quantitative real-time RT-PCR then data were normalized with  $\beta$ -2M mRNA levels in order to calculate fold induction. Data from 8 independent experiments using different donors expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-test. \* = P value  $\leq 0.05$  considered as significant when compared with normoxia.

Table 4.10 shows a comparison between array and real-time RT-PCR data. Two sets of total RNA samples were used for the two microarray experiments (shaded columns

with green and orange colours for 1<sup>st</sup> and 2<sup>nd</sup> array respectively). These two RNA samples were also used for RT-PCR to verify the microarray data. Another six sets of samples obtained from healthy donors were used only for RT-PCR (3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, and 9<sup>th</sup> lots in un-shaded columns). The mean values of the array and RT-PCR data are shown in bold. Six synergy genes from table 4.3, TRAIL, wnt5a, DDIT4, CA12, MT1M, and NIACR2, were confirmed by RT-PCR as seen in figures 4.11, 4.12, and 4.13.

**Table 4.10: Microarray and RT-PCR data comparison of six selected synergy genes from table 4.3, TRAIL, wnt5a, DDIT4, CA12, MT1M, and NIACR2, were analysed by RT-PCR:** Two lots of total RNA samples (the 1<sup>st</sup> and 10<sup>th</sup> lots) were used for the two microarray experiments (shaded columns). These two RNA samples were also used for RT-PCR to verify microarray data. Another six sets of samples obtained from healthy donors were used only for RT-PCR (3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, and 9<sup>th</sup> lots). The means of array and RT-PCR data are shown in bold. The standard deviation ( $\sigma$ ) was calculated in each treatment for each gene.

Gene	Condition	Array data		Mean	RT-PCR data								Mean	Standard Deviation ( $\sigma$ )
		1 <sup>st</sup> array	2 <sup>nd</sup> array		1 <sup>st</sup> lot	3 <sup>rd</sup> lot	4 <sup>th</sup> lot	5 <sup>th</sup> lot	6 <sup>th</sup> lot	8 <sup>th</sup> lot	9 <sup>th</sup> lot	10 <sup>th</sup> lot		
TRAIL	H	1.53	1.8	<b>1.66</b>	1.33	3.61	1.50	2.23	3.12	1.33	2.25	0.79	<b>2.02</b>	<b>2.29</b>
	LPS	3.93	5.09	<b>4.51</b>	2.33	1.69	0.89	2.90	3.36	1.88	3.52	2.90	<b>2.43</b>	<b>0.84</b>
	H+LPS	21.51	11.78	<b>16.64</b>	9.11	8.97	0.51	6.27	1.28	5.48	10.74	8.39	<b>6.34</b>	<b>3.5</b>
Wnt5a	H	1.01	0.92	<b>0.96</b>	2.08	7.90	1.05	2.32	1.43	4.16	47.59	5.45	<b>8.99</b>	<b>14.74</b>
	LPS	1.89	2.84	<b>2.36</b>	8.98	125.34	3.32	28.22	2.04	123.1	294.2	188.3	<b>96.68</b>	<b>99.35</b>
	H+LPS	12.99	6.11	<b>9.55</b>	16.91	1251	5.10	113.55	1.60	415.6	1369	4071	<b>905.5</b>	<b>1302.98</b>
DDIT4	H	2.90	4.56	<b>3.73</b>	0.75	3.32	1.39	1.96	2.42	2.72	5.78	2.81	<b>2.64</b>	<b>1.414</b>
	LPS	1.39	3.09	<b>2.24</b>	0.63	1.47	1.34	1.98	2.57	1.10	1.83	3.26	<b>1.77</b>	<b>0.785</b>
	H+LPS	8.44	15.99	<b>12.21</b>	2.76	7.44	1.08	4.71	1.09	4.85	4.62	8.23	<b>4.34</b>	<b>2.472</b>
CA12	H	3.37	4.63	<b>4</b>	1.18	3.18	0.49	9.08	3.57	0.22	13.80	2.45	<b>4.24</b>	<b>4.460</b>
	LPS	3.21	3.64	<b>3.42</b>	0.57	2.66	0.23	6.14	3.45	0.33	4.37	3.13	<b>2.61</b>	<b>1.986</b>
	H+LPS	9.57	15.15	<b>12.36</b>	2.15	32.88	0.40	11.18	2.32	1.82	4.71	1.74	<b>7.15</b>	<b>10.221</b>
MT1M	H	5.74	6.7	<b>6.22</b>	139.1	0.15	1.58	1.44	3.20	7.40	1.18	5.22	<b>19.90</b>	<b>45.105</b>
	LPS	2.9	4.89	<b>3.89</b>	10.16	2.95	1.11	3.48	2.59	11.85	3.10	2.32	<b>4.69</b>	<b>3.725</b>
	H+LPS	23.79	14.26	<b>19.02</b>	145.3	6.80	0.62	20.13	1.99	20.12	3.53	9.84	<b>26.04</b>	<b>45.630</b>
NIACR2	H	1.53	1.8	<b>1.66</b>	2.5	1.08	0.94	0.54	4.01	1.28	2.80	0.90	<b>1.75</b>	<b>1.133</b>
	LPS	3.88	5.19	<b>4.53</b>	0.57	5.92	0.97	4.41	2.60	0.82	0.77	2.40	<b>2.30</b>	<b>1.776</b>
	H+LPS	9.32	13.83	<b>11.57</b>	2.22	34.99	0.43	8.12	2.01	2.01	5.88	1.30	<b>7.12</b>	<b>10.805</b>

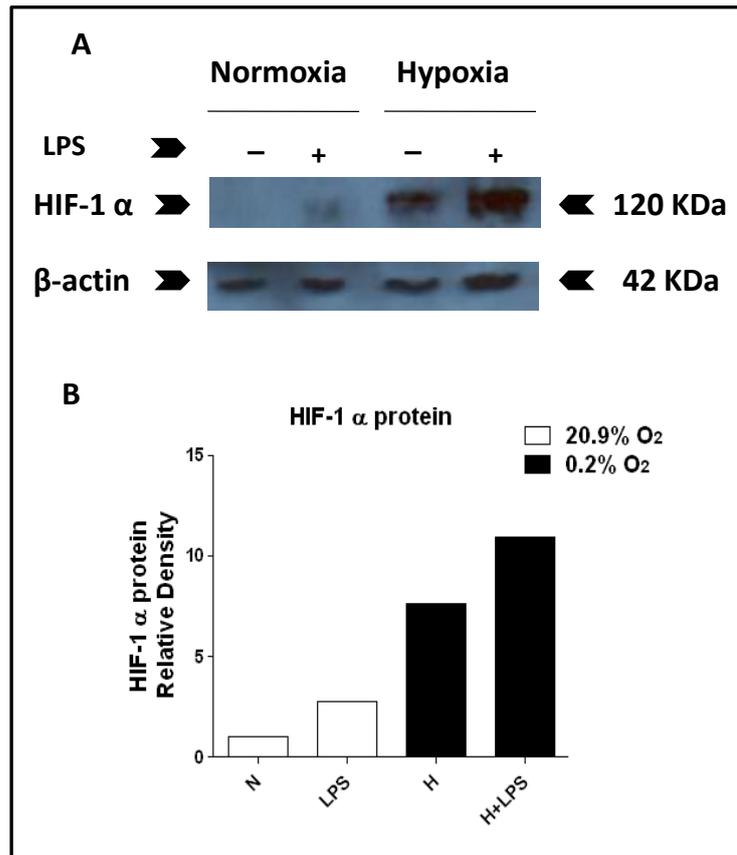
### **4.3.6 Investigation of synergy between hypoxia and LPS in mediating HIF-1 $\alpha$ protein and mRNA up-regulation in primary human macrophages**

A logical starting place to examine the possible mechanisms responsible for the apparent synergistic effects of hypoxia and LPS on expression of numerous genes was to determine the effects on HIF-1 $\alpha$  levels. Extensive evidence has demonstrated that induction and activation of HIF-1 occurs in response to many non-hypoxic stimuli (Dery *et al*, 2005). LPS was found to up-regulate HIF-1 $\alpha$  protein levels, and this was shown to be mediated by transcriptional up-regulation of the HIF-1 $\alpha$  gene, (Blouin *et al*, 2004). This represents a distinct mechanism from hypoxic up-regulation of HIF-1 $\alpha$ , which occurs primarily by protein stabilisation due to inhibition of proteolytic degradation initiated by prolyl hydroxylases which require oxygen (Semenza, 2009). Treatment with LPS in normoxia causes increased expression of many hypoxia-inducible genes that are known to be regulated by HIF-1 in hypoxia (Frede *et al*, 2006; Oh *et al*, 2008; Westra *et al*, 2010).

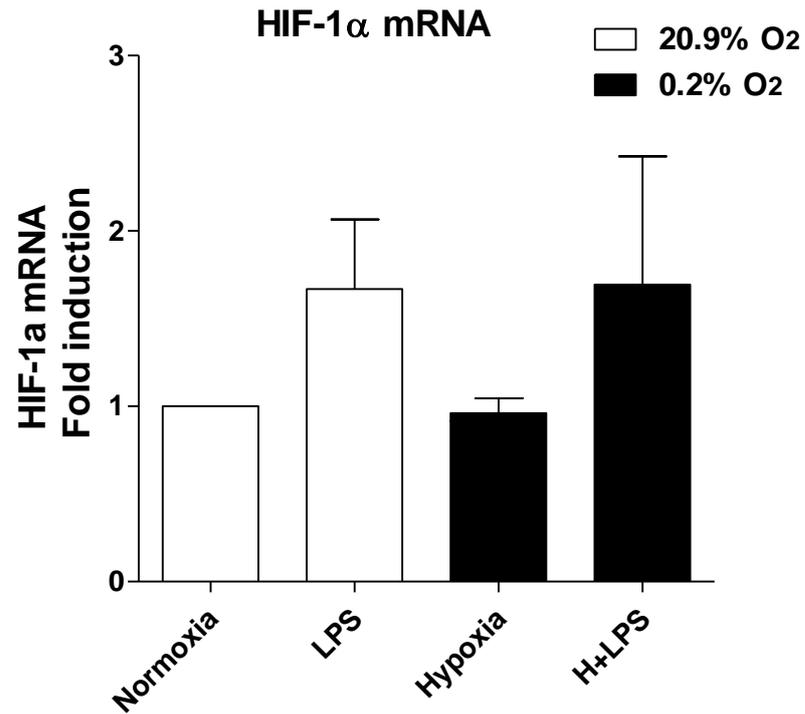
HIF-1 $\alpha$  protein is present at low levels in most cells, including macrophages (Burke *et al.*, 2002) under normal oxygen tension. As expected in (figure 4.14 panel A), hypoxia markedly up-regulated HIF-1 $\alpha$  protein in primary hMDM cells in comparison with normoxia. No band was detected in normoxia which means that the HIF-1 $\alpha$  protein level was very low, as expected. The molecular weight of HIF-1 $\alpha$  protein is about 120 kDa and  $\beta$ -actin (42 kDa) is shown as a loading control for each sample. LPS increased levels of HIF-1 $\alpha$  protein in normoxia. HIF-1 $\alpha$  protein induction by LPS in normoxia is consistent with previous studies (Blouin *et al*, 2004). However it should be noted that levels of the HIF-1 $\alpha$  protein in response to LPS in normoxia are not at as high as under

hypoxia without LPS treatment. Importantly, cells treated with combined hypoxia plus LPS show more HIF-1 $\alpha$  protein than with hypoxia alone, suggesting a co-operative effect of hypoxia and LPS in these cells. It was important to determine the effects of hypoxia and LPS on HIF-1 $\alpha$  protein levels in my hMDM model, since PBMC isolation and cell culture media and incubation conditions could affect this. It is interesting to note that even the severe hypoxia used in this study (0.2%O<sub>2</sub>) did not produce maximal HIF-1 $\alpha$  protein accumulation; addition of LPS to hypoxic cultures was able to further increase the already high levels.

In order to examine the effect of these treatments on mRNA level, HIF-1 $\alpha$  mRNA levels were also analyzed in hMDMs by RT-PCR. As figure 4.14 B shows, the HIF-1 $\alpha$  mRNA levels in both normoxia and hypoxia are similar which indicates, as previously reported, that HIF-1 $\alpha$  regulation by hypoxia predominately occurs at the protein level (Semenza, 2009). However, LPS increased HIF-1 $\alpha$  mRNA levels in normoxic and hypoxic samples, consistent with previous reports in primary mouse bone MDMs, rat alveolar macrophage-derived cell line cells (Blouin *et al*, 2004) and in the mouse macrophage cell line ANA-1 (Mi *et al*, 2008).



**Figure 4.14: Effect of hypoxia and LPS on HIF-1 $\alpha$  protein in primary human macrophages:** Primary human macrophages (hMDM) were incubated under normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours with or without treatment with 100ng/ml LPS. (A) Equal amounts of total cellular protein was examined for presence of HIF-1 $\alpha$  protein by Western blot analysis. The same membrane was re-probed for  $\beta$ -actin as loading control. (B) The intensity of HIF-1 $\alpha$  bands was measured by densitometry and normalised against the intensity of  $\beta$ -actin bands. Representative data obtained from 1 donor are shown.



**Figure 4.15: Effect of hypoxia and LPS on HIF-1 $\alpha$  mRNA in primary human macrophages:** Primary human macrophages (hMDM) were incubated under normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours with or without treatment with 100ng/ml LPS. HIF-1 $\alpha$  mRNA expression was measured by quantitative real-time RT-PCR. Data was normalized against the housekeeping gene  $\beta$ -2M. Data from 6 independent experiments using different donors are shown, expressed as means  $\pm$  SEM.

## 4.4 Discussion

Using microarray technology, the current study set out to investigate whether combined treatment of primary human macrophages with hypoxia plus LPS can induce expression of certain genes which were not highly induced when only one treatment was applied.

Macrophages accumulate in poorly vascularised tissues characterized by hypoxia (low oxygen tension) including human tumours, myocardial infarction, atherosclerotic plaques, rheumatoid arthritis, and sites with bacterial infection (Lewis *et al*, 1999; Vaupel *et al*, 2001). In serious wounds, hypoxia is often present due to the damage to the blood supply and high consumption of oxygen by microorganisms and leukocytes including macrophages (Lewis *et al*, 1999). To elucidate the interactions between two of the important stimuli found in such sites, hypoxia and microbial compounds such as LPS, in this chapter, the effect of short-term (18 hours) hypoxia in the presence of LPS on global gene expression was investigated in human MDMs.

The working hypothesis was that there would be some synergy between LPS and hypoxia in regulating gene expression in primary human macrophages.

The use of microarray technology is a complex, multistep process which allows gene expression levels in cellular samples to be quantified. As with any complex process, each step can contribute variability to the final measurements. Some of this variability is intrinsic to the biological properties of the samples being studied, and other variability is due to sample processing such as the quality and amount of RNA extracted from the sample and the length of time between collecting and processing the sample. In addition, the complexity of the microarray instruments used may

introduce a large amount of technical variability. Properly analysed microarray data is important to build a solid foundation for subsequent further investigation, therefore, I collaborated with an expert, Dr. Wenbin Wei from The University of Birmingham, who agreed to analyse the raw microarray data I obtained. In addition, definite criteria were formulated for gene selection in both arrays as stated in section 4.3.4. Genes were defined as being differentially regulated by hypoxia, LPS, or hypoxia plus LPS if they exhibited  $\geq 2$  fold increase, or a  $\leq 0.5$  fold decrease compared to untreated normoxic cultures. Based on those criteria, the data showed that relatively few genes were differentially expressed in arrays 1 and 2 compared to the number of transcripts analysed by the whole genome arrays used (figure 4.8). A slightly lower number of genes were found to be modulated in common between both arrays. This indicates that some modulated genes observed in only one array are random, occurring due to methodological and technological variation, or related to differences between the donors. However, considering the fact that there are 28,869 genes represented on the arrays used, the number of non-reproducible transcripts found between the two array experiments is very small, and the degree of overlap of the two gene sets from array 1 and 2 is considerable (greater than 50% genes in common between both arrays in all treatment conditions: Figure 4.8), indicating that the data is in general quite reproducible and reliable. Even assuming a “random” hit rate of 1% (higher than that observed in our data, as indicated in figure 4.8), ie 1 in 100, analysis of probability indicates that it is unlikely that more than a handful of genes (1 in 100 x 100, ie 1 in 10,000) would be found to be consistently up- or down-regulated between both arrays. For arrays analysing expression of 28,869 genes, this should equate on average

to 2.9 genes, in each treatment condition, which would be incorrectly found to be regulated in both of the two entirely independent array experiments.

The average of both arrays was calculated as shown in figure 4.9. My data showed that 55 genes were up-regulated in common by hypoxia in both arrays and 384 genes were found to be up-regulated by combined treatment by hypoxia plus LPS. Among these two treatments, only 36 genes were found on both. This indicates that some hypoxia inducible genes were suppressed when LPS was added, and these are 19 genes in total. The same scenario was found with LPS inducible genes (277 genes), when hypoxia was added only 234 genes were up-regulated in both arrays, which mean there are 43 LPS-inducible genes which were suppressed by hypoxia. These findings suggest that many genes in hypoxia plus LPS are regulated by LPS not by hypoxia. Furthermore, this could suggest that there is a new mechanism of regulation by which LPS or hypoxia block the up-regulation of hypoxia- or LPS-inducible genes respectively. This data indicate that human macrophages have a selective gene expression due to the presence of hypoxia or/and LPS. Interestingly, my data have shown that only 8 genes were up-regulated independently by hypoxia or LPS alone (MT1M, MT1G, MT1F, MT1JP, CP, ADM, CA12, and VEGF). In addition, the same 8 genes were also found up-regulated by hypoxia, LPS, and hypoxia plus LPS, (figure 4.9 A). This indicates these genes up-regulated by combined hypoxia plus LPS are consistent in up-regulation by the hypoxia or LPS alone.

For the first time, microarray data in the current study has shown that treatment of HMDMs by hypoxia plus LPS together for short period of time can synergizes to induce certain genes which were not induced by neither hypoxia nor LPS alone or just slightly induced by either hypoxia or LPS as seen in table 4.3.

Two types of synergy effect were observed in microarray data; additive and multiplying (Table 4.3). The multiplying type is more reliable and more significant than the additive type, and possibly indicates that these two factors (hypoxia plus LPS) were needed together to augment induction, suggesting that hypoxia and LPS are capable of intracellular cross-talk or cooperation to multiply their effect on macrophage gene expression. Hypoxia and LPS can activate a lot of transcription factors (TFs) to induce a diverse array of genes in mammalian cells (Cummins & Taylor, 2005). Most carefully selected synergy genes of my interest in table 4.3 are showing multiplying effect by combined treatment of hypoxia plus LPS.

As table 4.3 shows, mucin 1 (MUC1) is one of the selected synergy genes which shows no induction by either hypoxia or LPS treatment. However, when the hypoxia was combined with LPS, the two factors synergistically up-regulated mucin 1 expression by 6.41 fold change relative to normoxia. NOD2 (Nucleotide-binding oligomerization domain containing 2) and P4HA2 (Prolyl 4-hydroxylase, alpha polypeptide II) shows very slight up-regulation by hypoxia or LPS, and this slight elevation might be due to the intensity variation that usually occurred with such low intensity. However, when a combination of hypoxia plus LPS conditions were exposed to primary human MDMs, expression of both genes increased by 4.04 and 3.62 fold change for NOD2 and P4HA2 respectively. P4HA2 enzyme catalyzes the formation of 4-hydroxyproline that is essential to the proper hydroxylation processes of several proteins such as alpha subunit of HIF-1 $\alpha$  (Myllyharju, 2009).

Ceruloplasmin, is the main copper transport protein in the plasma (Mukhopadhyay *et al*, 1998) and it is known as a HIF-1 target gene in many cell type including

macrophages (Mukhopadhyay *et al*, 2000). The induction of ceruloplasmin was found due to the iron deficiency needed for HIF-1 $\alpha$  hydroxylation by PHDs hydroxylases, this subsequently leads accumulation of HIF-1 $\alpha$  protein and then translocation to the nucleus and dimerization with the constitutively expressed subunit HIF1- $\beta$ , to form functional active HIF1 transcription factor. Furthermore, Kimmura and colleagues have shown that levels of ceruloplasmin increased in the serum of rabbit subjected to LPS (Kimura *et al*, 1994). In this project, microarray data has shown for the first time that hypoxia and LPS can synergise together to induced ceruloplasmin expression mRNA in primary human macrophages. The up-regulation of ceruloplasmin by hypoxia plus LPS was 4 times more than either condition (17.11 fold change), see table 4.3. Since the ceruloplasmin is regulated by HIF-1 pathway under hypoxia, and LPS can accumulate HIF-1 $\alpha$  in normoxia (Blouin *et al*, 2004), so the synergistic up-regulation of ceruloplasmin in human macrophages might be also through the HIF-1 pathway.

BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3) was found up-regulated by hypoxia in my microarray data as well as in previous study (Igwe *et al*, 2009) in the macrophages cell line (RAW 264.7). In addition, another study has shown BNIP3 up-regulation in human embryonic kidney (HEK 293) cells (Kothari *et al*, 2003). According to my array data, LPS has no effect on BNIP3 expression in human macrophages, however, when LPS-treated cells were incubated under hypoxia, a significant synergistic effect was found on BNIP3 expression, suggesting that LPS may play a role in BNIP3 activation only under hypoxia (Table 4.3). DNA-damage-inducible transcript 4 (DDIT4), also called REDD1 (regulated in development and DNA damage response), is a stress-related protein triggered by adverse environmental conditions including

hypoxia. DDIT4 is expressed in multiple human tissues including macrophages, and it is a known hypoxia-inducible gene which is up-regulated under acute hypoxia through HIF1 pathway (Shoshani *et al*, 2002). Schwarzer and colleagues showed evidence of the anti-apoptotic role of DDIT4 in two different cell lines, in HaCaT keratinocytes and PC-3 prostate cancer cells. Their data revealed that down-regulated DDIT4 expression induced apoptosis in cancer cells as well (Schwarzer *et al*, 2005). DDIT4 was found to be moderately induced by LPS in the microarray data, and this up-regulation is consistent with real time RT-PCR data (figure 4.12 A). Furthermore, DDIT4 also was up-regulated by combined treatment (hypoxia plus LPS) and reached a level by 12.21 fold change relative to normoxic controls, suggesting a cooperative and synergistic effect of hypoxia plus LPS in this type of cells.

Four synergy genes were found unchanged by hypoxia, NIACR2, TRAIL, PLAC8, and wnt5a. However, in the presence of LPS they responded markedly by up-regulation of mRNA levels in primary human MDMs, this indicates that there is an intracellular link between hypoxia and LPS which can enhance the expression of these genes in that cell type (Table 4.3).

TRAIL (TNF-related apoptosis-inducing ligand), also called Apo-2L is a member of TNF superfamily (Beutler & van Huffel, 1994). TRAIL expression has been demonstrated in various tissues and organs (Pitti *et al*, 1996) including macrophages (Griffith *et al*, 1999), and can be found as membrane bound or soluble secreted forms that can bind to its specific receptors known as death receptors 4 and 5 (DR4/5) on the surface of target cells (Kimberley & Screatton, 2004). Various groups of researchers have shown that macrophages up-regulate expression of TRAIL at mRNA and protein by LPS,

interferon-alpha (IFN-alpha), -beta and -gamma (Halaas *et al*, 2004; Zheng *et al*, 2005; Genc *et al*, 2003), and this agreed with my microarray data in LPS finding. As seen in table 4.3, TRAIL markedly induced by exposure to LPS in primary human MDMs. However, hypoxia had a minor effect on TRAIL compared to LPS. Interestingly, hypoxia in combined with LPS synergistically up-regulated TRAIL expression in this type of cells. Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor controlling the transcription of many genes in response to hypoxia (Semenza, 1998). However, hypoxia also has been shown to activate a number of transcription factors such as activating protein-1 (AP-1) and Sp1 (Cummins & Taylor, 2005). For example, VEGF is a well known hypoxia-inducible gene regulated by HIF-1, and also has found to be partially regulated by AP-1 (Salnikow *et al*, 2002) and Sp1 (Lee *et al*, 2004) in response to hypoxia. The TRAIL promoter was identified as having binding sites for AP-1 and Sp1 (Wang *et al*, 2000), suggesting that this gene also regulated partially by hypoxia and this might be the cause of its slight induction under hypoxia in primary human MDMs.

Atherosclerosis is an inflammatory condition of arteries, and advanced atherosclerotic lesions are prone to rupture, leading to disability or death (Ross, 1999). Hypoxic areas are known to be present in human atherosclerotic lesions, and the lesion progression is associated with the formation of lipid-loaded macrophages which form what is called fatty streaks in the arterial wall, and this leads to increased local inflammation (de Villiers & Smart, 1999). Array data has shown significant up-regulation of gene encoded NIACR2 mRNA by combined hypoxia plus LPS. Several studies reported NIACR2 gene is involved in modulating inflammation by working as an anti-inflammatory agent to minimize exaggerated inflammatory injury (Yu & Zhao, 2007). A

study by Giri and colleagues has shown that NIACR2 in combination with taurine prevents the activation of NF- $\kappa$ B pathway, suggesting the role of macrophages to prevent dysfunction of endothelial cells at atherosclerotic lesions in the walls of arteries (Giri, 2003).

Wnt5a is a member of a large family of secreted protein that activate the highly conserved wnt pathway through their receptor Frizzled (Klaus & Birchmeier, 2008). Wnt–Frizzled signalling controls a wide variety of processes, including proliferation, differentiation, migration, and cell adhesion, playing a crucial role during development (Klaus & Birchmeier, 2008). Wnt signalling occurs through the canonical and non-canonical pathways. The canonical pathway has a central mediator,  $\beta$ -catenin. In the absence of wnt ligand,  $\beta$ -catenin is phosphorylated and ubiquitinated by the activity of a multiprotein destruction complex leading to degradation. However, binding of wnt to the Frizzled receptor leads to inhibition of the destruction complex through activation of the protein Dishevelled. Then,  $\beta$ -catenin is stabilized and translocated into the nucleus. This allows binding of  $\beta$ -catenin to transcription factors such as lymphoid enhancing factor-1 and T-cell factor, and leads to transcriptional activation of multiple target genes such as matrix metalloprotease 7 (MMP7) (Clevers, 2006).

Carbonic anhydrase XII (CA12) participates in a variety of biological processes, including respiration, and plays a role in acid-base balance in mammalian cells. Disturbed acid-base balance can cause low extracellular PH level and it is a feature of hypoxic tumour tissues due to increased high-lactate activity of hypoxic tumour cells. Significant expression of CA12 was found in many cancers including breast carcinomas and this was revealed by immunohistochemical staining (Watson *et al*, 2003). Using

microarray technology, other previous studies (Bosco *et al*, 2006; Fang *et al*, 2009) have also shown that CA12 is up-regulated by short-term hypoxia, and this data is consistent with the current study. Interestingly, hypoxia plus LPS synergistically up-regulated CA12 mRNA expression in primary human MDMs (Table 4.3).

PLOD2 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2) is a hydroxylase enzyme that is localized to the cisternae of the rough endoplasmic reticulum in eukaryotic cells, and plays an essential role in the formation and the stabilization of the intermolecular collagen cross-links. The excessive accumulation of collagen causes what is called fibrosis which is mainly modulated by hyperactivity of PLOD2 gene expression (AJ, 2003). Hofbauer and colleague have found that PLOD2 mRNA is up-regulated in rat vascular smooth muscle cell line A7r5 under hypoxia and also in cells treated by hypoxia mimics deferoxamine and cobalt chloride. Furthermore, they have shown the essential role of HIF in triggering PLOD2 by using mouse embryonic fibroblasts lacking the HIF-1 $\alpha$  subunit gene which showed suppressed induction of PLOD2 mRNA comparing with wild type (Hofbauer *et al*, 2003). As shown in table 4.3, primary human MDMs also responded to acute hypoxia but not to LPS by up-regulating PLOD2 mRNA. However, when both (Hypoxia plus LPS) were combined, its expression was synergistically enhanced and reached up to 12.09 fold change relative to normoxic controls.

Metallothionein 1M (MT1M) is among the differentially expressed genes synergistically regulated by hypoxia plus LPS. The metallothionein (MT) gene family encodes proteins with high affinity to metals such as zinc and cadmium and are regulated by metal ion concentrations (Andrews, 1990). These proteins contribute to a number of

fundamental processes involved in metal metabolism and detoxification, chemoresistance, cell proliferation, apoptosis, and protection against several types of cell damage (Miles *et al*, 2000). Several previous studies demonstrated that chronic hypoxia induces the expression of metallothionein (Kojima *et al*, 2009). Tumour hypoxic sites in human prostate cancer tissue are characterized by high expression of metallothionein and the down-regulation of MT by siRNA resulted in inhibition of cell growth and induced apoptosis (Yamasaki *et al*, 2007).

Three different test conditions were used in this project: hypoxia, LPS, and combined hypoxia plus LPS, and this generated a huge amount of data (Tables 4.4 to 4.9). When the microarray data in this project is compared with the previous work, some similarities were found. For example, ADM, BNIP3, Glut-1, CA12, and VEGFA were found up-regulated by hypoxia in the studies of (Bosco *et al*, 2006; Burke *et al*, 2003; Fang *et al*, 2009; Fong *et al*, 2007) as well as the present study. In addition to that, genes such as DDIT4, FCGR2B, ALDOC, P4HA1, Glut-3, Glut-5, and ENO2 were found up-regulated only by Fang, and Bosco *et al*, and also this project. The array data in this present work shows that human macrophages respond to hypoxia by up-regulating various genes with fold changes ranging from 2.2 to 5.9 fold change. The consistency of up-regulated genes under hypoxia listed above with previous studies goes some way to indicate that the microarray data is reliable.

It should be noted that there are some differences between data presented here and the data from previous reports. For example, some genes that are involved in the inflammatory response such as IL-1 $\beta$ , IL-8, NCF, IL-6, ADORA2A, SERPINB2, IL-1 $\alpha$ , and CCR7 were found up-regulated by LPS here in this data (Table 4.6). However, one of

the previous papers (Fang *et al*, 2009) has shown these genes to be up-regulated by hypoxia. This could be due to contamination of medium with LPS, not the effect of hypoxia alone, and might be due to synergy. Also, one of the two arrays data presented in the Fang *et al*. study gives higher fold change than the other. For example, IL-8 (give 2 and 43 fold change) and IL-6 (give 62 and 7 fold change) in response to hypoxia by Affymetrix and Illumina arrays respectively (Fang *et al*, 2009). These data indicate that using different array platforms to assess gene expression may give different aberrant levels of up-regulation for the same tested genes.

Also, the differences seen between the data in this project comparing with previous studies could be due to the severity of hypoxic conditions and the period of time applied on the cells. For example, in this current study 18h of 0.2% O<sub>2</sub> was used, however 8h with 1% O<sub>2</sub>, 6h with 0.1% O<sub>2</sub>, and 18h with 0.1% O<sub>2</sub> were used in the studies of (Bosco *et al*, 2006; Burke *et al*, 2003; Fang *et al*, 2009) respectively.

Cells in hypoxia tend to maintain survival by induction of several genes known to be involved in glycolytic energy production. As shown in table 4.4, glucose transporter genes such as Glut-1, 3, and 5, and glycolytic enzymes like Aldolase (ALDOC) and lactate dehydrogenase A (LDHA) were up-regulated by hypoxia which helps to compensate inhibition of oxidative phosphorylation to produce energy. These genes are known to be up-regulated through HIF-1 (Iyer *et al*, 1998). Angiogenesis and adhesion genes such as VEGF, ADM, TNS1, and VCAN play a crucial role in vasculature of hypoxic tissue specifically tumour tissues and movement of macrophages in the tissue toward the hypoxic tissues.

As seen in figure 4.4, some selected hypoxia-inducible genes have been confirmed by RT-PCR using the same total RNA samples that were used for microarray experiments. RT-PCR data shows that mRNA levels of well known hypoxia-inducible genes such as Glut-1, VEGF, MMP-7, and VCAN were up-regulated by hypoxia which indicates human macrophages responded to acute hypoxia. The fold induction of these genes ranged from 3-7 relative to normoxic sample (figure 4.3), these data indicate that macrophages were treated properly by hypoxia and that my microarray data is reliable.

HIF-1 $\alpha$  is a master regulator of transcriptional induction of most hypoxia inducible genes (Semenza, 2003). Binding of HIF-1 to HRE motifs in the promoters of these genes induces transcriptional activation. The expression of over 100 hypoxia-inducible genes are known to date, most of them found to be regulated by HIF-1 at the transcriptional level in different types of cells including macrophages (Semenza, 2001). Some known HIF-1-dependent genes such as DNA-damage-inducible transcript 4 (DDIT4) (Shoshani *et al*, 2002), VEGF, MT1M, and Glut-1 were detected in my array (Tables 4.3 and 4.4).

Studies (Blouin *et al*, 2004) have shown accumulation of HIF-1 $\alpha$  protein can be increased by a number of non-hypoxic stimuli in normal conditions and that these stimuli modulate the transcription of hypoxia-responsive genes (Frede *et al*, 2006). LPS is one of those stimuli, which induces expression of HIF-1 $\alpha$  at the transcriptional level in macrophages (Blouin *et al*, 2004).

My array data showed that HIF-1 $\alpha$  mRNA was down-regulated by hypoxia (table 4.5), with 2 fold down-regulation. Therefore, HIF-1 $\alpha$  mRNA levels were analyzed in hMDMs by RT-PCR. As figure 4.14 B shows, the HIF-1 $\alpha$  mRNA levels in both normoxia and hypoxia are similar which indicates, as previously reported, that HIF-1 $\alpha$  regulation by

hypoxia predominately occurs at the protein level (Semenza, 2009). However, my data showed that LPS increased HIF-1 $\alpha$  mRNA levels in normoxic and hypoxic samples, consistent with previous reports in primary mouse bone MDMs and rat alveolar macrophage-derived cell line cells (Blouin *et al*, 2004) and in the mouse macrophage cell line ANA-1 (Mi *et al*, 2008). Importantly, my immunoblotting data showed that cells treated with combined hypoxia plus LPS show more HIF-1 $\alpha$  protein than with hypoxia alone, suggesting a co-operative effect of hypoxia and LPS in these cells. However, as would be expected, given that hypoxia was not seen to up-regulate HIF-1 $\alpha$  mRNA, the HIF-1 $\alpha$  mRNA level does not show this synergy effect, and this result is consistent with previous work which showed that treatment of ANA-1 cells by LPS (10 ng/ml) under hypoxia for 4 hours gave no synergistic up-regulation when compared with either hypoxia or LPS alone (Mi *et al*, 2008).

LPS is a potent stimulator for macrophages by activation of signal transduction pathways which lead to altered gene expression patterns. Table 4.6 shows selected LPS-inducible genes. In this table, the majority of these genes are those related to immune response, and this is not surprising because the principal function of macrophages is to respond to bacterial infection by releasing humoral immune components such as cytokines, and by direct engulfment of organisms by phagocytosis (Ma *et al*, 2003).

In tissues infected with bacteria, macrophages respond rapidly by up-regulation of huge number of genes involved in the regulation of immune responses, cell chemoattraction, cell growth, and proliferation (Beutler & Rietschel, 2003). As seen in table 4.6, complement factor B (CFB), Interleukin 1 alpha (IL-1 $\alpha$ ) and Interleukin 1 beta

(IL-1 $\beta$ ) were highly up regulated by LPS. These immune components have crucial roles which help to resist and control microbial infections. Table 4.6 also shows up-regulation of CCL8 by LPS. CCL8 is a chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils to the site of infection (Proost *et al*, 1996). In addition to LPS, CCL8 secretion is enhanced by IL-1 $\alpha$  in different cell types.

Macrophages accumulate in areas of diseased tissues with bacterial infection which is characterised by low oxygen tension, and this condition has been shown to profoundly affect immunoregulatory responses by modulating the expression of genes coding for angiogenic factors, inflammatory cytokines, and extracellular matrix (ECM) components/regulators (Murdoch *et al*, 2004).

In the last ten years, many members of the chemokine superfamily have been identified. Chemokines are group of cytokines classified into CXC, CC, C, and CX3C families depending on the arrangement of the first two cysteines (Lira & Furtado, 2012). These Chemokines can bind to receptors differentially expressed in leukocytes which leads to activation of these cells (Rossi & Zlotnik, 2000). Microarray data have shown up-regulation of chemokines by LPS in human macrophages, including CCL20 (table 4.6). CCL20 (also known as MIP-3 $\alpha$ ) was found to selectively attract immature dendritic cells (iDC), effector/memory T lymphocytes, and naive B cells through its specific receptor, CCR6, expressed on these cells, (reviewed by (Schutyser *et al*, 2003)). One report investigated the hypoxia transcriptome of primary human monocytes demonstrated induction of genes encoding chemokines, and CCL20 was one of most important findings of their study (Bosco *et al*, 2006). This indicates that CCL20 expression by macrophages is indispensable in modulating immune response in ischaemic and infected tissues. In contrast, CCL20 did not respond to hypoxia in

primary HMDMs in our microarray (data not shown). The discrepancy between Bosco and my data may be due to the different level of hypoxia and level of LPS used in the current study. For example, in this current study 18h of 0.2% O<sub>2</sub> with 100 ng/ml of LPS were used, however just 8h with 1% O<sub>2</sub> in Bosco study.

The microarray data in the current study also demonstrated down-regulation of some genes by hypoxia, LPS, or hypoxia plus LPS, as seen in tables 4.5, 4.7, and 4.9 respectively. The fold change number of down-regulated genes by each condition ranges from -2 to -6.25, which is relatively low in comparison with the fold change of up-regulated genes. However, the most interesting finding in these down-regulated gene lists is that the mRNAs of the two most important hypoxia-inducible transcription factors, HIF-1 $\alpha$  and HIF-2 $\alpha$  (EPAS1) are suppressed by hypoxia. HIF-2 $\alpha$  (EPAS1) mRNA expression was also found decreased under short and long hypoxia by other investigators which is in consistent with current study (Staples *et al*, 2010).

The expression patterns of six selected synergy genes were investigated by using qRT-PCR (figures 4.11, 4.12, and 4.13). Only two of them, TRAIL and DDIT4 showed significant up-regulation by hypoxia plus LPS. The variation in results between array and RT-PCR data could have been due to the inherent genetic differences between individuals, although this was disproved because when the same donors were re-tested by RT-PCR, different results were obtained (data not shown). Genetic variation could however be one of the reasons for some of the variations in fold change between the 1<sup>st</sup> and 2<sup>nd</sup> arrays. Some of the variation could also be due to the different methodologies used to examine gene expression (microarray versus real-time RT-PCR) (Vengellur *et al*, 2005). It is likely that these discrepancies between the two microarrays and real-time RT-PCR data may be reduced if it were possible to increase

the number of samples used in microarray experiments from different donors. However, this was not possible in this study. As mentioned in section 4.1, the microarray chip used in this project interrogates more than 28,000 transcripts, so there is a possibility that when looking at a large number of transcripts one may get some aberrant false synergy in some genes, by chance, in both array experiments, when doing only two array experiments. Discrepancies between array and RT-PCR data in some genes were found in a previous study (Vengellur *et al*, 2005). Vengellur and colleagues showed big differences between RT-PCR and array quantification of gene expression modulated by hypoxia, CoCl<sub>2</sub>, DFO, and nickel. For example, INHBN and MCT3 were shown as up-regulated genes by microarray, whereas by RT-PCR showed them to be down-regulated. In addition, 15PGDH was shown down-regulated by microarray, but RT-PCR shows up-regulation. This indicates that there are some marked anomalies in their data, however overall there is correlation between microarray and RT-PCR in other genes (Vengellur *et al*, 2005).

The data presented in this chapter show that hypoxia and LPS activate a broad range of genes in human MDMs. Some of these genes were previously known to be regulated by hypoxia, such as VEGF, Glut-1, LDHA, ADM, and VCAN, and others were known to be regulated by LPS, such as IL-1 $\alpha$ , CFB, IL-1 $\beta$ , TNF, and NF $\kappa$ B, which underscores the validity of the data. In addition, combined treatment with hypoxia plus LPS synergized to induce a group of genes which were not induced or just slightly induced when only one condition was applied. These findings of synergistically regulated genes indicate that cross talk occurs between hypoxia and LPS in human macrophages. In order to further understand the mechanism of synergistic up-

regulation, TRAIL and DDIT4 genes were selected for further investigation in the studies described in chapters 5 and 6 respectively.

## CHAPTER 5: Results

### **TNF-related apoptosis-inducing ligand (TRAIL) gene regulation in primary human macrophages**

#### **5.1 Introduction**

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a 40-kDa type II trans-membrane protein that is a member of the TNF family of proteins which includes TNF, lymphotoxin, CD30 ligand, TNFRSF9 (Tumour necrosis factor receptor superfamily, member), CD40 ligand, CD27 ligand, and Fas ligand (Nagata & Golstein, 1995). It is able to induce apoptosis of tumour cells without harming normal cells. TRAIL was identified and characterized by Wiley and college by virtue of its sequence homology to CD95 (Apo1) ligand and TNF (Wiley *et al*, 1995). This protein was independently discovered by another group of investigators and named Apo2L (Pitti *et al*, 1996; Wiley *et al*, 1995). TRAIL also occurs in a soluble form following proteolytic cleavage from the membrane (Gruss, 1996). TRAIL activates apoptosis through the death receptors including Fas, TNFR-1, DR3, DR4, DR5 and DR6. These death receptors contain an intracellular region that is called a “death domain”. The death domain is an important structure that plays a key role in the activation of apoptotic signals in target cells (Jin & El-Deiry, 2005) (Sheikh & Huang, 2004). TRAIL also binds two other membrane bound receptors which are designated as decoy receptors, DcR1 and DcR2, which have incomplete death domains, and therefore do not trigger apoptosis (Degli-Esposti *et al*, 1997b) (Degli-Esposti *et al*, 1997a). These receptors are thought to serve as decoys, competing for TRAIL’s binding with other intrinsic death receptors (LeBlanc & Ashkenazi, 2003). Increased expression of these decoy receptors on normal cells was

thought to be the major factor responsible for protecting them from TRAIL-induced apoptosis (Marsters *et al*, 1997).

TRAIL is expressed in most human cells including the spleen, lung, and prostate (Pan *et al*, 1997) (Wiley *et al*, 1995). The regulation of TRAIL mRNA and protein expression is limited by few stimuli and transcription factors. TRAIL is induced by different members of the interferon family such as IFN- $\alpha$ ,  $\beta$ , and  $\gamma$  (Papageorgiou *et al*, 2007) (Choi *et al*, 2003) (Miura *et al*, 2006). The human TRAIL promoter has been described and several putative consensus transcription factor sites have been identified such as NFAT, GATA, C/EPB, Sp1, Ap1, Ap3, CF-1 and ISRE which are important for other TNF family members (Wang *et al*, 2000) (Gong & Almasan, 2000). Some researchers have reported that IFN $\gamma$  and IFN $\beta$  stimulate Stat1 activation and increased secretion of soluble TRAIL (Miura *et al*, 2006) (Choi *et al*, 2003). Other investigators have documented that IFN $\alpha$  induce rapid activation of Stat1 and accumulation of IRF-1 and increase DNA binding to the TRAIL promoter (Papageorgiou *et al*, 2007). siRNA targeting both transcription factors was able to inhibit IFN $\alpha$ -induced expression of TRAIL, implying that both transcription factors are required for inducible expression of TRAIL (Papageorgiou *et al*, 2007). IRF-3 and IRF-7 have also been implicated in regulating TRAIL expression (Kirshner *et al*, 2005) (Romieu-Mourez *et al*, 2006).

Several investigators have shown that LPS induces expression of both membrane-bound and soluble forms of TRAIL protein in human monocytes and MDMs (Halaas *et al*, 2000) (Ehrlich *et al*, 2003). Genc and colleagues found that expression of TRAIL mRNA in N9 murine microglia cells is induced by 100 ng/ml LPS in a dose-dependent manner and peaks at 4 h followed by down-regulation after 36 h. Furthermore, they

showed that LPS and IFN $\gamma$  synergistically induced TRAIL mRNA expression, which suggest some crosstalk between cellular signaling pathways of LPS and IFN $\gamma$  (Genc et al, 2003).

A -1523bp (relative to transcription start site) fragment of the TRAIL promoter was cloned and characterized by Wang and colleagues (Wang *et al*, 2000). They chose this region because it contains a number of important putative transcription binding sites needed for the expression of other TNF family members such as NFAT (nuclear factor of activated T-cells). Using the TRANSFAC (transcription factor binding site) program, promoter analysis revealed that this sequence (-1523bp) is lacking "TATA" and "CAAT" boxes, suggesting the essential consensus sequence may be located further upstream of the initiation transcription site (Gong & Almasan, 2000). However, consensus sequences for several putative transcription factor binding motifs were identified in this region, including sites for GATA (-175 to -180, -711 to -717, -792 to -798), C/EBP (-477 to -485, -674 to -681), SP-1 (-773 to -781), AP1 (-633 to -639) (Gong & Almasan, 2000).

## 5.2 Aim

I wanted to understand the mechanisms regulating TRAIL expression when human MDMs are exposed to hypoxia and LPS. Specifically, I aimed to determine the DNA elements containing binding sites for hypoxia/LPS induced transcription factors in the TRAIL promoter by cloning sections of the promoter into a luciferase reporter plasmid, followed by transfection of these constructs into human MDMs to measure luciferase activity under hypoxia in the presence or absence of LPS.

## 5.3 Results

### 5.3.1 Analysis of the TRAIL promoter for hypoxia and LPS-response transcription factor binding sites

In this project, a TRAIL promoter reporter construct was made, because I wanted to determine the part of the promoter responsible for the up-regulation of TRAIL under hypoxia and LPS. A 685bp construct containing the (-585/+100) promoter region was made, because previous studies have identified several essential putative consensus transcription factor sites within this region (Wang *et al*, 2000). In addition, I included 100bp downstream of the transcription start site because some genes have been found to be partially regulated by the downstream region (Jacobson *et al*, 2006).

In order to get an idea of the transcription factors that may play roles in regulating the TRAIL promoter, the sequence of the DNA fragment to be cloned (-585/+100) was searched for putative transcription factor binding sites using Genomatix MatInspector and TESS software. As figure 5.1 shows, the analysis indicated a number of potential binding sites for transcription factors associated with induction by hypoxia or LPS. The binding sites for some hypoxia-responsive transcription factors other than HIF are present in this region. For example, there are five and seven potential binding sites for AP-1 and Sp-1, respectively. Furthermore, the analysis showed nine binding sites for the NF-1 transcription factor and only one for NFkB. Some studies have shown that the presence of binding sites for AP-1 with other transcription factors such as NFkB in the promoter region is necessary to reach full activation of some hypoxia-sensitive genes (Shi *et al*, 1999). cAMP responsive element binding (CREB) and CCAAT enhancer binding protein-beta (C/EBP $\beta$ ) sites, which are involved in regulating many hypoxia-

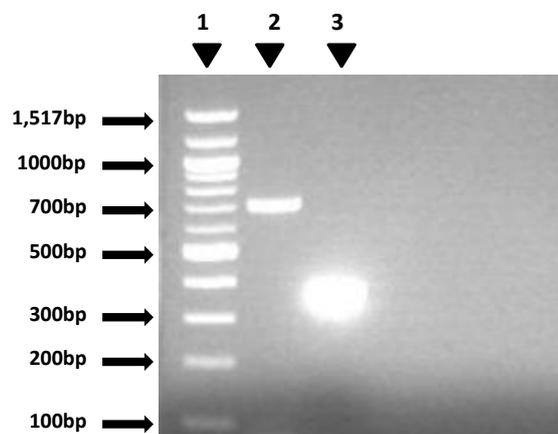
induced genes, were found in this region. Sp-1 is also involved in the expression of some LPS and hypoxia-induced genes (Liu *et al*, 2007).

	<b>NF-1+GATA</b>	<b>GATA</b>	
-585	GGGATGGAGATCTGAGAAGGAGATTAGAATTTGTGTCTGAAGGTTTGCA		
	<b>ETSF</b>	<b>CREB</b>	<b>AP1</b> <b>NF-1</b>
-536	AAGAGGAAGAAGTCGTCAATATTTAGATTCTGACATTCAAGATGGAGTT		
	<b>C/EBP<math>\beta</math></b>		<b>IRFF+NF-1</b>
-487	ATGTAGCAAGACCATTGCTATGAGACAGTATTTCTATTTTCCTTTATCC		
	<b>Sp-1</b>	<b>ETSF+Sp-1</b>	
-438	ACTCCCACCCTGCCCTCTTCCCACCCTCACAGTAGCATGAGAAAAACCA		
	<b>NF-1+ETSF</b>	<b>AP1</b>	<b>GATA</b>
-389	CATATGGAAGTTTCAGGTCATAAAAATTATCTTATAATTTAGAAAACAG		
	<b>Sp-1</b>	<b>AP1 NF-1</b>	
-340	GCCTTGTGCCTATGACAGCCAGGCCATGAGGCTTAGAGCTCTGTGGTAG		
		<b>NF<math>\kappa</math>B</b>	<b>NF-1</b>
-291	AATGAGGATATGTTAGGGAAAAGCAAAGAAAATCCCTCCCCTCTTTGGC		
	<b>GATA</b>		
-242	TGAGGACATTATCAAAAGGAGAGCAAGAAAGAGAAGAGAGAAATGGGCT		
	<b>Sp-1+GATA</b>	<b>Sp-1+NF-1</b>	
-193	TGAGGTGAGTGCAGATAAGGGGTGCATGGATCCTGAGGGCAAGGAGAGG		
		<b>IRFF</b>	<b>NF-1</b>
-144	AGCTTCTTTTCAGTTTCCCTCCTTTCCAACGACTACTTTGAGACAAGAGC		
	<b>Sp-1</b>		
-95	TGTCCCTGGGCAGTAGGAAAGGGGAGGGACAGTTGCAGGTTCAATAGAT		
	<b>Sp-1+NF-1</b>		
-46	GTGGGTGGGGCCAAGGCCACAGAACCCAGAAAAACAACCTCATTCGCTTT		<b>AP1</b> $\rightarrow$ +1
	<b>C/EBP<math>\beta</math></b>	<b>AP1</b>	<b>AP1</b>
+4	CATTCCTCACTGACTATAAAAGAATAGAGAAGGAAGGGCTTCAGTGAC		
	<b>NF-1</b>		
+53	CGGCTGCCTGGCTGACTTACAGCAGTCAGACTCTGACAGGATCATGGC		

**Figure 5.1: The human TRAIL promoter sequence (-585/+100) with putative transcription binding sites:** The promoter sequence was analysed using Genomatix MatInspector and TESS software. The data shows many regulatory element sites for transcription factors activated by hypoxia and LPS. Nuclear factor 1 (NF-1), activator protein 1 (AP1), cAMP responsive element binding (CREB), nuclear factor kappa B (NF $\kappa$ B), Sp1, interferon regulatory factors (IRFF), CCAAT enhancer binding protein-beta (C/EBP $\beta$ ), GATA binding protein (GATA), and human ETS1 factors (ETSF).

### 5.3.2 PCR amplification of 5`-upstream promoter region of the TRAIL gene

To determine the DNA sequences required for hypoxic expression of TRAIL in the presence or absence of LPS in primary human MDM, a 685bp promoter region was PCR-amplified from human genomic DNA, using -585bp TRAIL-5` (forward) and +100bp TRAIL-3` (reverse) primers: Then the PCR products were run on a 2% agarose gel in parallel with a previously prepared 343bp DNA segment of the MMP-7 promoter PCR-amplified as a positive control for PCR amplification and 100bp DNA molecular weight ladder to confirm that the correct size of DNA fragment was amplified. As seen in figure 5.2, the gel electrophoresis image showed two bands of DNA fragments around 700 and 350bp for (TRAIL and MMP-7) respectively comparing with the 100bp molecular ladder. This indicates the right piece of TRAIL DNA promoter was successfully amplified by PCR.

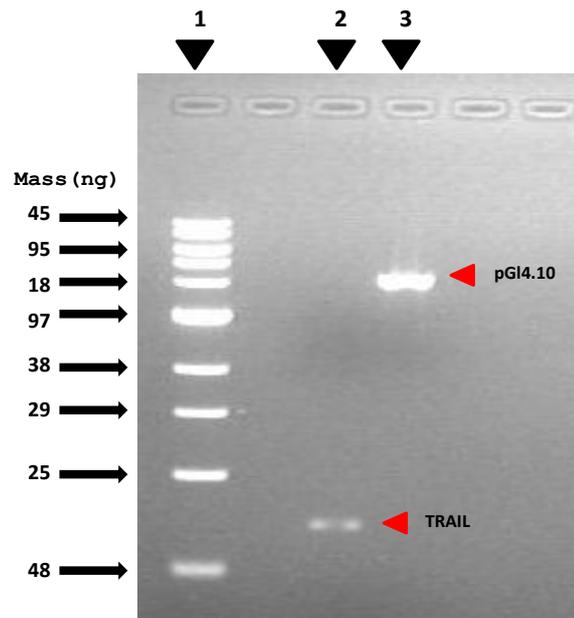


**Figure 5.2: TRAIL promoter PCR-amplification:** 2% agarose gel electrophoresis of PCR amplification of a 685bp (-585bp/+100bp relative to transcription start site) TRAIL promoter region from human genomic DNA. Lane 1= 100bp DNA ladder, lane 2= TRAIL 685bp, PCR product and lane 3= Amplified MMP-7 DNA (343bp) used as a control for PCR amplification and DNA size.

### 5.3.3 Cloning of DNA fragment of TRAIL promoter (-585/+100bp) into the pGL4.10 luciferase reporter vector

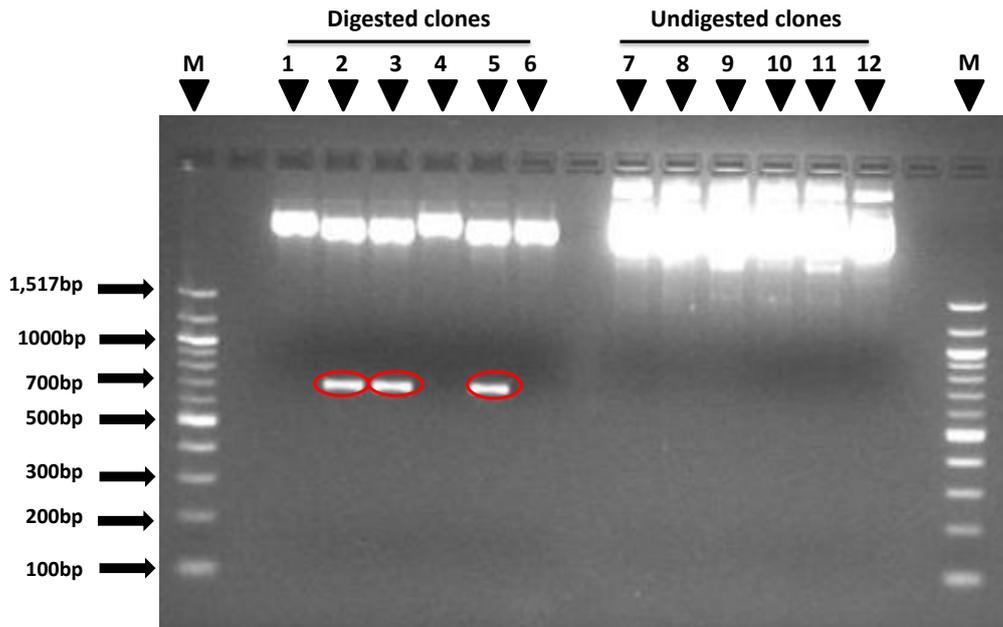
The pGL4.10 [luc2] vector is a modern improved firefly luciferase vector designed primarily to accept a putative promoter element for investigation of regions controlling gene transcription in mammalian cells with high robust expression and reduced aberrant transcription. To insert the amplified region of the TRAIL promoter into the pGL4.10 [luc2] plasmid vector, both DNAs were digested using the *SfiI* restriction enzyme. This digestion step creates symmetrical ends of TRAIL and pGL4.10 [luc2] plasmid that make them compatible to be ligated to each other. In addition the primers used to amplify TRAIL DNA fragment contain a sequence that recognized by *SfiI* restriction enzyme. The digested TRAIL promoter DNA and pGL4.10 [luc2] vector were run on agarose gel and extracted with the QIAquick Gel Extraction Kit (figure 5.3).

After gel extraction, small amounts of TRAIL-685bp and pGL4.10 [luc2] vector were run on another gel to confirm successful DNA extraction. In order to estimate the concentration of both digested DNAs (TRAIL insert and pGL4.10 vector), they were run on 1% agarose gel and roughly comparing the intensity of the bands with the intensity of known molecular standard marker run simultaneously in the same gel as seen in figure 5.3.



**Figure 5.3: 1% agarose gel electrophoresis of TRAIL and pGL4.10 purified DNA:** Two purified DNA, TRAIL and pGL4.10 plasmid run in gel against molecular weight standard. Only 0.5  $\mu\text{g}$  of the standard was loaded in the gel. The approximate mass of DNA in each of the bands of the standard is provided in front of each band. 1= molecular weight standard (100bp DNA ladder), 2=TRAIL DNA, and 3=pGL4.10 DNA.

The purified product of digested DNA fragment extracted from the gel was ligated into pGL4.10 [luc2]. In order to confirm the correct cloned construct was carrying the TRAIL promoter (-585/+100bp) insert, all six putative constructs generated were digested using *the Sfi I* restriction enzyme and run on agarose gels in parallel with undigested counterpart constructs. As seen in figure 5.4, only three clones (clones 2, 3, and 5) showed the correct insert size at 685bp. The digestion of these three clones generated two DNA fragments, one is the pGL4 vector, and the other one is the insert as indicated by the circles in figure 5.4.



**Figure 5.4: 2% agarose gel electrophoresis of restriction digested plasmid preps of 685bp (-585/+100bp) TRAIL promoter pGL4.10 constructs:** Six aliquots of putative TRAIL 695bp promoter clone digested with *Sfi*I and run in parallel with undigested counterpart aliquots (constructs). Only three clones (lane 2, 3, and 5) were successful 685-bp TRAIL pGL4.10 constructs (with inserts). The inserts (TRAIL) have been indicated by a red circle. M= 100bp molecular marker.

The DNA sequence was performed (<http://www.le.ac.uk/mrctox/pnacl/>) for only one cloned insert (Digested clone number 2 in figure 5.4). The sequence was checked for similarity to the published sequence using BLAST (<http://blast.ncbi.nlm.nih.gov.ezproxy.lib.le.ac.uk/>). Only one base pair mismatch was found as indicated by the red box in figure 5.5.

```

> ref|NT\_005612.16| D Homo sapiens chromosome 3 genomic contig, GRCh37.p5 Primary
Assembly
Length=100537107

Features in this part of subject sequence:
  tumor necrosis factor ligand superfamily member 10 isoform 1
  tumor necrosis factor ligand superfamily member 10 isoform 2

Score = 1260 bits (682), Expect = 0.0
Identities = 684/685 (99%), Gaps = 0/685 (0%)
Strand=Plus/Minus

Query 1      GGGATGGAGATCTGAGAAGGAGATTAGAATTTGTGTCTGAAGGTTTGCAAAGAGGAAGAA 60
             |||
Sbjct 78737000 GGGATGGAGATCTGAGAAGGAGATTAGAATTTGTGTCTGAAGGTTTGCAAAGAGGAAGAA 78736941

Query 61     GTCGTCAATATTTAGATTCTGACATTC AAGATGGAGT CATGTAGCAAGACCATTGCTATG 120
             |||
Sbjct 78736940 GTCGTCAATATTTAGATTCTGACATTC AAGATGGAAAT CATGTAGCAAGACCATTGCTATG 78736881

Query 121    AGACAGTATTTCTATTTTCCTTTATCCACTCCCACCCTGCCCTCTTCCCACCCTCACAGT 180
             |||
Sbjct 78736880 AGACAGTATTTCTATTTTCCTTTATCCACTCCCACCCTGCCCTCTTCCCACCCTCACAGT 78736821

Query 181    AGCATGAGAAAAACCCACATATGGAAGTTTCAGGTCATAAAAAATTATCTTATAATTTAGAA 240
             |||
Sbjct 78736820 AGCATGAGAAAAACCCACATATGGAAGTTTCAGGTCATAAAAAATTATCTTATAATTTAGAA 78736761

Query 241    AACAGGCCTTGTGCCTATGACAGCCAGGCCATGAGGCTTAGAGCTCTGTGGTAGAATGAG 300
             |||
Sbjct 78736760 AACAGGCCTTGTGCCTATGACAGCCAGGCCATGAGGCTTAGAGCTCTGTGGTAGAATGAG 78736701

Query 301    GATATGTTAGGGAAAAGCAAAGAAAATCCCTCCCCTCTTTGGCTGAGGACATTATCAAAA 360
             |||
Sbjct 78736700 GATATGTTAGGGAAAAGCAAAGAAAATCCCTCCCCTCTTTGGCTGAGGACATTATCAAAA 78736641

Query 361    GGAGAGCAAGAAAGAGAAGAGAGAAAATGGGCTTGAGGTGAGTGCAGATAAGGGGTGCATG 420
             |||
Sbjct 78736640 GGAGAGCAAGAAAGAGAAGAGAGAAAATGGGCTTGAGGTGAGTGCAGATAAGGGGTGCATG 78736581

Query 421    GATCCTGAGGGCAAGGAGAGGAGCTTCTTTTCAGTTTCCCTCCTTTCCAACGACTACTTTG 480
             |||
Sbjct 78736580 GATCCTGAGGGCAAGGAGAGGAGCTTCTTTTCAGTTTCCCTCCTTTCCAACGACTACTTTG 78736521

Query 481    AGACAAGAGCTGTCCCTGGGCAGTAGGAAAGGGGAGGACAGTTGCAGGTTCAATAGATG 540
             |||
Sbjct 78736520 AGACAAGAGCTGTCCCTGGGCAGTAGGAAAGGGGAGGACAGTTGCAGGTTCAATAGATG 78736461

Query 541    TGGGTGGGGCCAAGGCCACAGAACCCAGAAAAACAACACTCATTGCG TTCATTTCCTCACT 600
             |||
Sbjct 78736460 TGGGTGGGGCCAAGGCCACAGAACCCAGAAAAACAACACTCATTGCG TTCATTTCCTCACT 78736401

Query 601    GACTATAAAAGAATAGAGAAGGAAGGGCTTCAGTGACCGGCTGCCTGGCTGACTTACAGC 660
             |||
Sbjct 78736400 GACTATAAAAGAATAGAGAAGGAAGGGCTTCAGTGACCGGCTGCCTGGCTGACTTACAGC 78736341

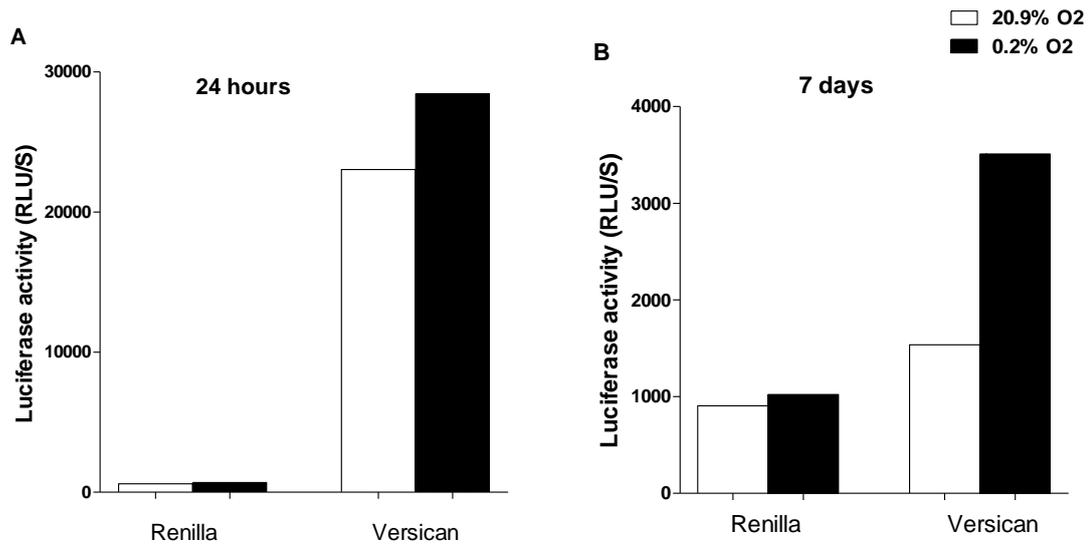
Query 661    AGTCAGACTCTGACAGGATCATGGC 685
             |||
Sbjct 78736340 AGTCAGACTCTGACAGGATCATGGC 78736316

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**Figure 5.5: A nucleotide alignment of cloned fragment of TRAIL-585/+100bp sequence with published sequence:** The cloned DNA promoter of TRAIL (-585/+100bp) which is 685bp long was BLAST searched and only one base mismatch was identified in the whole sequence, indicated by red box. The green highlighted base with arrow is the transcription start site, (Wang *et al*, 2000) and (Kirshner *et al*, 2005).

### **5.3.4 Optimization of the internal control (*Renilla* phRL-TK) luciferase reporter plasmid for transfection experiments and verifying reliability for data normalization**

Choosing the right control is very crucial to get reliable and accurate data in any experiment. The phRL-TK *Renilla* luciferase reporter vector was used as an internal control in combination with experimental reporter vectors in this project. Hypoxia is one of the two main stimulatory factors in every experiment, therefore testing the induction of the *Renilla* reporter by hypoxia was crucial to check its suitability. Primary human MDMs were transfected with the versican 240bp (-56/+184) pGL4.10 firefly luciferase construct. This reporter construct was a kind gift from my fellow PhD student colleague Fattah Sotoodehnejadnematalahi and was known to be hypoxia inducible. The human MDMs were co-transfected with the *Renilla* phRL-TK plasmid and then treated with hypoxia (0.2% O<sub>2</sub>) for 24hrs and 7 days. Then the values of firefly luciferase were normalized with *Renilla* luciferase values. As indicated from the luciferase activity in figure 5.6 A and B, *Renilla* was not induced by hypoxia in comparison with normoxic samples in both periods of incubation (24 hrs and 7 days); therefore, it can be used for normalizing the luciferase activity of the experimental reporters in hypoxic conditions (figure 5.6). It should be mentioned that although the *Renilla* phRL-TK was not activated by hypoxia, the raw luciferase values were well above the background. The normalization with the *Renilla* control is needed to minimize experimental variability caused by such things as differences in cell numbers due to pipetting error. All transfection data shown was normalised against the *Renilla* luciferase expression unless otherwise indicated.



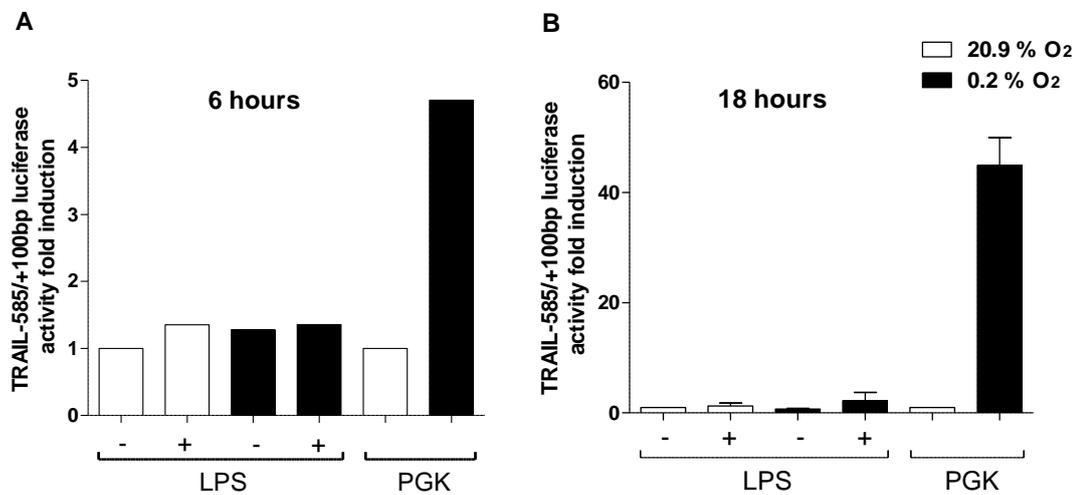
**Figure 5.6: Optimization of internal luciferase control *Renilla* phRL-TK:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells were transfected with 1  $\mu$ g of versican 240bp (-56/+184) promoter construct inserted in pGL4.10 firefly luciferase construct, and co-transfected with 25 ng of *Renilla* (phRL-TK) normalization plasmid using 3.2  $\mu$ l of JetPEI reagent according manufacturer's instruction. Transfected cells were then incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for (A) 24h or (B) 7 days. The graph represents luciferase data from 1 experiment.

### 5.3.5 Effect of hypoxia and LPS on TRAIL (-585/+100bp) promoter reporter construct expression in primary HMDMs

To determine whether the cloned region of the human TRAIL promoter (-585/+100) is important for gene expression, the effect of hypoxia in the presence or absence of LPS on the TRAIL reporter construct was investigated in primary human MDMs. Wells were seeded with PBMCs at a cell density of  $2 \times 10^6$  per well, and the media changed after 2 hours. Adherent cells were further incubated for five days and then transfected with 1  $\mu$ g of TRAIL 685bp reporter construct and co-transfected with 25 ng *Renilla* phRL-TK using 3.2  $\mu$ l of JetPEI transfection reagent. Then human MDMs were incubated in normoxia (20.9% O<sub>2</sub>) and hypoxia (0.2% O<sub>2</sub>) with or without 100 ng/ml of LPS for 6 hours. As seen in figure 5.7 A (left panel), the TRAIL promoter (-585/+100) reporter construct shows little activity fold induction by hypoxia, LPS, or by combined treatment with LPS and hypoxia for 6 hours. However, as expected, hypoxia induced the level of

luciferase expression in HMDMs transfected with Phosphoglycerate kinase (PGK), a hypoxia-inducible positive control luciferase reporter construct (Ameri *et al*, 2002).

Based on the above data, I decided to try increasing the incubation period of transfected cells up to 18h. As shown in 5.7 B, unfortunately again 18hrs of incubation could not activate this TRAIL (-585/+100bp) promoter construct under this time period as well in human MDMs.



**Figure 5.7: Effect of hypoxia and LPS on TRAIL (-585/+100bp) promoter reporter construct activity:** Primary human MDMs were incubated under normal oxygen condition for 5 days, and then transfected with 1  $\mu$ g of TRAIL (-585/+100bp) promoter reporter construct DNA and 25 ng of *Renilla* phRL-TK normalization plasmid using 3.2  $\mu$ l of JetPEI transfection reagent according to manufacturer's instruction. Cells were incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2 % O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 6h (A) and 18h (B) prior to luciferase assay. For 18 hours incubation, luciferase raw values data from two independent experiments using different donors are expressed as means  $\pm$ SEM, whereas for 6 hours incubation, only one experiment was performed. Luciferase activity of PGK construct was used as a control for hypoxic inducibility in human MDMs.

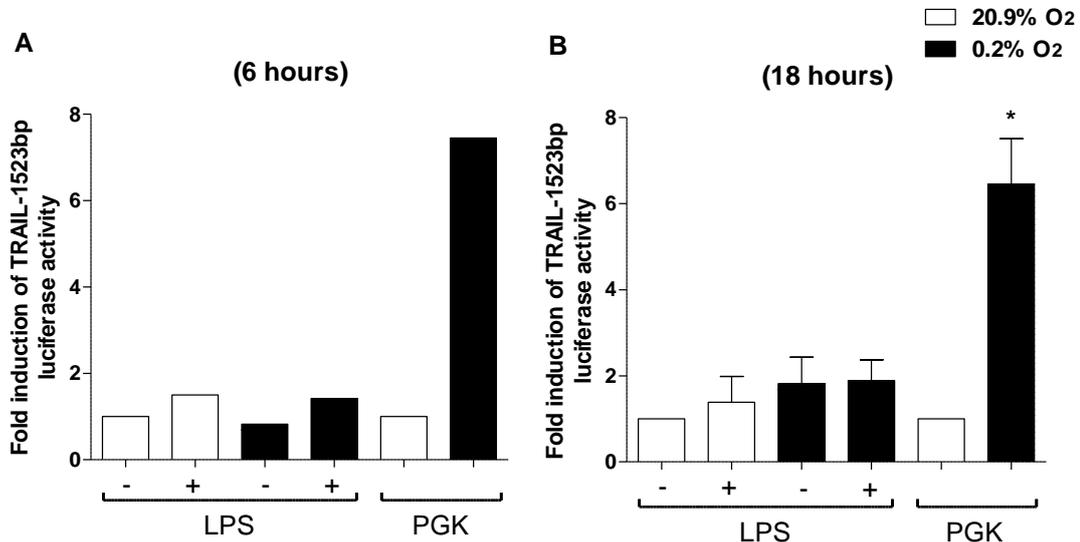
### 5.3.6 Effect of hypoxia and LPS on TRAIL-1523bp promoter reporter construct expression in primary HMDMs

As described above, no luciferase activity was detected after 6 or 18hrs of transfection of TRAIL (-585/+100bp) reporter construct in primary human MDMs.

Several reports have documented that NFAT is an important transcription factor which has a critical role for TRAIL expression as seen in intestinal-derived cells (HT29), (Wang *et al*, 2000). In addition, previous studies had identified a number of putative transcription factor binding sites including NFAT and Sp1 sequences in TRAIL promoter (Wang *et al*, 2000) (Kirshner *et al*, 2005). Therefore, in this project, it was decided to use a longer TRAIL reporter construct with 1523bp of DNA promoter that includes binding sites for NFAT and Sp1. This TRAIL long construct was a kind gift from Professor B. Mark Evers (Wang *et al*, 2000). The plasmid was prepared and purified and checked by a suitable host strain restriction digestion and sequence analysis before being used in the transfection experiments (Data not shown).

As with the short TRAIL construct, human MDMs were transfected with 1 µg of TRAIL-1523bp reporter construct and 25 ng *Renilla* phRL-TK using 3.2 µl of JetPEI transfection reagent for each transfection reaction. Then cells were incubated in normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) with and without 100 ng/ml LPS for 6 or 18 hours. Luciferase activity in each condition was measured and normalized with *Renilla* luciferase values. As seen in figure 5.8 A, the TRAIL-1523bp reporter construct did not show any significant activity by hypoxia, LPS, or by combined treatment with LPS and hypoxia at 6h, however, when the transfection incubation period was extended to 18 hours, the TRAIL-1523bp reporter construct had a low fold induction of luciferase activity under hypoxia and hypoxia plus LPS (figure 5.8 B). Despite the low activity of this construct,

this data indicate that TRAIL-1523bp reporter construct is partially functioning under those conditions.

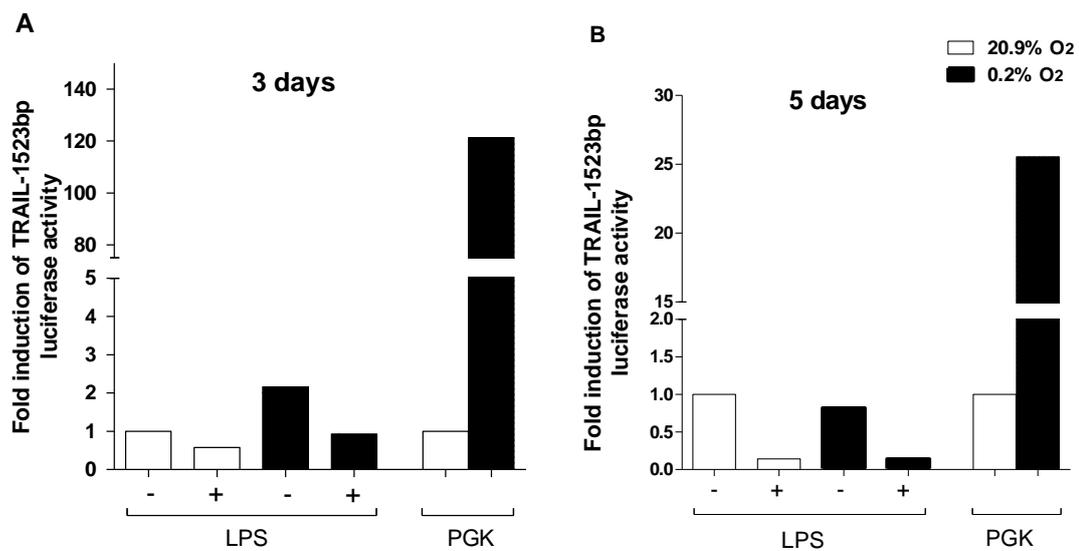


**Figure 5.8: Effects of hypoxia and LPS on TRAIL-1523bp promoter construct activity:** Primary human MDMs were incubated under normal oxygen condition for 5 days, and then transfected with 1  $\mu$ g of TRAIL-1523 promoter reporter construct DNA and 25 ng of *Renilla* phRL-TK normalization plasmid using 3.2  $\mu$ l of JetPEI transfection reagent according to manufacturer's instruction. Cells were incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2 % O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 6h (A) and 18h (B) prior to luciferase assay. For 6 hours incubation, only one experiment was performed, whereas 18 hours incubation, luciferase raw values data from 3 independent experiments using different donors are expressed as means  $\pm$ SEM. Data were further analysed for significant induction using paired one-tailed t-tests. \* =  $p < 0.05$  when compared to the normoxic PGK.

The data presented in figure 5.8 B with 18 hrs encouraged me to further investigate the TRAIL-1523bp reporter construct by extending the transfection period to 3 and 5 days under the same conditions of hypoxia and LPS. Human MDMs were transfected with the same amount of DNA with both TRAIL-1523bp reporter construct and Renilla normalization plasmid. As figure 5.9 A shows, the TRAIL -1523 promoter reporter construct showed just a slight induction when incubated under hypoxia for 3 days in comparison with normoxia. In contrast, neither LPS nor (hypoxia plus LPS) produced any fold induction, but slightly suppressed in human MDMs by the TRAIL-1523 reporter

construct. However, this finding must be treated with caution, as it is based on results from just one transfection experiment.

Extending the incubation period of transfected human MDMs by TRAIL-1523 reporter construct for 5 days failed to activate this reporter construct as seen in figure 5.9 panel B. Moreover, 5 days of incubation appeared to suppress the activity of TRAIL-1523 reporter construct after exposure to either hypoxia or LPS in comparison with normoxia. It is noticeable that after both 3 and 5 days, the activity of the hypoxia-inducible control PGK construct was highly induced under acute hypoxia when compared with those obtained by either 18 or 6 hrs incubation (figure 5.8).



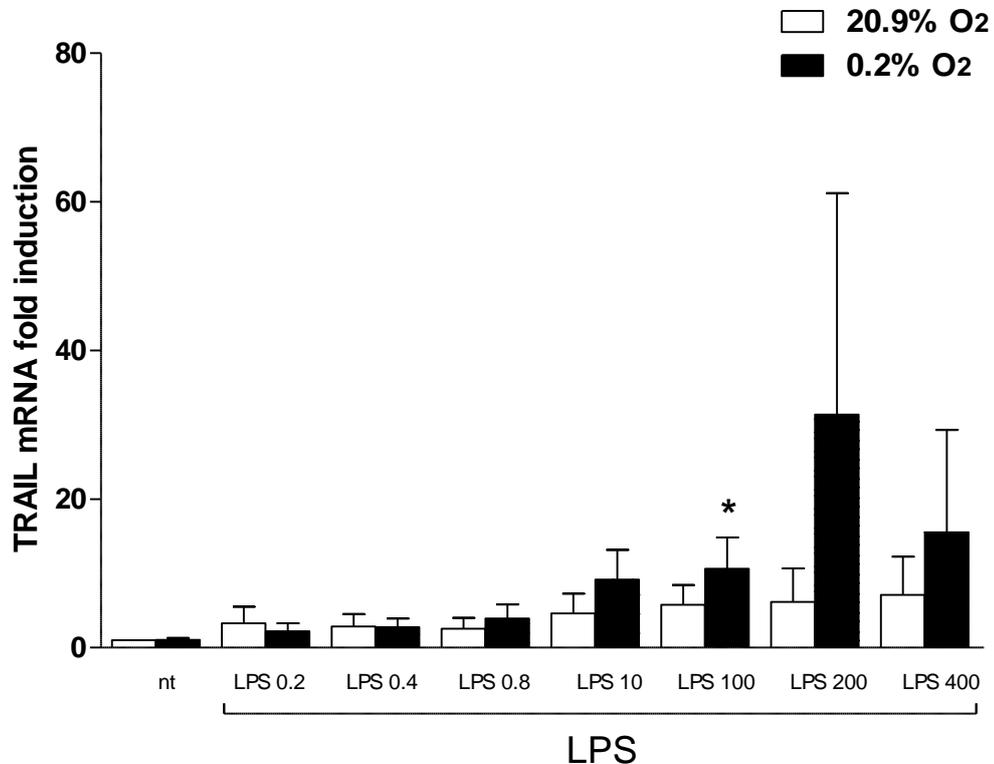
**Figure 5.9: Effects of hypoxia and LPS on TRAIL-1523bp promoter construct activity:** Primary human MDMs were incubated under normal oxygen condition for 5 days, and then transfected with 1  $\mu$ g of TRAIL-1523 promoter reporter construct DNA and 25 ng of *Renilla* phRL-TK normalization plasmid using 3.2  $\mu$ l of JetPEI transfection reagent according to manufacturer's instruction. Cells were incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2 % O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 3 days (A) and 5 days (B) prior to luciferase assay. For both graphs, only one experiment was performed. Luciferase activity of PGK construct was used as a control for hypoxic inducibility in human MDMs.

### **5.3.7 Effect of different concentrations of LPS on TRAIL mRNA expression under hypoxia in primary human MDMs**

TRAIL like other TNF family members are involved in immune regulations which induce apoptosis in cells expressing specific receptors (Wiley *et al*, 1995). Several studies have shown that TRAIL mRNA and protein expressed in activated monocytes and macrophages (Griffith *et al*, 1999). The basal level of TRAIL mRNA in resting monocytes is low, however, it was rapidly increased by LPS within 2 hours after addition of LPS, and declines after overnight incubation (Halaas *et al*, 2000). In the present study, mRNA expression level of TRAIL gene was assessed by RT-qPCR in human MDMs stimulated with different concentrations of LPS. The aim of doing this was to test the optimal dose of LPS needed to induce TRAIL expression and give the highest synergy effect with hypoxia. Primary human MDMs were incubated for 5 days in normoxia, and then cells were treated with 0.2, 0.4, 0.8, 10, 100, 200, and 400 ng/ml of LPS. Cells were exposed to normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for further 18 hrs. The level of TRAIL mRNA expression was quantified by real time RT-PCR and the data were normalised with internal control  $\beta$ -2m.

As figure 5.10 shows, LPS-treated cells up-regulated TRAIL mRNA in all the different concentrations tested. Furthermore, the synergy effect of hypoxia plus LPS also induced TRAIL mRNA expression in dose-dependent manner, however cells treated with high LPS (100 ng/ml and more) under hypoxia had the same levels of induction, although there was high variability in combined hypoxia plus LPS fold induction among these donors. It is noticeable that three low LPS doses (0.2, 0.4, and 0.8 ng/ml) produced a similar pattern of TRAIL expression in either LPS or hypoxia plus LPS

conditions, indicating that this level of LPS stimulation may be too low to elicit a marked synergistic response.



**Figure 5.10: Expression of TRAIL mRNA in primary human macrophages under hypoxia after treatment with different LPS concentrations:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells were treated with different concentrations of LPS 0.2, 0.4, 0.8, 10, 100, 200, and 400 ng/ml and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior the RNA isolation. TRAIL mRNA expression was quantified by real-time RT-PCR then data were normalized with  $\beta$ -2M mRNA levels in order to calculate fold induction. Data from 5 independent experiments using different donors expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-test. \* = P value  $\leq$ 0.05 considered as significant when compared with normoxia (nt).

## 5.4 Discussion

My array data, which showed high up-regulation of TRAIL under hypoxia plus LPS (16.65 fold change), encouraged me to look more closely at its regulation by these stimuli in human MDMs. My data has shown slight up-regulation by hypoxia (1.67 fold change) with moderate induction by LPS (4.51 fold change) (table 4.3). This data shows that TRAIL is markedly induced by LPS but only slightly by hypoxia, which is concordant with what is found in the literature (Halaas *et al*, 2000) (Halaas *et al*, 2004) (Zheng *et al*, 2005). Based on these findings and my real-time RT-PCR data on TRAIL mRNA expression (figure 4.11) which showed a synergistic effect by combined hypoxia plus LPS, in this chapter I set out to understand how TRAIL expression is regulated by LPS and hypoxia in primary macrophages, principally by cloning a 685bp DNA fragment of the TRAIL promoter (-585/+100) which was placed upstream of a luciferase reporter gene in the pGL4.10 [luc2] vector. The computer analysis of the TRAIL promoter (-585/+100) relative to the transcription start site showed many putative binding sites for transcription factors activated by hypoxia, LPS, or both (figure 5.1). The analysis showed one binding site for NF- $\kappa$ B transcription factor in TRAIL promoter sequence which is located between -254 and -263bp relative transcription start site. Recent technology has allowed the application of novel methods such as chromatin immunoprecipitation (ChIP) to the identification of transcription factor binding regions on DNA (Dey *et al*, 2012). ChIP experiments require a variety of molecular biology methods including crosslinking, cell lysis (protein-DNA extraction), nucleic acid shearing, antibody-based immunoprecipitation, DNA sample clean-up and PCR. Many protein-DNA interactions are transient, so ChIP assays begin with stabilization

(crosslinking) of protein-DNA complexes normally by formaldehyde. The second step is cell lysis which extracts the crosslinked protein-DNA complexes from the nucleus. In order to analyze protein binding sequences, the extracted genomic DNA must be sheared into smaller fragments by either mechanical sonication or enzymatic digestion with nucleases. The fourth stage involves precipitation of fragmented protein-bound DNA. In this step, specific antibodies bind to the transcription factor of interest, allowing removal of other unrelated cellular material. Before the specific DNA products of a CHIP can be amplified and measured, the crosslinks between protein and DNA must be reversed, which can be done for example by digestion of the protein component with proteinases. Finally, the purified DNA products are quantified by quantitative PCR (Hudson & Snyder, 2006; Wu *et al*, 2006).

LPS is a potent stimulator for macrophages which activates the NF- $\kappa$ B-signalling pathway (Kawai & Akira, 2007). Several reports have shown that TRAIL expression increased with LPS stimulation in many cell types including macrophages, suggesting this activation of TRAIL might be mainly through the NF- $\kappa$ B pathway (Zheng *et al*, 2005) (Halaas *et al*, 2000). Hypoxia also stimulates NF- $\kappa$ B activation and the transcription of homology of other TNF family member, TNF- $\alpha$  gene (Koong *et al*, 1994). No published work to date has investigated the effect of acute hypoxia on TRAIL gene expression in any cell type. The TRAIL promoter lacks HIF binding sites, hypoxia response element (HRE). However, an NF- $\kappa$ B binding site is present in close proximity to the transcription start site, and binding of activated NF- $\kappa$ B leads to TRAIL up-regulation, (Kwon & Choi, 2006).

The TRAIL promoter construct in this study was transfected into human MDMs and exposed to hypoxia and LPS, and luciferase was measured after 6 hrs and 18 hrs. As seen in figure 5.7, unfortunately both incubation periods could not activate the luciferase activity of the TRAIL promoter construct, although the transfection and hypoxia treatment were successfully achieved as indicated by PGK, a hypoxia-inducible positive control luciferase reporter construct. There are a few reasons which may explain the failure of this construct to be activated under those conditions. First, it is possible that this region of TRAIL promoter (-585/+100) is not enough to activate gene expression, which may need a longer promoter region containing more transcription factor binding sites that are essential for TRAIL expression. Second, the incubation period of human MDMs with TRAIL construct (-585/+100) may not be optimal. Halaas and colleagues showed that TRAIL mRNA transcription in monocytes was rapidly increased by LPS within 2 hours and the transcription levels increased over time up to 8 hrs, then a down-regulation was observed after overnight culture (Halaas *et al*, 2000). Since the MDMs were incubated for 18 hrs, this may explain my finding in which TRAIL luciferase reporter expression showed no increase after 18 hours.

A longer TRAIL promoter construct was tested in human MDMs under hypoxia plus LPS (Wang *et al*, 2000). As described in section 5.3.6, human MDMs were transfected with this TRAIL-1523bp reporter construct and incubated in normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) with and without 100 ng/ml LPS for different time periods (6h, 18h, 3d, and 5d) in several experiments. However, no synergy effect of hypoxia plus LPS was obtained with this longer construct either. Overall, luciferase activity of the TRAIL-1523bp construct was low after 18 hrs of hypoxia and LPS incubation in human MDMs.

There are several possible reasons: TRAIL induction may result from the activation of several signaling pathways, therefore TRAIL expression needs the full promoter sequence longer than that contained within the TRAIL-1523bp construct. In addition, the low luciferase activity of the TRAIL-1523bp construct could be due to the fact that this TRAIL construct was found to be activated in cancer cells like HT29, Caco-2 and HEC-1B, (Wang *et al*, 2000) (Kirshner *et al*, 2005), rather than primary macrophages. Thus, cell type may determine the activity of the TRAIL promoter construct.

The activation status of macrophages also possibly could be one of the reasons for the failure of the TRAIL constructs. For example, the concentration of LPS used in the current study may be much higher than the level needed to stimulate macrophages. Thus, I questioned whether the concentration of LPS (100 ng/ml) used in this project was the right amount or not. Therefore, LPS-dose response experiments were performed in order to investigate that approach. Figure 5.10, shows two groups of samples which have similarity in their expression patterns. The first group are samples treated with low doses (0.2, 0.4, and 0.8 ng/ml of LPS) and the second group are samples treated with high doses (10, 100, 200, and 400 ng/ml) of LPS. All samples in both groups share the feature of up-regulation of TRAIL mRNA by LPS and hypoxia plus LPS, however, the second group have more fold induction. If the low and high doses groups are compared with each other, the TRAIL mRNA expression shows a dose-dependent response, and this is concordant with a previous report (Alexander *et al*, 2001). In addition, the higher LPS dose group showed evidence of the synergy effect from combined treatment (hypoxia plus LPS), although this synergy is not statistically significant. Despite the non-significance, there is clear evidence that both LPS and

hypoxia are able to regulate TRAIL expression, and signs of an upward trend when the two are combined. This, and the high degree of variability inherent in experiments such as these and the array experiments, using primary human cells from different donors, is a possible explanation for the cooperative effect between LPS and hypoxia on TRAIL expression not reaching statistical significance.

Several investigators have shown that IKK $\gamma$  and NF- $\kappa$ B play an essential role in expression of TRAIL in T cells (Baetu *et al*, 2001), which may suggest that LPS-induced expression of TRAIL in macrophages may also be mediated through the NF- $\kappa$ B pathway. In addition to LPS, my project has shown that hypoxia also has a strong effect on the regulation of TRAIL expression in macrophages.

In conclusion, the data in this chapter have shown that TRAIL mRNA is markedly up-regulated by hypoxia plus LPS, but not by hypoxia alone. As array and RT-PCR showed that according to my criteria hypoxia has no effect on TRAIL expression, the synergy between hypoxia and LPS could be due to an effect of hypoxia which would only have effect in the presence of LPS. For example, synergy could be due to the increased expression of the LPS receptor (TLR4) on the macrophage cell surface under hypoxia, since TLR4 has been found to be up-regulated by hypoxia (Kim *et al*, 2010), so that when the LPS was added, the macrophages, the LPS could bind to more receptors which might result in enhanced cell activation and expression of TRAIL.

## CHAPTER 6: Results

### DNA-damage-inducible transcript 4 (DDIT4) gene regulation by hypoxia and LPS in primary human macrophages

#### 6.1 Introduction

Macrophages respond to hypoxia or LPS by up-regulating a broad array of genes. DNA-damage-inducible transcript 4 (DDIT4) is a gene I found to be up-regulated by combined treatment of hypoxia plus LPS in microarray data (Table 4.3) and this data was confirmed by real-time PCR in figure 4.12 A. This gene is expressed in many human tissues at low level. However, in response to hypoxia its transcription is rapidly increased (Shoshani *et al*, 2002).

In addition to hypoxia, DDIT4 is also induced by oxidative stress, energy depletion, and DNA damage (Lin *et al*, 2005b; Lin *et al*, 2005a). A study by Regazzetti and colleagues has shown that prolonged treatment with insulin induces DDIT4 mRNA and protein expression in murine and human adipocytes. Interestingly, this induction by insulin is enhanced under hypoxia through the HIF-1 pathway, and this synergy between hypoxia and insulin could be explained by the finding that hypoxia activates HIF-1 by stabilizing the  $\alpha$ -subunit, whereas insulin up-regulates HIF-1 by enhancement of HIF-1 $\alpha$  translation (Regazzetti *et al*, 2010).

Brugarolas and colleagues reported that DDIT4 induced by hypoxia results in down regulation of mTOR through the activation of the up-stream protein complex, tuberous sclerosis tumour suppressor complex (TSC1/TSC2) (Brugarolas *et al*, 2004). Other studies have shown that overexpression of DDIT4 inhibits the invasive activity of cancer cells such as H1299 through suppression of mammalian target of rapamycin

(mTOR), the master regulator of protein translation (Jin *et al*, 2011). The suppression of mTOR results in deactivation of p70-S6 kinase 1 which leads to decreased protein synthesis, and subsequently decrease cell activity and invasiveness (Zhong *et al*, 2000).

## 6.2 Aim

In this chapter, I wanted to:

1. Determine the effect of different levels of LPS on DNA-damage-inducible transcript 4 (DDIT4) mRNA expression in primary HMDMs under hypoxia.
2. Determine the mechanism responsible for DDIT4 regulation in primary HMDMs when exposed to hypoxia and LPS, by using promoter reporter constructs and specific transcription factor inhibitors.

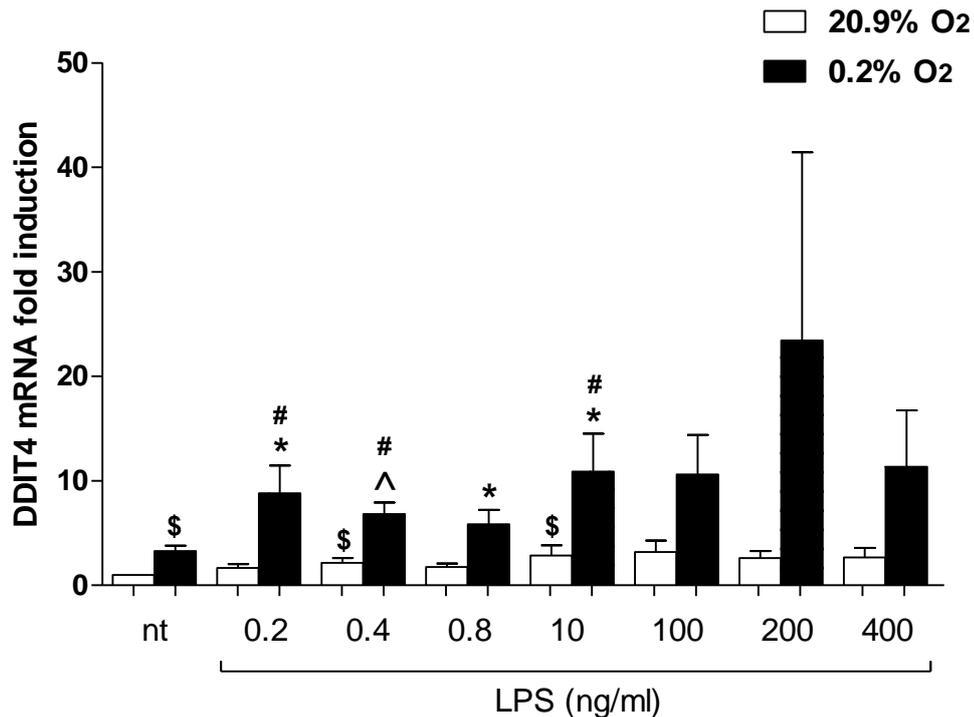
## 6.3 Regulation of DDIT4 expression *in vitro*

### 6.3.1 Hypoxia synergises with different concentrations of LPS to up-regulate DDIT4 mRNA expression in primary human macrophages *in vitro*

When macrophages are activated with LPS, they immediately respond by releasing several mediators. The microarray data presented in chapter 4 were obtained from the analysis of gene expression of primary HMDMs in response to hypoxia with and without 100 ng/ml of LPS. In this chapter, I wanted to investigate the effect of hypoxia on DDIT4 mRNA expression in combination with different levels of LPS. The aim of these experiments was to find out whether using low concentration of LPS, less than that used in my microarray experiments (100 ng/ml) would give significant synergy with hypoxia or not. Therefore, cells were treated with different amounts of LPS and

incubated under hypoxia for 18 hours. Primary human MDMs were incubated for 5 days, then cells were treated with 0.2, 0.4, 0.8, 10, 100, 200, and 400 ng/ml of LPS, then cells were exposed to normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours. mRNA expression was quantified by semi-quantitative real time RT-PCR and the data were normalised with the internal control  $\beta$ -2m.

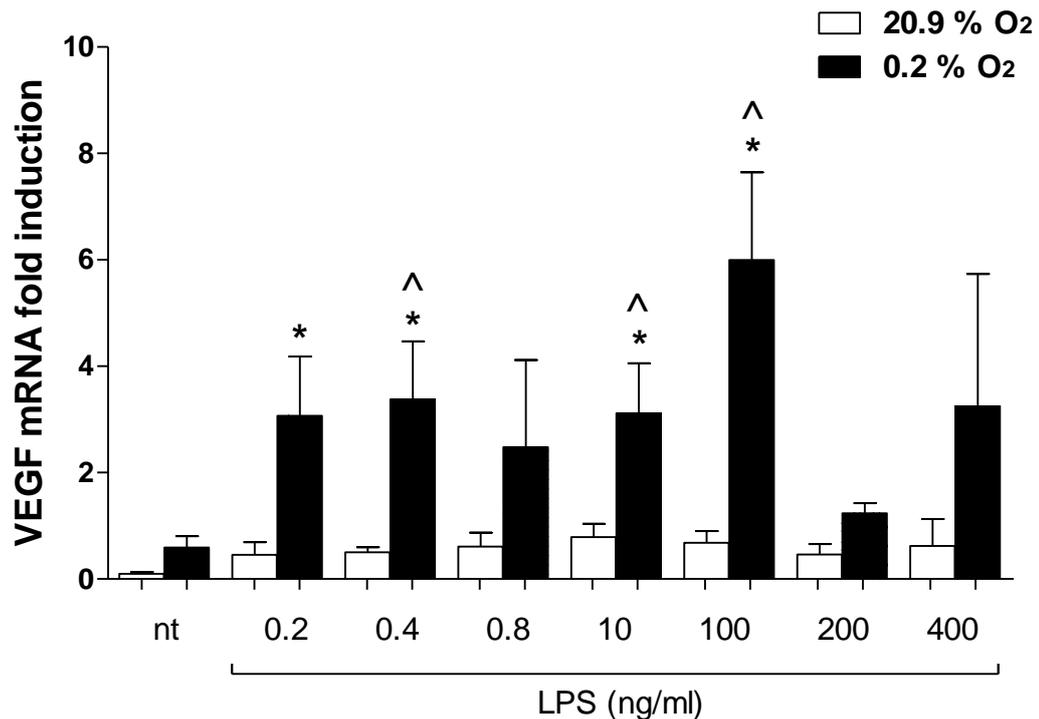
As expected (Shoshani *et al*, 2002), DDIT4 mRNA was up-regulated significantly ( $\leq 0.05$ ) by hypoxia, 4 fold when compared with normoxia (figure 6.1). In addition, figure 6.1 shows that DDIT4 responds to different concentrations of LPS in normal oxygen tension. However, only two LPS-treated samples (treated with 0.4 and 10 ng/ml of LPS) are statistically significantly different ( $\leq 0.05$ ) from the normoxia untreated samples. In this LPS-dose response experiment, hypoxia synergised with LPS to increase DDIT4 mRNA expression. The combined treatments with hypoxia plus LPS have roughly the same fold induction with all different LPS concentrations. This also suggests that the synergy effect of LPS with hypoxia is not dose-dependent because increasing the LPS doses had no effect on the synergistic fold induction. Furthermore, this RT-PCR data indicates that very low concentrations of LPS (down to 0.2 ng/ml) are able to cooperate with hypoxia to significantly ( $\leq 0.05$ ) up-regulate DDIT4 mRNA expression in a synergistic manner in primary HMDMs.



**Figure 6.1: DDIT4 mRNA expression in primary human macrophages under hypoxia with different LPS concentrations:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells were treated with different concentrations of LPS (0.2, 0.4, 0.8, 10, 100, 200, and 400 ng/ml) and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior to RNA isolation. DDIT4 mRNA expression was quantified by semi-quantitative real-time RT-PCR then data were normalized with  $\beta$ -2M mRNA levels in order to calculate fold induction. Data shown is from 4 independent experiments using different donors expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-tests. \* = P value  $\leq$ 0.05 considered as significant when compared with sample in normoxia with the same LPS concentration. ^ = P value  $\leq$ 0.01 with respect to 0.4 LPS alone in normoxia. \$ = P value  $\leq$ 0.05 with respect to normoxic sample (nt). # = P value  $\leq$ 0.05 when compared with hypoxic sample (nt).

My microarray data revealed up-regulation of two known HIF-inducible genes, VEGF and Glut-1 (Burke *et al*, 2003), as shown in table 4.4. VEGF was found to be up-regulated synergistically by combined treatment with hypoxia plus LPS (Table 4.3). However, Glut-1 was shown to be induced by hypoxia but not by LPS. In order to examine whether the synergistic induction of DDIT4 mRNA by hypoxia plus LPS would be replicated by these two well characterised genes, as a control to further validate the interesting results I had obtained in figure 6.1, VEGF and Glut-1 mRNA was

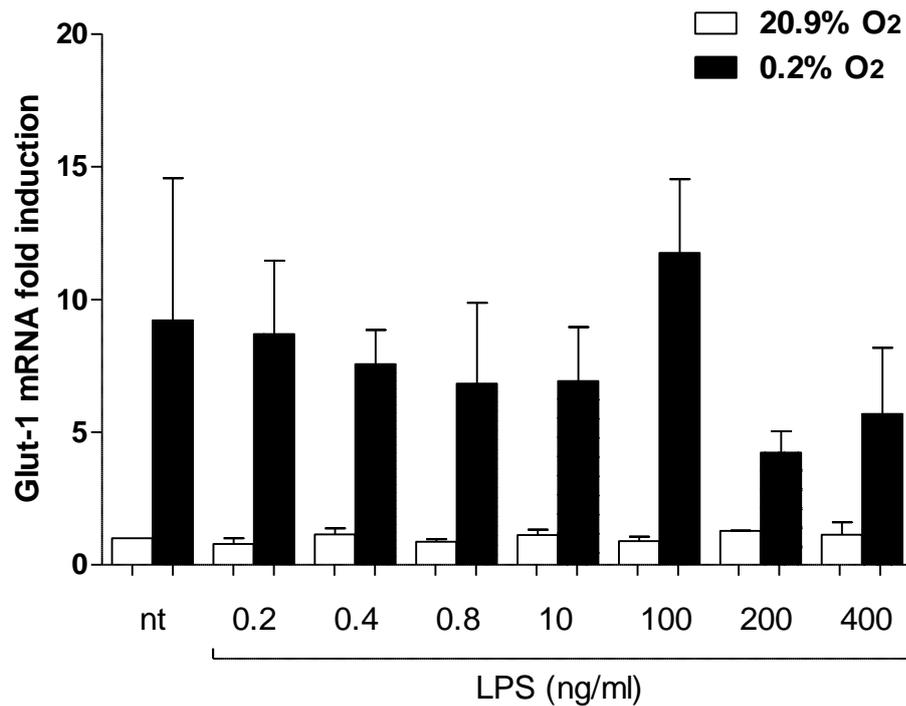
quantified in the same samples used to generate this figure. Several previous reports have shown that LPS induces VEGF mRNA and protein expression (Itaya *et al*, 2001; Botero *et al*, 2003). As figure 6.2 showed, human MDMs respond to LPS by up-regulation of VEGF mRNA. This data also showed that VEGF is induced by acute hypoxia. Moreover, hypoxia plus LPS synergizes to induce VEGF mRNA at several different LPS concentrations. However, as seen with DDIT4, increasing LPS concentration apparently has no consistent effect on VEGF mRNA expression. This synergistic up-regulation was found statistically significant ( $\leq 0.05$ ), compared to both hypoxia alone and the same concentration of LPS alone, in hypoxic samples treated with 0.4, 10, and 100 ng/ml of LPS.



**Figure 6.2: VEGF mRNA expression in primary human macrophages under hypoxia with different LPS concentrations:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells were treated with different concentrations of LPS (0.2, 0.4, 0.8, 10, 100, 200, and 400 ng/ml) and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior to RNA isolation. VEGF mRNA expression was quantified by semi-quantitative real-time RT-PCR then data were normalized with  $\beta$ -2M mRNA levels in order to calculate fold induction. Data from 4 independent experiments using different donors expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-test. ^ = P value  $\leq$  0.05 considered as significant when compared with sample in normoxia with the same LPS concentration. \* = P value  $\leq$  0.05 with respect to hypoxic sample (nt).

Next I tested the samples for Glut-1 mRNA expression. As figure 6.3 indicates, Glut-1 is sharply up-regulated by hypoxia in comparison with normoxia (nt). In addition, Glut-1 mRNA expression was also up-regulated in samples treated with hypoxia plus LPS, however, this up-regulation is similar to the hypoxic sample (nt), i.e. no synergy occurred (figure 6.3) which indicates that Glut-1 responded to hypoxia but not to LPS, which is consistent with the microarray data. Overall, using the same cDNA samples, the induction and synergy pattern is very similar for DDIT4 (figure 6.1) and VEGF (figure 6.2), but distinctly different for Glut-1, a hypoxia-regulated gene known not to be LPS-

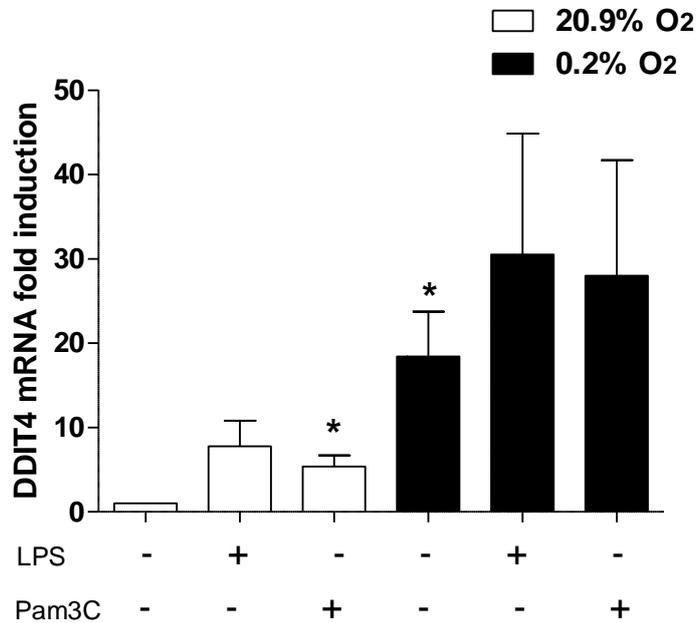
responsive in primary HMDMs. This strongly suggests that the synergy observed is genuine and is not due to a non-specific global effect on expression of all genes across all the samples.



**Figure 6.3: Expression of Glut-1 mRNA in primary human macrophages under hypoxia with different LPS concentrations:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells were treated with different concentrations of LPS (0.2, 0.4, 0.8, 10, 100, 200, and 400 ng/ml) and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior to RNA isolation. Glut-1 mRNA expression was quantified by semi-quantitative real-time RT-PCR then data were normalized with  $\beta$ -2M mRNA levels in order to calculate fold induction. Data from 4 independent experiments using different donors except for 200 and 400 ng/ml treated samples with just 2 different donors were used, and data expressed as means  $\pm$  SEM.

### **6.3.2 Regulation of DDIT4 mRNA in activated primary human macrophages by toll-like receptor (TLR) ligands**

In this project, I wanted to investigate whether other TLR ligands able to activate macrophages are able to synergize with hypoxia to induce the expression of DDIT4 mRNA, or whether the effect is limited to LPS. Therefore, the TLR ligand Pam 3 Cys was used. Pam 3 Cys is a synthetic lipopeptide and a potent activator of pro-inflammatory transcription factor NF- $\kappa$ B in monocytes and macrophages. Unlike LPS, which is recognized by TLR4, Pam 3 Cys is recognized by TLR2 (Heuking *et al*, 2009). Primary human MDMs were incubated for 5 days in normoxia, and then treated with either 100 ng/ml of LPS or Pam 3 Cys, then incubated in normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2 % O<sub>2</sub>) for 18 hours prior to RNA isolation. As seen in figure 6.4, the stimulation of human macrophages by LPS or Pam 3 Cys induced DDIT4 mRNA expression at similar levels. Moreover, both ligands appeared to synergize with hypoxia to up-regulate DDIT4 mRNA expression. However, this increase was not statistically significant due to high variability between these different donors. As expected, hypoxia alone was able to significantly up-regulate DDIT4 mRNA expression (figure 6.4).

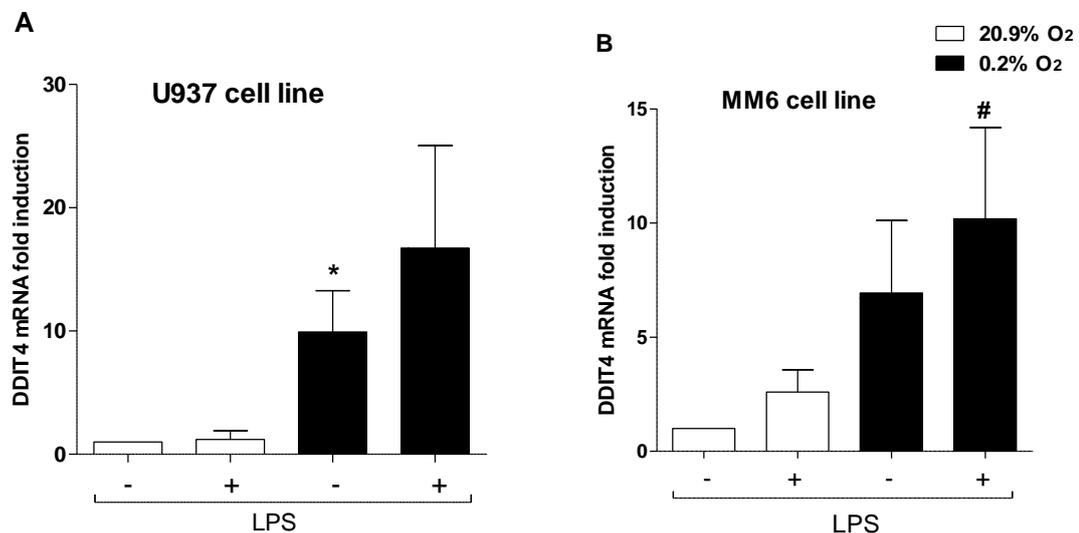


**Figure 6.4: Induction of DDIT4 mRNA in primary human macrophages treated with LPS and Pam3Cys in normoxia and hypoxia:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells were treated with 100 ng/ml of LPS or 100 ng/ml of Pam3C and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior to RNA isolation. DDIT4 mRNA expression was quantified by real-time RT-PCR and data were normalized against  $\beta$ -2M mRNA values. Data are from 3 independent experiments using different donors, and data are expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-tests. \* = P value  $\leq$ 0.05 is significant when compared to normoxic sample (nt).

### 6.3.3 Combined treatment with hypoxia and LPS induces DDIT4 mRNA expression in monocyte cell lines (U937 and MM6) *in vitro*

To further investigate the synergistic effect of hypoxia and LPS on DDIT4 mRNA expression in human monocytic cell lines, U937 and MM6 cells were used. Six well plates were seeded with 2 ml of U937 and MM6 cell suspensions at a density of  $2.0 \times 10^5$  cells/well, and treated with 100 ng/ml of LPS and incubated under normoxia or hypoxia for 18h prior RNA isolation. The analysis of my real-time PCR data shows that

DDIT4 mRNA levels were sharply induced by hypoxia in both cell lines. In addition, LPS moderately induce DDIT4 mRNA expression in MM6 but not U937 cells. However, when LPS and hypoxia were applied together, high levels of DDIT4 mRNA were induced in both cell types (figure 6.5). This latter data is consistent with primary macrophages, and indicates that combined treatment with hypoxia plus LPS seems to synergize to up-regulate DDIT4 expression in these cells. The synergistic effect of LPS plus hypoxia is significant ( $\leq 0.05$ ) in the MM6 cell line when compared with hypoxia alone. However, the synergy effect was not significant in U937 cells due to the marked experiment to experiment variation (figure 6.5 A), but when hypoxia alone was compared with LPS treated samples, statistical analysis shows a significant difference ( $\leq 0.05$ ). Overall, the fold induction of DDIT4 mRNA in U937 cells is higher than in MM6 cells.



**Figure 6.5: Regulation of DDIT4 mRNA in monocyte cell lines U937 and MM6 in response to LPS and hypoxia:** U937 and MM6 were plated at  $2 \times 10^6$  cells per well and treated with 100 ng/ml of LPS and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior to RNA isolation. DDIT4 mRNA expression was quantified by semi-quantitative real-time RT-PCR and data were normalized with  $\beta$ -2M mRNA levels. Data obtained from 3 independent experiments and expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-tests. \* = P value  $\leq 0.05$  when compared to LPS; # = P value  $\leq 0.05$  when compared to hypoxia, both therefore significantly different.

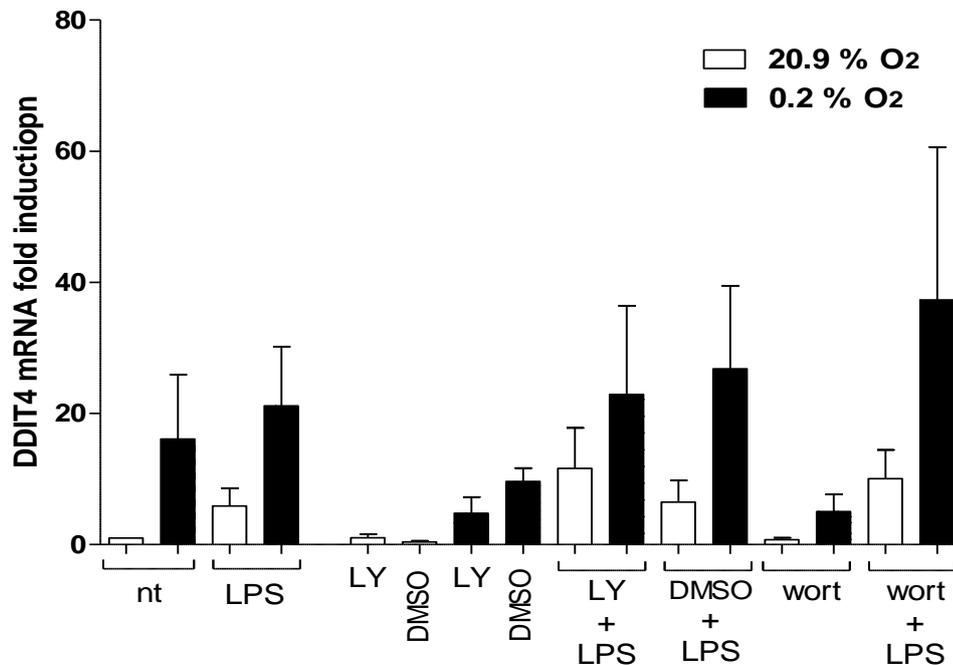
### **6.3.4 PI3-Kinase inhibitors reduce hypoxic induction of DDIT4 mRNA, but not induction by LPS or hypoxia plus LPS**

DDIT4 is a hypoxia-inducible gene under the regulation of HIF-1 (Shoshani *et al*, 2002). Schwarzer and colleague have shown that DDIT4 expression is depend on HIF-1 and PI3-kinase activity in human prostate cancer cells (PC-3) (Schwarzer *et al*, 2005). They used CoCl<sub>2</sub> to activate the hypoxic response in PC-3 cells, also they treated cells with the PI3-K inhibitor, LY294002. Both DDIT4 and HIF-1 $\alpha$  protein were found to be significantly increased by CoCl<sub>2</sub>, and this induction was blocked by either CoCl<sub>2</sub> inhibitors, or by LY294002. Another study by Jin and colleagues showed that the expression of DDIT4 mRNA and protein are dependent on HIF-1 $\alpha$  and Sp-1 in HeLa cells. In addition, inhibition of the PI3-K pathway by LY294002 reduces the expression of both DDIT4 mRNA and protein, and the activation of HIF-1 and Sp-1 by CoCl<sub>2</sub> were also found reduced by the PI3K inhibitor (Jin *et al*, 2007).

In this project, I wanted to investigate whether the synergistic effect of hypoxia plus LPS on DDIT4 mRNA expression in primary HMDMs is mediated through the activation of PI-3K pathway. Primary human MDMs were incubated for 5 days in normoxia, and then cells were treated with 10  $\mu$ M LY294002 or 200 nM wortmannin. Then cells were treated with or without 100 ng/ml of LPS, and incubated in normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hrs prior to RNA isolation. mRNA expression was quantified by real time RT-PCR and normalised with internal control  $\beta$ -2m values.

As expected, DDIT4 mRNA was up-regulated by hypoxia, LPS, and hypoxia plus LPS (figure 6.6). However, the expression of DDIT4 mRNA by hypoxia appeared to be slightly down-regulated in cells treated with the two specific inhibitors of PI3-Kinase, 10  $\mu$ M LY294002 and 200 nM wortmannin (with 3-fold reduction by each inhibitor)

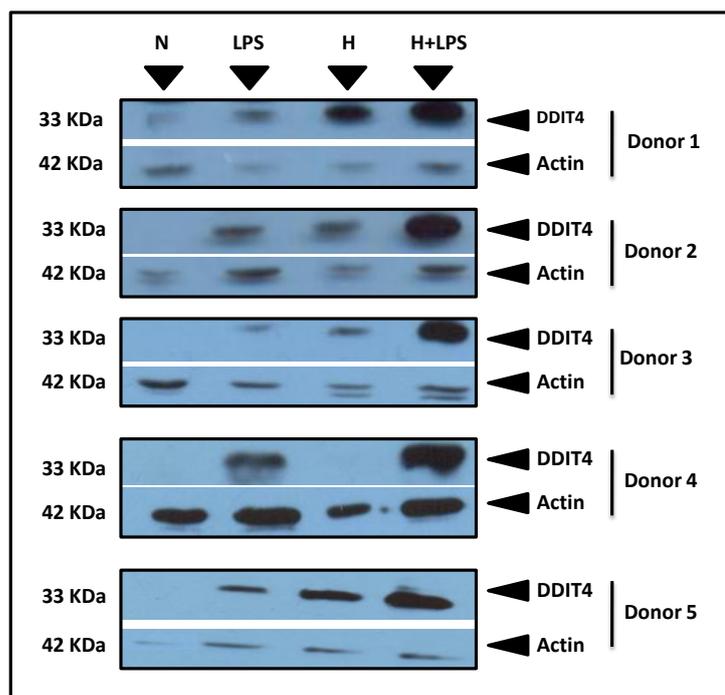
although this data were not statistically significant due to high variability in hypoxic untreated sample fold induction among these different donors. Furthermore, the possible inhibitory effect of LY294002 and wortmannin was lost in hypoxic cells when LPS was added. As previously mentioned in this thesis, LPS up-regulated DDIT4 mRNA expression in normoxia. Here my data have shown that both inhibitors have no effect on LPS-induced DDIT4 expression. It should be noted that DMSO itself causes a slight reduction in DDIT4 expression under hypoxia in comparison with untreated hypoxic cells (figure 6.6).



**Figure 6.6: Effect of PI3-kinase inhibitors on DDIT4 mRNA level in adherence-purified human MDMs:** The expression of DDIT4 mRNA after hypoxia and LPS treatment was quantified by real-time PCR to determine the effect of the PI3-kinase inhibitors, LY294002 and wortmannin. Plates were seeded with  $2 \times 10^6$  cells per well and treated with LY294002 or wortmannin at final concentrations of 10  $\mu$ M and 200 nM, respectively. Both inhibitors were dissolved in DMSO as a carrier. The same volume of DMSO was added to some cells as a control. Cells were then exposed to normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) for 18 hours prior to RNA isolation. Data from at least 5 independent experiments except for wortmannin and wortmannin plus LPS samples which represent only 2 independent experiments. All experiments were performed using different donors and DDIT4 RT-PCR data were normalized with  $\beta$ -2M mRNA values and expressed as means  $\pm$  SEM.

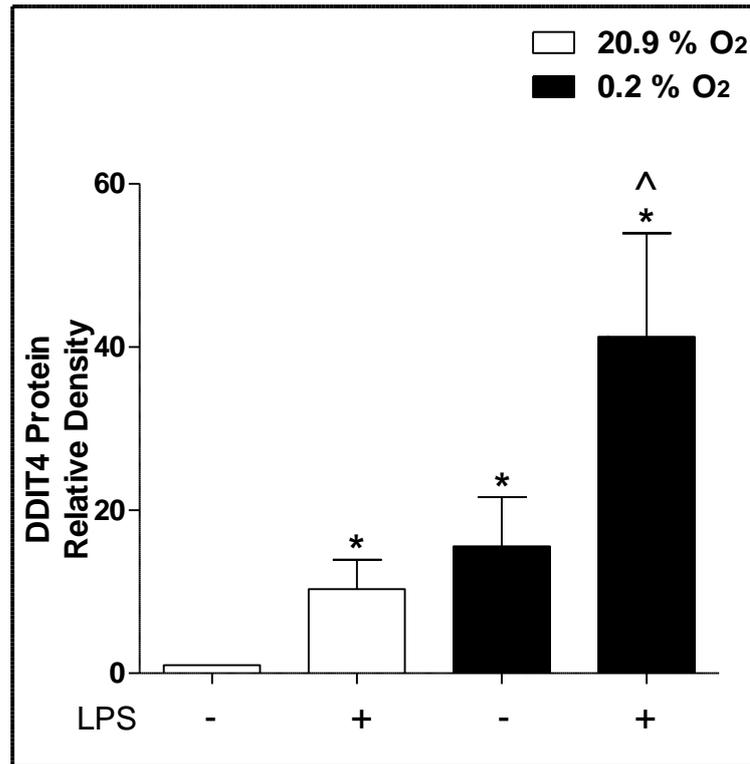
### **6.3.5 Analysis of the effect of hypoxia and LPS on DDIT4 protein expression in primary human macrophages *in vitro***

Several studies have shown that expression of DDIT4 protein is induced by different stimuli including hypoxia, in a variety of cell types (Shoshani *et al*, 2002; Ellisen *et al*, 2002). In this project, regulation of DDIT4 protein by hypoxia and LPS in primary HMDMs was investigated by immunoblotting. Cells were treated with and without 100 ng/ml LPS, and then incubated in normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2 % O<sub>2</sub>) for 18 hours prior to total protein extraction and samples separation by gel electrophoresis. As seen in figure 6.7, the effect of hypoxia and LPS on DDIT4 protein expression was investigated in HMDMs from 5 different healthy donors. On all the blotting membranes, no DDIT4 protein band was detected in normoxia cells, except for donor number 1 which showed very low expression. However, treatment of cells with LPS or hypoxia showed moderate induction of DDIT4 protein in all extracts, except for donor number 4 which showed no accumulation under hypoxia. In contrast to LPS or hypoxia samples, the treatment of macrophages with combined hypoxia plus LPS produced strong induction of DDIT4 protein in all 5 donors samples used. This indicates that expression of DDIT4 protein, as well as mRNA (as shown earlier) in primary macrophages responds synergistically to hypoxia plus LPS. The molecular mass shown here for DDIT4 is 33 KDa (Ellisen *et al*, 2002), and blots were re-probed for  $\beta$ -actin which is about 42 KDa, as a loading control for each blot.



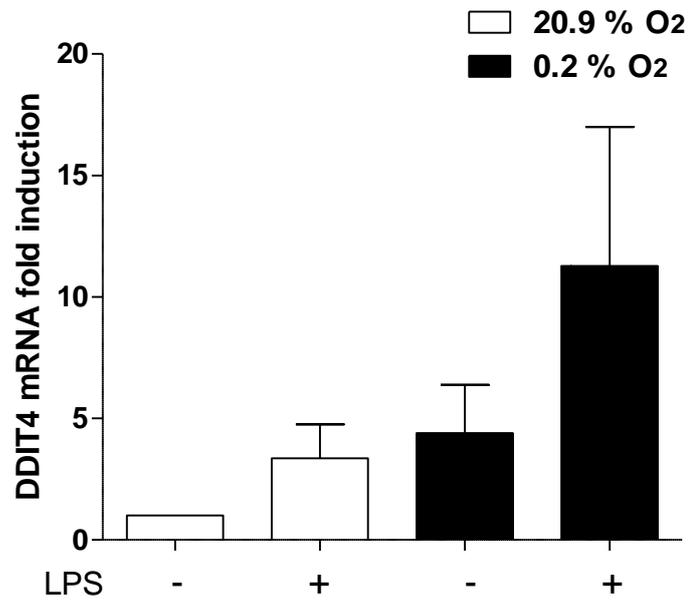
**Figure 6.7: Effect of hypoxia and LPS on DDIT4 protein expression in primary human MDMs:**  $2 \times 10^6$  cells of primary human macrophages were seeded with 2 ml of filtered Iscove's medium per well. Cells were treated with and without of 100 ng/ml LPS and incubated under normoxia (20.9 %  $O_2$ ) or hypoxia (0.2 %  $O_2$ ) for 18 hours prior to total protein extraction. 100  $\mu$ g of total protein was then immunoblotted to assess the presence of DDIT4 and actin proteins using 1: 1000 and 1:2500 dilutions of primary antibody, respectively, followed by 1:2500 dilutions of secondary antibody. Blots of 5 protein extractions obtained from different donors in independent experiments were performed.

In order to get accurate quantification of DDIT4 protein expression in primary HMDMs, the intensity of each band in membrane was estimated by densitometry, which enables statistical evaluation. The intensity of DDIT4 protein bands was measured and normalized against intensity of the internal loading control, actin. As figure 6.8 indicates, the expression of DDIT4 protein is significantly increased by LPS, hypoxia, and hypoxia plus LPS condition in comparison with normoxia. The densitometer data shows that DDIT4 protein expression is synergistically increased by combined treatment with hypoxia plus LPS in comparison with either LPS or hypoxia alone. However, this synergy effect is statistically significant only when compared with LPS treated samples.



**Figure 6.8: Densitometer analysis of DDIT4 protein expression in primary human MDMs:** The intensity of DDIT4 bands in immunoblotting membranes was measured by densitometer and normalised against the intensity of actin bands. Data obtained from 5 independent experiments using different donors and expressed as means  $\pm$  SEM. Data were further analysed for significant induction using paired t-tests. \* = P value  $\leq$ 0.05 considered as significant when compared with normoxic sample. ^ = P value  $\leq$ 0.05 is significant when compared with LPS treated sample.

In order to make a clear comparison between DDIT4 mRNA and protein expression in primary HMDMs, the mRNA level of DDIT4 was also analysed by real-time PCR in treated cells obtained from the same 5 donors used in immunoblotting for protein detection. As seen in figure 6.9, the real-time PCR data indicates a synergistic effect of hypoxia plus LPS on DDIT4 mRNA expression in these samples, although these data were not statistically significant due to high variability in hypoxic fold induction among these different donors. The consistency in pattern similarity of expression found between protein and mRNA induction in each condition indicates that the up-regulation of DDIT4 by combined treatment (hypoxia plus LPS) occurred at the transcriptional and post-transcriptional levels in human MDMs, figure 6.9.



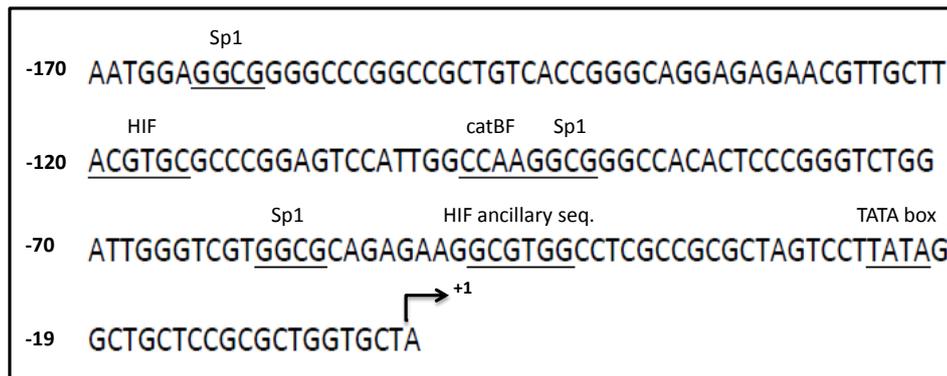
**Figure 6.9: Effect of hypoxia and LPS on DDIT4 mRNA expression in primary human MDMs:** Primary human macrophages were obtained from the same donors that used in western blot experiments. Only 4 donors blood were used in this real-time PCR, n=5. Cells were treated with normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2 % O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS and incubated under for 18 hours. Total RNA was isolated, and DDIT4 mRNA was quantified by semi-quantitative real-time RT-PCR, normalized against  $\beta$ -2M mRNA levels to calculate fold induction, expressed as means  $\pm$  SEM.

### **6.3.6 Effect of hypoxia and LPS on DDIT4 promoter reporter construct activity in primary human macrophages *in vitro***

Several investigators have analysed the regulation of DDIT4 promoter by different treatments. Some have shown many transcription factor binding sites involved in the induction of DDIT4 expression in response to different extracellular stimuli including hypoxia. Hypoxia-inducible factor (HIF-1) has been identified as the main regulator for DDIT4 in hypoxia (Shoshani *et al*, 2002). Lee and colleagues have used serial deletion of constructs carrying the DDIT4 DNA promoter in front of luciferase to identify the essential motif that important for its activity under hypoxia (Lee *et al*, 2004). They showed that DDIT4 promoter is mediated mainly by Sp1 and HIF-1 in 293 cells, and it is possible that Sp1 and HIF-1 synergize to induce the DDIT4 promoter under hypoxia.

Plasmid-based gene reporter systems are valuable tools to study the expression of a given gene both *in vitro* and *in vivo* for monitoring intrinsic promoter expression in animal cells. Therefore, to further understand DDIT4 gene regulation in primary HMDMs, four constructs were used; pGL3-Basic (empty vector), DDIT4 (495bp (-197/+298)), and two (495bp (-197/+298)) constructs mutated at either the HIF-1 or Sp1 binding sites. These 495bp constructs were a kind gift from Professor Sung Wan Kim (Lee *et al*, 2004). All four plasmids were grown up and purified and checked by restriction digestion and sequence analysis before being used in transfections experiments (Data not shown). The 5'-flanking sequence of the wild-type promoter construct was analysed using Genomatix MatInspector and TESS software for putative transcription binding sites (figure 6.10). The analysis indicated a number of potential binding sites for transcription factors associated with induction by hypoxia or LPS, including the binding site for HIF-1 transcription factor and the HIF ancillary sequence.

The HRE mutated construct is mutated at the HIF binding site between (-115 to -120). In addition, there are three binding sites for Sp-1; the mutated Sp1 construct has a mutated binding site between -161 to -164bp. A TATA box sequence also was found close to the transcription start site (figure 6.10).



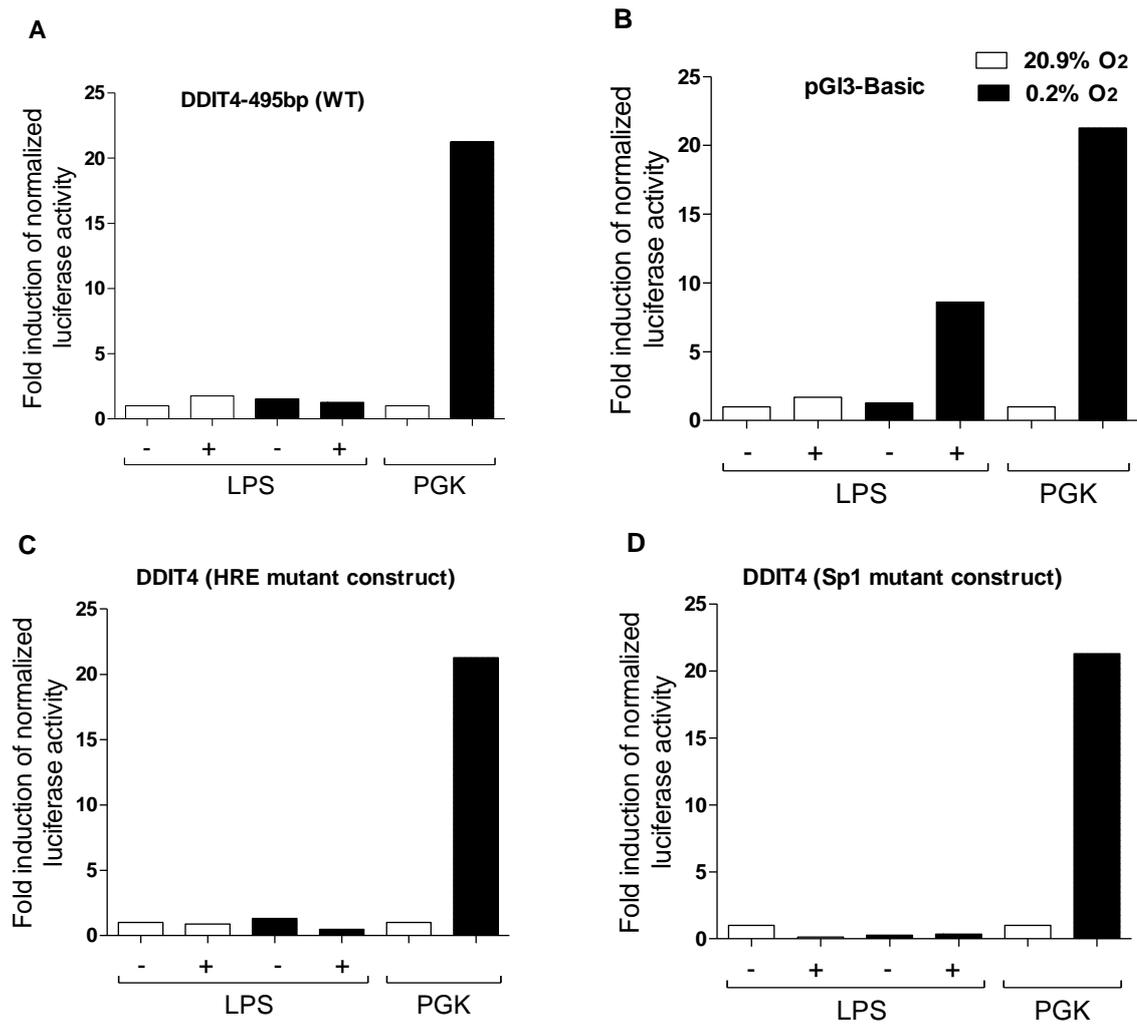
**Figure 6.10: The human DDIT4 promoter sequence showing putative transcription factor binding sites:** Human DDIT4 promoter sequence was analysed using Genomatix MatInspector and TESS software. The data shows regulatory element sites for transcription factors activated by different stimuli. Hypoxia-inducible Factor (HIF), Sp-1 transcription factor, TATA box, HIF ancillary sequence, and CAT binding factor (CAT BF).

Macrophages were transfected with 1 µg of DDIT4 reporter construct and co-transfected with 25 ng *Renilla* phRL-TK using 3.2 µl of JetPEI transfection reagent, then incubated in normoxia (20.9% O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) with or without 100 ng/ml of LPS for 6 hours. Luciferase activity of the constructs in each condition was measured and the data of each DDIT4 reporter construct activity was normalized with *Renilla* values. Table 6.1 shows the luciferase values in normoxia and hypoxia of both firefly and *Renilla* control plasmids after subtracting the background values of LAR II and Stop & Glo reagent.

As figure 6.11 shows, no fold induction was obtained from the wild type DDIT4-495bp construct under hypoxia or/and LPS. In contrast, the basic (empty vector) was induced by hypoxia plus LPS. However this induction is not reliable because of the very low luciferase values it is based on, because this construct has no promoter, and because the data is from only one experiment. The DDIT4 gene is known to be regulated by HIF-1 (Shoshani *et al*, 2002). In addition, Sp-1 and HIF-1 were found to be essential for DDIT4 regulation in different cell types including HUVEC (human umbilical vascular endothelial cell), A7R5 (rat smooth muscle cell), NIH3T3 (mouse fibroblast cell), and HepG2 (human hepatocyte) (Lee *et al*, 2004). Therefore, to investigate the role of Sp-1 and HIF-1 under combined treatment of macrophages by hypoxia and LPS, another two mutated reporter promoter constructs in either the HIF or Sp-1 binding sites were used to transfect primary HMDMs and incubated under hypoxia in the presence or absence of LPS for 6 hrs. As seen in figure 6.11, similar to the wild type construct, the DDIT4-HRE mutated construct does not show any fold induction under any condition used in primary HMDMs. As with the previous transfections, a PGK construct was also used as a control for cell transfection and hypoxic activity. As expected, hypoxia induced luciferase expression in HMDMs transfected with PGK, a hypoxia-inducible positive control luciferase reporter construct (Ameri *et al*, 2002). It was noticed that the luciferase activity of DDIT4-Sp-1 mutated construct markedly decreased when LPS was added into samples under normoxia and hypoxia as showed in table 6.1, which may indicate a negative effect of LPS on this construct. Furthermore, DDIT4-Sp-1 mutated construct showed a decreased fold induction under every treated condition used in primary HMDMs.

Normoxia (6h)		
Sample	Luciferase (RLU/s)	Renilla (RLU/s)
pGI3-Basic	43	1493
pGI3-Basic+LPS	27	556
DDIT4-495	8386	561
DDIT4-495+LPS	3784	145
HRE-mutant	7060	320
HRE-mutant+LPS	3178	161
Sp1-mutant	19302	405
Sp1-mutant+LPS	399	55
PGK N	115	103
Hypoxia (6h)		
Sample	Luciferase (RLU/s)	Renilla (RLU/s)
pGI3-Basic	54	1484
pGI3-Basic+LPS	110	443
DDIT4-495	16710	730
DDIT4-495+LPS	3411	180
HRE-mutant	9936	347
HRE-mutant+LPS	2123	201
Sp1-mutant	13299	1018
Sp1-mutant+LPS	3629	213
PGK H	23165	975

**Table 6.1: Luciferase values of DDIT4 promoter reporter constructs:** Primary human macrophages were incubated under normoxia for 5 days. Cells were transfected with 1 µg of DDIT4 promoter constructs. All constructs were co-transfected with 25 ng of *Renilla* normalization plasmid, using JetPEI reagent. Transfected cells were incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 6hrs. Luciferase activity of firefly and *Renilla* were measured for all DDIT4 promoter constructs. Background RLU obtained using only LAR II and Stop & Glo reagent, in the absence of cell lysate, were subtracted from the Firefly and Renilla values.



**Figure 6.11: DDIT4 promoter reporter construct luciferase activity in primary human macrophages:** Cells were incubated under normoxia for 5 days, and transfected with 1  $\mu$ g of (A) DDIT4-495bp promoter construct, (B) pGL3-basic, (C) HRE mutated DDIT4-495bp promoter construct, and (D) Sp1 mutated DDIT4-495bp promoter construct. All constructs were co-transfected with 25 ng of *Renilla* normalization plasmid. Transfected cells were incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 6 hrs. Luciferase data obtained from 1 experiment.

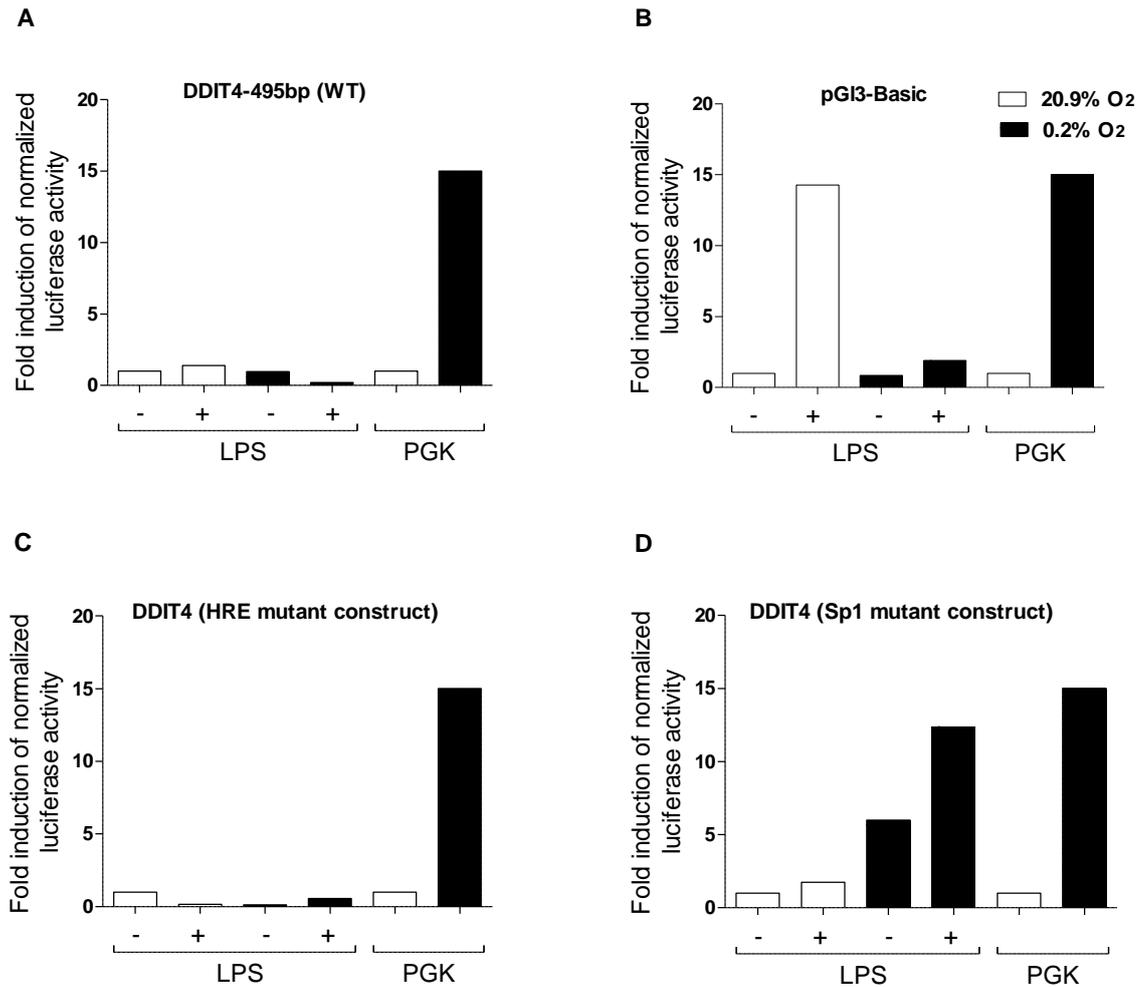
Based on the data generated with 6 hrs of incubation (Table 6.1 and figure 6.11), I decided to perform further experiments with longer incubation periods. Some previous investigations on the hypoxia-inducible gene versican had shown that hypoxia-inducible mRNAs can be up-regulated much more highly by extending the hypoxic incubation period (B. Burke, unpublished data). Therefore, I investigated the

luciferase activity of the same set of DDIT4 reporter constructs under 18h of hypoxia. Primary HMDMs were transfected as before and incubated under hypoxia in the presence or absence of LPS for 18 hrs. Table 6.2 shows that the luciferase values of the Renilla control plasmids are promising in that the levels of luciferase expression are above the background which can be used for normalization (Table 6.2).

As seen in figure 6.12, similar to the wild type construct, the DDIT4-HRE mutated construct does not show any fold induction under any condition used in primary HMDMs. Empty vector and Sp-1 mutated constructs showed induction by LPS, and hypoxia, and hypoxia plus LPS. However this data is opposite to what I expected because pGL3-basic has no promoter and Sp-1 mutated construct should not be activated under hypoxia because DDIT4 expression has been previously shown to depend on Sp-1 in accompanied with HIF (Shoshani *et al*, 2002). Furthermore, since the wild type construct was not induced, it is difficult to understand how the Sp1 mutant construct could be induced unless Sp1 exerts a negative effect on the promoter.

Normoxia (18h)		
sample	Luciferase (RLU/s)	Renilla (RLU/s)
pGI3-Basic	37	1185
pGI3-Basic+LPS	86	193
DDIT4-495	19488	651
DDIT4-495+LPS	4427	107
HRE-mutant	39232	747
HRE-mutant+LPS	2584	328
Sp1-mutant	2235	307
Sp1-mutant+LPS	1421	111
PGK N	5305	403
Hypoxia (18h)		
sample	Luciferase (RLU/s)	Renilla (RLU/s)
pGI3-Basic	63	2367
pGI3-Basic+LPS	46	772
DDIT4-495	8704	301
DDIT4-495+LPS	976	153
HRE-mutant	4459	682
HRE-mutant+LPS	6763	230
Sp1-mutant	37142	851
Sp1-mutant+LPS	22210	246
PGK H	136740	692

**Table 6.2: Luciferase values of DDIT4 promoter reporter constructs:** Primary human macrophages were incubated under normoxic condition for 5 days. Cells were transfected with 1 µg of DDIT4 promoter constructs. All constructs were co-transfected with 25 ng of *Renilla* normalization plasmid, and incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 18h. Firefly and *Renilla* luciferase activity were measured for all constructs. Background RLU obtained using only LAR II and Stop & Glo reagent, in the absence of cell lysate, were subtracted from the Firefly and Renilla values.

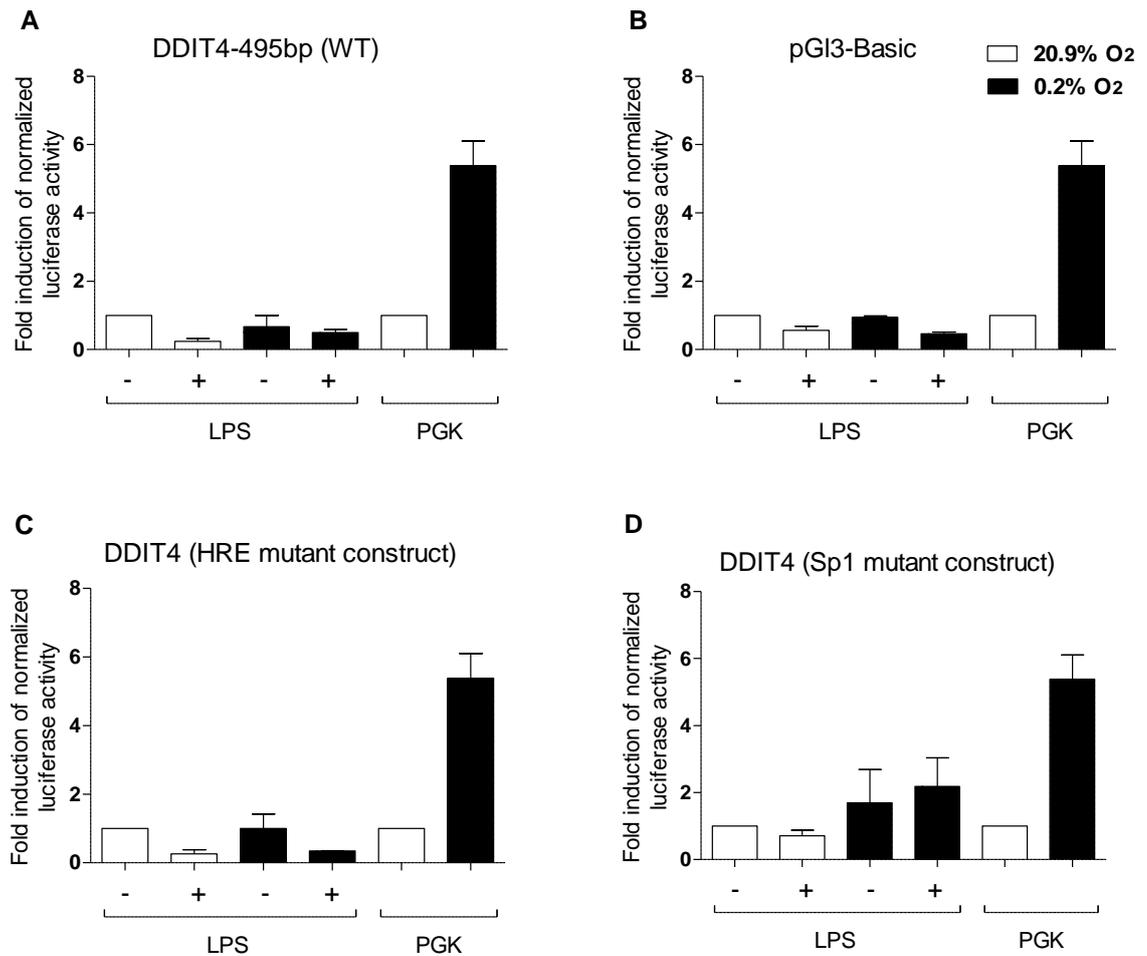


**Figure 6.12: Luciferase activity of DDIT4-495bp reporter constructs:** Primary human macrophages were incubated under normoxia for 5 days. Cells were transfected with 1  $\mu$ g of (A) DDIT4-495bp promoter construct, (B) with 1  $\mu$ g pGL3-basic, (C) with 1  $\mu$ g of HRE mutated DDIT4-495bp promoter construct, and (D) with 1  $\mu$ g Sp1 mutated DDIT4-495bp promoter construct, and all constructs were co-transfected with 25 ng of *Renilla* normalization plasmid. Transfected cells were incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 18 hrs. Luciferase data obtained from 1 experiment.

In order to evaluate the reproducibility of the above experiments, I decided to repeat the transfections with the same incubation period (18 hrs), because accurate judgment cannot be built on only one experiment (n=1), and repeated transfections are required. Therefore, another two transfection experiments were performed on

different occasions using the same amount of DNA and incubation period used in figure 6.12. In these two extra transfections, the data could not be normalized by internal control due to very low level of Renilla luciferase expression. So to solve this problem and get reproducible data, luciferase values of firefly were normalized to the protein concentrations instead of Renilla values (raw data not shown).

As figure 6.13 shows, no fold induction was obtained from DDIT4-495bp construct under hypoxia or/and LPS. Similar to wild type construct, DDIT4-HRE mutated construct does not show up-regulated fold induction under any condition used in primary HMDMs. However, hypoxia and hypoxia plus LPS were possibly slightly able to activate Sp-1 mutated construct but this aberrant up-regulation seems to be not real because as mentioned above, Sp-1 mutated construct should not be activated under hypoxia. The pGl3-basic construct does not show induction by hypoxia and this is expected because it has no promoter. However, transfected samples treated with LPS and hypoxia plus LPS showed down-regulation in respect with untreated, but also this is not real because pGl3-basic construct has no promoter. As with all the previous transfections, PGK construct was also used as a control for cell transfection and hypoxic activity (figure 6.13).



**Figure 6.13: DDIT4 promoter reporter construct luciferase activity in primary human macrophages:** Primary human macrophages were incubated under normoxia for 5 days. Cells were transfected with 1  $\mu$ g of (A) DDIT4-495bp promoter construct, (B) with 1  $\mu$ g pGL3-basic, (C) with 1  $\mu$ g of HRE mutated DDIT4-495bp promoter construct, and (D) with 1  $\mu$ g Sp1 mutated DDIT4-495bp promoter construct. Transfected cells were incubated under normoxia (20.9 % O<sub>2</sub>) or hypoxia (0.2% O<sub>2</sub>) in the presence or absence of 100 ng/ml LPS for 18 hrs. Data from 2 independent experiments using different donors are expressed as means  $\pm$ SEM.

## **6.4 The role of hypoxia-inducible factor (HIF) in DDIT4 regulation in macrophages**

The hypoxia-inducible factors (HIFs) 1 and 2 are the predominant transcription factors mediating the effects of hypoxia on gene expression in mammalian cells (Majmundar *et al*, 2010). In tumours, atherosclerotic plaques, and rheumatoid arthritis, these two transcription factors play crucial roles to control many functions of macrophages under hypoxia, such as angiogenesis and glycolysis (Semenza, 2003).

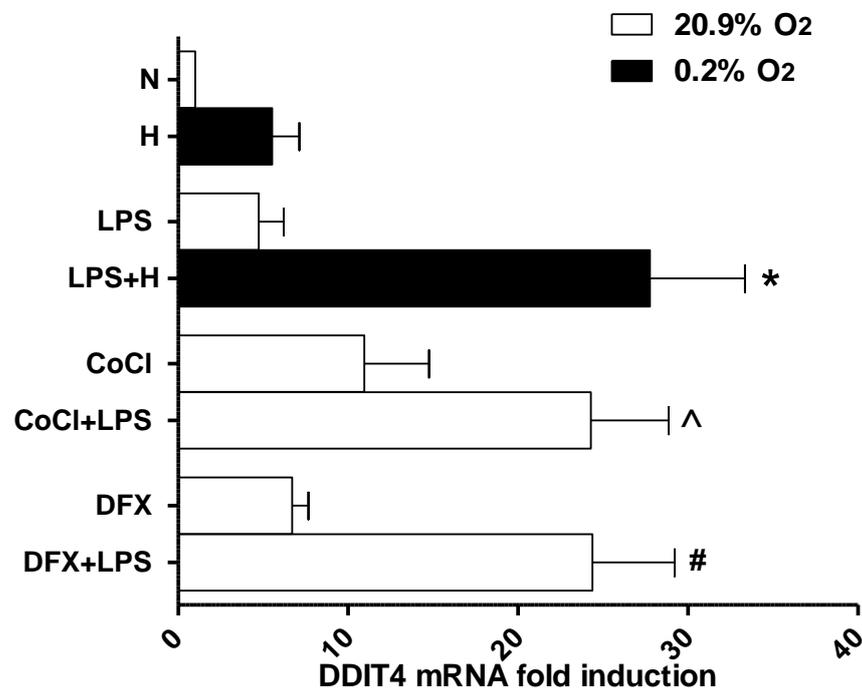
Gene-specific silencing methods are widely used by scientists to determine the effect of gene expression in mammalian cells. Transfection of cells by interference RNA (iRNA) is a novel approach to inhibit HIF expression in primary macrophages in order to see effects on downstream genes (Tuschl & Borkhardt, 2002).

In the following sections, attempts were made to investigate the contribution of HIF in DDIT4 expression in macrophages under hypoxia and LPS. The first attempt was to investigate the effect of HIF in normoxia on DDIT4 induction by using “hypoxia mimetic” agents. I also tried to use two different types of RNAi, short hairpin RNA (shRNA) transduced by a viral vector (lentivirus) and also small interfering RNA (siRNA) synthetic oligonucleotides delivered by chemical carrier (HyperFect).

### **6.4.1 Hypoxic mimetic agents, cobalt chloride and desferrioxamine induce DDIT4 mRNA expression in primary human macrophages**

HIF-1 is a main regulator of many important functions under hypoxia including angiogenesis and glycolysis (Semenza, 2003). The DDIT4 gene was identified to be regulated through the HIF-1 pathway in different cell types under hypoxia (Shoshani *et*

*al*, 2002). In addition, many studies demonstrated that HIF-1 $\alpha$  can be up-regulated by non-hypoxic stimuli such as LPS, and by the “hypoxia mimetic” agents cobalt chloride (CoCl<sub>2</sub>), and desferrioxamine (DFO), under normal oxygen concentrations (Frede *et al*, 2006; Dery *et al*, 2005). To determine whether hypoxia plus LPS-induced DDIT4 mRNA expression is dependent specifically on HIF-1 $\alpha$ , primary HMDMs were treated with cobalt chloride, desferrioxamine, or hypoxia in the presence or absence of 100 ng/ml LPS for 18 hours. As expected, DDIT4 is up-regulated by LPS alone and by hypoxia alone (figure 6.14). Interestingly, CoCl<sub>2</sub> or DFX also induced up-regulation of DDIT4 mRNA expression in primary HMDMs (figure 6.14). This up-regulation reached a level similar to hypoxia. Furthermore, CoCl<sub>2</sub> or DFX synergized with LPS in up-regulation of DDIT4 mRNA expression at a level close to that achieved by combined treatment by hypoxia plus LPS. These data suggest that DDIT4 is regulated (directly or indirectly) through HIF in primary HMDMs, and that this also mediates the LPS – Hypoxia synergy effect.



**Figure 6.14: Hypoxia mimetic agents and LPS synergistically induce DDIT4 mRNA expression:** Primary human macrophages were treated with CoCl<sub>2</sub> (300 μM), and DFX (200 μM) in the presence or absence of 100 ng/ml LPS and incubated under normoxia and hypoxia for 18 hours. Total RNA was isolated, and DDIT4 mRNA expression was quantified by semi-quantitative real-time RT-PCR and normalized with β-2M mRNA values. Data were obtained from 3 independent experiments using different donors and expressed as means ± SEM. Data were further analysed for significant induction using paired t-tests. \* = P value ≤0.05 considered as significant when compared to LPS or hypoxic samples. ^ = P value ≤0.05 with respect to LPS treated sample. # = P value ≤0.05 when compared to DFX or LPS samples.

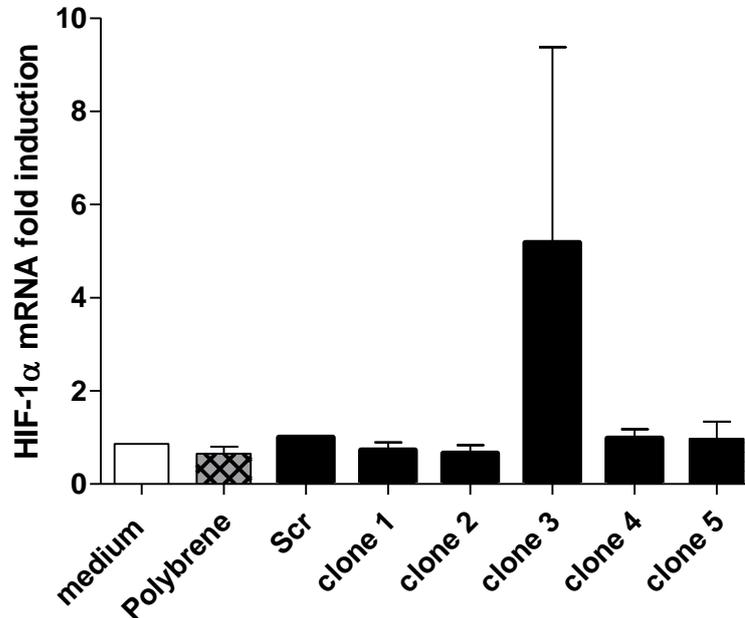
#### 6.4.2 HIF-1α gene silencing in primary human macrophages using shRNA delivered by lentivirus particles

In the last few years, several mechanisms for gene silencing have been developed utilizing vector-base strategies that contain stem-loop constructs encoding hairpin RNAs (Brummelkamp *et al*, 2002). Recently, lentiviruses have been used to introduce shRNA into a wide range of cell lines (Sliva & Schnierle, 2010). The shRNA is produced

and shuttled out into the cytoplasm where it is cleaved by the enzyme complex Dicer to produce double stranded RNA (dsRNA). Once this cleavage is completed, one strand of this double-stranded molecule is loaded into the RNA induced silencing complex (RISC), where it seeks complementary mRNA targets for degradation. Lentiviral particles are ideal for transducing into animal cells. MISSION<sup>®</sup> lentiviral particles (Sigma Aldrich) allow transduction of a wide range of cells. In addition, lentiviral particles stably integrate the shRNA into the genomes of both dividing and non-dividing cell lines. In this project, we used these commercial lentivirus HIF-1 $\alpha$  shRNA clones to attempt to induce silencing of HIF-1 $\alpha$  to observe the effect on DDIT4.

In this project, five clones of shRNA with different targeting sequences were used against HIF-1 $\alpha$  mRNA. In addition, one shRNA nonsense control was used as a negative control in transfection experiments. Primary human macrophages were incubated under normoxia for 5 days. Cells were cultured for 4 hrs with polybrene in combination with viral infection. Polybrene neutralizes charge interactions to increase binding between the viral capsid and the cellular membrane. The optimal concentration of polybrene depends on cell type and may need to be empirically determined (usually in the range of 2-10  $\mu$ g/ml). I found that 2 mg/ml of polybrene was the best to use without causing toxicity to cells. Excessive exposure to polybrene (>12 hrs) can be toxic to some cells. The multiplicity of infection (MOI) used was 6 virions per cell. Cells were incubated in normoxia for a further 3 days prior to RNA isolation. As shown in figure 6.15, all HIF-1 $\alpha$  shRNA clones failed to suppress the expression of HIF-1 $\alpha$  mRNA levels in primary MDMs in comparison with untreated or non-target control vector cells.

However, this data should be considered with care because only two transfection experiments were performed.



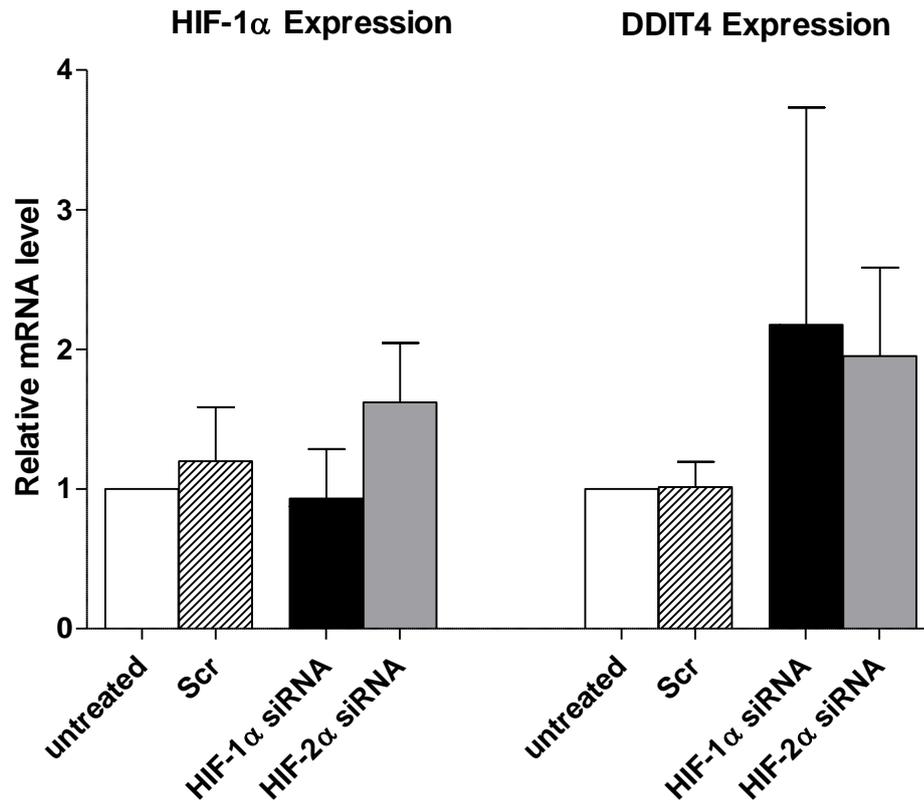
**Figure 6.15: Attempted HIF-1 $\alpha$  knockdown in primary human macrophages by shRNA:** Primary human macrophages were incubated under normoxia for 5 days. Cells were treated with 2 mg/ml of polybrene and transduced with shRNA using MOI=6. shRNA targeting HIF-1 $\alpha$  mRNA was delivered by five different lentivirus clones, a negative control clone, and with medium as the untreated control. After transduction cells were incubated in normoxia for three days prior to RNA isolation. HIF-1 $\alpha$  mRNA expression was quantified by semi-quantitative real-time RT-PCR and data were normalized against  $\beta$ -2M mRNA values. Data obtained from 2 independent experiments using different donors are expressed as means  $\pm$  SEM.

#### 6.4.3 Attempts at HIF-1 $\alpha$ and HIF-2 $\alpha$ gene silencing in MM6 cells by transient transfection with small interfering RNA (siRNA)

Another approach for HIF knockdown was used in this project. Small interfering RNA (siRNA) synthetic RNA oligonucleotides were shown to be able to enhance mRNA degradation in living cells (Caplen, 2003). Several amounts of siRNA were first used in order to find the best amount needed without causing toxicity to the cells. I found that cells stay healthy when 150 ng/ml of siRNA was used with 12 $\mu$ l of HiPerFect

transfection reagent as a carrier. In addition, scrambled siRNA was also used as a nonsense negative control for transfection experiments. Different post transfection incubation periods were tested and it was found that 96 hrs in normoxia is the best time period to avoid affecting the cell viability. RNA was isolated and RT-PCR performed for HIF-1 $\alpha$ . As shown in figure 6.16, the scrambled siRNA showed a slight effect on HIF-1 $\alpha$  expression, and this is expected due to the stress of the transfection on the cells. The level of HIF-1 $\alpha$  mRNA was unchanged when compared with untreated sample taking into account the variation indicated by the error bars, and this indicate that siRNA has no effect on HIF-1 $\alpha$  mRNA suppression. In addition, cells transfected with siRNA target HIF-2 $\alpha$  showed no significant reduction in HIF-2 $\alpha$  mRNA. This data indicate that both siRNA oligonucleotides did not suppress either HIF-1 $\alpha$  or HIF-2 $\alpha$  mRNA expression.

In order to elucidate the effect of siRNA oligonucleotides on DDIT4 transcription, DDIT4 mRNA levels were also quantified by real-time RT-PCR in the same transfected samples. As figure 6.16 shows, DDIT4 mRNA expression was not reduced by HIF-1 $\alpha$  siRNA.

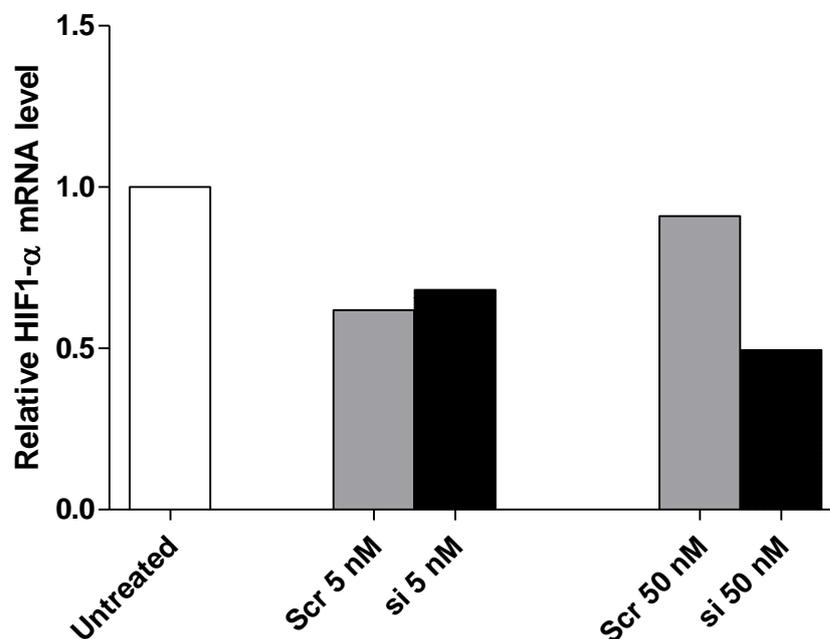


**Figure 6.16: HIF-1/2- $\alpha$  knockdown in the MM6 cell line by siRNA:** Macrophages cell line (MM6) has transfected with siRNA targeted HIF-1 $\alpha$  and HIF-2 $\alpha$  genes. Cells also transfected with siRNA negative (non-coding) control and with medium as untreated cells. siRNA was delivered by HiPerFect transfection reagent mixture. After transfection, cells were incubated in normoxia for 96 hours, then total RNAs have isolated. HIF-1 $\alpha$  and DDIT4 mRNA expression were quantified by real-time RT-PCR, and data were normalized with  $\beta$ -2M mRNA levels. Data obtained from 4 independent experiments and expressed as means  $\pm$  SEM.

#### 6.4.4 Attempts to knock down HIF-1 $\alpha$ mRNA in primary human macrophages using transient transfection with small interfering RNA (siRNA)

Following failing in the knockdown experiment of HIF-1 $\alpha$  in MM6 cell line, I wanted to attempt the silencing approach on primary HMDMs. In this experiment, only siRNA oligonucleotides targeting HIF-1 $\alpha$  were used. The PBMC were plated at  $2 \times 10^6$  cells per well and incubated under normoxia (20.9 % O<sub>2</sub>) for 5 days. Cells were transfected with two different amounts of siRNA, 5 and 50  $\mu$ g using HiPerFect transfection reagent, and

then incubated for 96 hours in normoxia. As shown in figure 6.17, HIF-1 $\alpha$  mRNA was reduced by 50 % in comparison with untreated sample when cells were transfected with 50 nM of siRNA. However, we should treat this data with care because only one experiment was performed, and because the level of HIF-1a mRNA at 50nM was not markedly different from the mRNA level in cells treated with 5 nM of the scrambled nonsense siRNA.



**Figure 6.17: HIF-1 $\alpha$  knockdown in primary human macrophages by siRNA:** Primary human macrophages were transfected with siRNA targeting HIF-1 $\alpha$  mRNA. Two concentrations were used, 5 and 50 nM as a final concentration, with scrambled as negative control. siRNA was delivered by HiPerFect transfection reagent. After transfection, cells were incubated in normoxia for 96 hrs prior to RNA isolation. HIF-1 $\alpha$  mRNA expression level was quantified by semi-quantitative real-time RT-PCR, and data were normalized with  $\beta$ -2M mRNA levels. Data obtained from only one transfection experiment.

## 6.5 Discussion

DDIT4 (DNA-damage-inducible transcript 4), also known as REDD1 (regulated in development and DNA damage responses 1), Dig2 (Dexamethasone-induced gene 2), or RTP801, is a stress response regulator in animal cells (Sofer *et al*, 2005). DDIT4 was identified as a gene induced by hypoxia and DNA damage (Shoshani *et al*, 2002; Ellisen *et al*, 2002). Other environmental stresses such as energy stress, glucocorticoid treatment and reactive oxygen species have also been reported to induce DDIT4 transcription (Lin *et al*, 2005b; Sofer *et al*, 2005) .

The DDIT4 gene is present in human, mouse and *Drosophila*, and is ubiquitously expressed in most mammalian tissues including brain, heart, colon, liver, muscle, stomach, and lung (Ellisen *et al*, 2002; Shoshani *et al*, 2002). DDIT4 encodes a 232-amino-acid cytosolic protein which lacks any identifiable enzymatic activity or functional domains. Initial studies into the function of DDIT4 suggested either a pro- or an anti-apoptotic role during the stress response, depending on the cell context (Ellisen *et al*, 2002; Shoshani *et al*, 2002). A significant advancement in the understanding of DDIT4 function was facilitated by work in *Drosophila* from Reiling and Hafen, who elucidated that it is a crucial regulator of the mammalian target of rapamycin (mTOR) signalling pathway during hypoxic stress (Brugarolas *et al*, 2004; Reiling & Hafen, 2004).

Previous reports have shown that DDIT4 mRNA is constitutively expressed at very low levels in most cell types, but under hypoxia its transcription is sharply increased both *in vitro* and *in vivo* (Shoshani *et al*, 2002; Jin *et al*, 2007). However, the effect of LPS on DDIT4 mRNA expression under hypoxia has not been investigated in primary human

MDMs. My microarray data have shown that DDIT4 is synergistically up-regulated by hypoxia plus LPS in primary HMDMs, table 4.3. These data were confirmed by real-time PCR as seen in figure 4.12 A, and statistical analysis indicated that DDIT4 mRNA expression was significantly increased by hypoxia plus LPS when compared with normoxic samples. Moreover, this increase caused by hypoxia plus LPS is also statistically significant when compared with either hypoxia or LPS.

Based on the above findings, I decided to use different concentrations of LPS to determine the optimal LPS dose which can give the highest synergy level of DDIT4 mRNA. The LPS-dose response experiment revealed that low levels of LPS such as (0.2, 0.4, 0.8, and 10 ng/ml) were able to induce DDIT4 mRNA transcription in synergy with hypoxia.

Vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells. This gene is also one of the synergy genes elucidated in my microarray data. It was previously found that VEGF is up-regulated by LPS in macrophages (Botero *et al*, 2003; Itaya *et al*, 2001) as well as in other cell types (Kim *et al*, 2008), and this up-regulation was documented to be through the NFkB pathway (Kiriakidis *et al*, 2003). I have studied the expression of VEGF mRNA by human macrophages in response to different concentrations of LPS under hypoxia, to allow comparison with the DDIT4 expression pattern. As figure 6.2 showed, VEGF mRNA is increased synergistically by hypoxia plus LPS. Similar to what was found with DDIT4, the data shows low doses of LPS, 0.2, 0.4 and 10 ng/ml and 100ng/ml in combination with hypoxia can up-regulate VEGF mRNA, and that this synergy induction is statistically significant when compared with hypoxia or LPS samples. This finding by RT-PCR supports my array data obtained by using 100

ng/ml of LPS. The above RT-PCR data of synergy genes DDIT4 and VEGF were also compared with another known hypoxia-inducible gene, Glut-1. Glut-1 is known to be induced by hypoxia in a range of cell types, including macrophages (Chen *et al*, 2001a; Cramer *et al*, 2003). Furthermore, Glut-1 was found to be induced by LPS in rat alveolar macrophage-derived cell line, NR8383 (Blouin *et al*, 2004), but no study shows this induction in primary human macrophages, which suggest that up-regulation of Glut-1 by LPS is cell-type specific. My microarray data have shown no effect of LPS on its transcription in primary macrophages. As indicated in figure 6.3, there is no difference in fold induction between hypoxia treated samples and hypoxia plus LPS samples, indicating that LPS causes no further up-regulation of Glut-1 mRNA in HMDMs, in contrast with VEGF and DDIT4. This raises the question that if LPS is the main mediator of the effect in DDIT4 and VEGF, why was Glut-1 not also affected. The data suggest that HIF-1 may not be the only, or even the major, mediator of the LPS/hypoxia synergy effect observed for DDIT4 and VEGF. TNF- $\alpha$  is known to activate the NF $\kappa$ B pathway. Work by Fang *et al* showed that exposure MDMs to 10 ng/mL recombinant human tumour necrosis factor- $\alpha$  for 18 hrs can affect the expression of Glut-1 and VEGFA. Exposure to TNF- $\alpha$  for 18 hrs significantly increased the expression of Glut-1 and VEGFA under normoxia but when hypoxia was included, it synergizes with TNF- $\alpha$  and showed even higher up-regulation of Glut-1 and VEGFA. These data show that, when macrophages experience hypoxia for 18 hrs, it can elicit profound changes in their gene expression profile through the NF $\kappa$ B pathway (Fang *et al*, 2009).

Lipopolysaccharide (LPS) is a potent activator of leukocytes including macrophages. Binding of LPS to TLR4 in macrophages results in production of inflammatory cytokines

such as TNF- $\alpha$  through the activation of transcription factor, NF- $\kappa$ B (Muller *et al*, 1993). To date, there is no published work showing the effect of LPS on DDIT4 up-regulation. In this chapter, I investigated the effect of using another exogenous TLR ligand (for TLR2), Pam 3 Cys (Heuking *et al*, 2009) to induce DDIT4 expression in macrophages, to determine if the effect was limited to LPS. As seen in figure 6.4, the stimulation of human macrophages by Pam 3 Cys up-regulated DDIT4 mRNA expression at similar levels to LPS. In addition, both Pam 3 Cys and LPS have the ability to synergize with hypoxia to up-regulate DDIT4 mRNA. However, this increase was not statistically significant due to high variability among these different donors. The above data indicates that macrophages can be activated through different TLRs to induce DDIT4 mRNA expression. My data show for the first time that DDIT4 can be induced by LPS and Pam 3 Cys, through TLR4 and TLR2, respectively.

Macrophages are involved in inflammatory sites such as atherosclerosis plaques (Bjornheden *et al*, 1999), rheumatoid arthritis (Hollander *et al*, 2001), myocardial infarcts (Jurgensen *et al*, 2004), healing wounds (Stevens *et al.*, 1991), malignant tumours (Murdoch *et al*, 2005), and sites of bacterial infection (Blouin *et al*, 2004) in which hypoxia is often present. In hypoxia, macrophages rely heavily on HIF transcription factors for their modulation of gene expression. Overexpression of HIF-1 $\alpha$  is a frequent event in many cancers (Zhong *et al*, 2000) and chronic activation of the PI3K pathway has been reported to enhance HIF-1 $\alpha$ -activated responses (Harris, 2002). Therefore, further investigation was carried out to investigate the role of PI3-kinase in up-regulation of DDIT4 mRNA by hypoxia plus LPS. Using two PI3-kinase pathway inhibitors, LY290042 and wortmannin, markedly reduced expression of DDIT4

mRNA by hypoxia in primary HMDMs cells in comparison with hypoxic untreated cells. Interestingly, the inhibition effect of these two specific inhibitors on hypoxic cells was lost when they were treated with LPS, figure 6.6. These data suggest that induction of DDIT4 under LPS and hypoxia occurs via two different pathways in this particular cell type. HIF-1 protein accumulates in macrophages by hypoxic and non-hypoxic stimuli (Semenza, 2009; Blouin *et al*, 2004). Since DDIT4 transcription is known to be regulated by HIF-1 under hypoxia (Shoshani *et al*, 2002), its up-regulation by LPS might be also through HIF-1 in HMDMs. However, as mentioned above, when LPS was added to hypoxic cells, the inhibition action of LY290042 and wortmannin on DDIT4 mRNA suppression was lost. This suggests that induction of DDIT4 under LPS and hypoxia have two different independent pathways. This idea fits with the observed difference between DDIT4 and VEGF, and Glut-1 expression, as described above, which indicated that HIF-1 was not the sole mediator of synergy in the LPS/hypoxia synergistic induction. This two pathway suggestion, involving PI3k, is supported by a study by Schwarzer *et al* who showed that inhibition of PI 3-kinase in PC-3 cells using LY290042 does not affect the expression level of HIF-1 $\alpha$ , in contrast to DDIT4 expression which was drastically reduced by the same inhibitor (Schwarzer *et al*, 2005). However, in different cell lines previous studies showed that PI3-Kinase activation by hypoxia increased HIF-1 $\alpha$  synthesis, (Mazure *et al*, 1997; Chen *et al*, 2001b; Lee *et al*, 2006), so the picture is complex and likely to be cell type dependent.

It was also of interest to examine the expression of DDIT4 mRNA in cell types other than primary human MDMs. Therefore, in this chapter, DDIT4 expression was investigated in two monocyte cell lines, U937 and MM6, which have many of the

phenotypic and functional characteristics of peripheral blood monocytes including phagocytosis, enzyme production, and expression of typical cell-surface marker, CD14 (Ziegler-Heitbrock *et al*, 1988). These cells grow rapidly and have been used by many investigators to elucidate the effect of hypoxia on gene expression (Han *et al*, 2006). Gery and colleague have shown that DDIT4 mRNA was induced by the differentiation inducers, retinoids, in U937 cells (Gery *et al*, 2007). As indicated in figure 6.5, the combined treatment of cells by hypoxia plus LPS synergistically induced DDIT4 mRNA in both cell types, MM6 and U937, and this data is consistent with real-time RT-PCR data from primary HMDMs (chapter 4). These data indicate that hypoxia and LPS may activate different transcription factors which promote high induction of DDIT4 mRNA expression.

In this chapter I also investigated DDIT4 protein abundance in macrophages. As expected, DDIT4 protein in normoxia was found at low levels, consistent with DDIT4 expression in other human tissues such as muscle and intestine (Shoshani *et al*, 2002). Donor number 1 shows very low protein under normal oxygen tension (Figure 6.7). All donors' samples have accumulation of DDIT4 protein under hypoxia or LPS, and this data was concordant with mRNA level determined by RT-PCR in this project. The exposure of human macrophages to hypoxia plus LPS strongly induced the accumulation of DDIT4 protein in primary HMDMs in a synergistic manner. Based on the band intensities of protein on membranes, the densitometer data indicated a significant synergy effect between hypoxia and LPS in comparison with hypoxia alone or LPS alone in this cell type (figure 6.8). DDIT4 protein has a predicted length of 232 amino acids, expected molecular mass 25 KDa. However, the detected protein

migrates at approximately 33 KDa on my gels (figure 6.7). The aberrant migration is possibly caused by amino acid composition or secondary structure of the protein (Ellisen *et al*, 2002; Shoshani *et al*, 2002).

Several studies have investigated the correlations between the mRNA expression levels and protein abundance in many cell types suggesting significant correlations in mRNA induction and protein accumulation (Chen *et al*, 2002; Guo *et al*, 2008; Gry *et al*, 2009; Maier *et al*, 2009). On the other hand, a report by Pascal and colleague has shown that mRNA level does not necessary reflect protein abundance for several genes (Pascal *et al*, 2008). Here, we found that the increased expression of protein is correlated with the induction of DDIT4 mRNA although the data were not statistically significant due to high variability in hypoxic fold induction among the different donors (figure 6.9). There are some differences in protein abundance between all 5 donors used in immunoblotting assay (figure 6.7).

The data presented in (figures 4.12, 6.1) and (figure 6.7) on real-time RT-PCR and protein expression respectively encouraged me to carry out further analysis at the transcriptional level. Analysis of DDIT4 promoter by MatInspector software shows a number of potential binding sites for transcription factors associated with induction by hypoxia or LPS. These are very important such as binding sites for Sp-1, HIF transcription factor, and a HIF ancillary sequence (HAS). One report suggested that the HAS plays a crucial role for HIF-mediated transcription, and it is an imperfect inverted repeat of HIF-1 binding site (HBS) (Kimura *et al*, 2001). Many genes have HAS in their promoters, such as Glut-1, EPO, LDHA, ALDA, and ENO-1 (Kimura *et al*, 2001). Another group have identified a critical region of the DDIT4 promoter responsible for

inducibility after treatment with the DNA-damaging agent, methyl methanesulfonate (MMS) (Lin *et al*, 2005a). MMS is able to activate a 2.5Kb DDIT4 promoter construct in HaCaT cells in a time-dependent manner. Moreover, short multiple constructs generated by serial deletion showed that the region between -1057 and -981 bp is essential for DDIT4 induction. This region of the promoter contains putative binding sites for Elk-1 and C/EBP which are thought to be responsible for its activity under MMS treatment (Lin *et al*, 2005a).

I investigated the effect of hypoxia plus LPS in the transcriptional regulation of DDIT4 promoter activity. Four luciferase reporter promoter constructs of 495bp located at the 5'-flanking sequence from the ATG translation initiation codon were used in this project (Lee *et al*, 2004). The wild type DDIT4-495bp promoter construct had been mutated in two regions, the HRE and Sp-1 binding sites (see section 6.3.6). These plasmids were amplified, purified, and transfected into primary human MDMs in accompanied with a *Renilla* normalizing plasmid. These plasmids were chosen for analysis in this project because it has previously been shown that a 495bp (-197/+298) sequence constitutes an active DDIT4 promoter (Lee *et al*, 2004). The luciferase activity of all DDIT4-495 constructs was investigated under two different incubation periods, 6h and 18h of hypoxia, as it has previously been found that extended periods of severe hypoxia in HMDMs produce higher mRNA fold induction in some hypoxia-inducible genes. The luciferase data in both transfections were normalized with *Renilla* luciferase values in order to calculate fold induction of each construct activity. As figure 6.11 shows, unfortunately no significant fold induction was obtained from the DDIT4-495bp (WT) construct under hypoxia or/and LPS as well as the mutated reporter

promoter constructs in of HIF and Sp-1 binding sites. When the incubation period of transfected cells was extended up to 18 hrs, the DDIT4-495bp (WT) construct again did not respond to any of the treatments. However, DDIT4-Sp-1 mutated constructs induced by hypoxia and hypoxia plus LPS, but this induction should not be seen, because Sp-1 is essential for DDIT4 induction. As scientific experiments, I cannot rely on just one experiment to conclude my results. Therefore, another two transfection experiments were performed with 18 hrs incubation (figure 6.13). However, firefly luciferase data could not be normalized by internal control (renilla) due to the very low expression. So, luciferase values of firefly were normalized to the protein concentrations instead of Renilla values. As seen in figure 6.13, DDIT4-495bp was suppressed by normoxia in HMDMs but no significant increased fold induction of this wild type construct was observed in any of these conditions, hypoxia, LPS, or hypoxia plus LPS in comparison with pGI3-basic plasmid which is has no promoter construct. In most hypoxia-inducible genes, HRE and Sp-1 sites are the most important binding sites in promoter activity. Therefore, macrophages were transfected with two different mutated reporter constructs at HRE and Sp-1 binding sites and exposed to hypoxia and LPS. As seen in figure 6.13, the DDIT4-HRE mutated construct did not show up-regulated fold induction under any condition used in the primary HMDMs. However, hypoxia and hypoxia plus LPS were possibly slightly able to activate Sp-1 mutated construct but this up-regulation seems to be not real because Sp-1 mutated construct should not be activated under hypoxia because it is needed with HIF-1 for DDIT4 induction (Shoshani *et al*, 2002). In all transfection experiments, control plasmid PGK showed up-regulation fold induction under hypoxia. PGK reporter construct is known to be regulated by HIF-1 (Carmeliet *et al*, 1998; Ameri *et al*, 2004). Overall, the above

findings may suggest that the DDIT4-495 construct is not functional in this particular cell type, or the conditions used in this experiments including incubation periods applied post transfection as well as the amount of DNA used need further optimization.

In this chapter, the role of HIF on DDIT4 regulation was investigated. DDIT4 mRNA expression is regulated mainly through HIF-1 pathway under hypoxia (Shoshani *et al*, 2002). To elucidate that DDIT4 mRNA up-regulation by hypoxia plus LPS may partially rely on HIF but not on other factor due to hypoxia, two known chemical agents, cobalt chloride (CoCl<sub>2</sub>), or iron chelator desferrioxamine (DFO) were used to stabilize the HIF- $\alpha$  subunit. It has been well documented that CoCl<sub>2</sub> or DFO cause the stabilization of the HIF- $\alpha$  protein (Maxwell *et al*, 1999; Wang & Semenza, 1993). These compounds increase HIF-1 $\alpha$  protein and therefore its transactivation function (Wang *et al*, 1995). My data have shown that CoCl<sub>2</sub> or DFO in combination with LPS up-regulated DDIT4 mRNA expression in primary HMDMs. This suggests that the hypoxia part of the Hypoxia/LPS synergistic induction of DDIT4 mRNA depends on HIF.

Another approach has been attempted in this chapter in order to understand DDIT4 regulation. RNA interference (RNAi) has recently emerged as a novel pathway that allows modulation of gene expression. Lentivirus transfection is a method has used as vector to intracellular transducing shRNA in to a wide range of cell lines (Sliva & Schnierle, 2010). I have used five clones of shRNA with different targeting sequences to HIF-1 $\alpha$ . Unfortunately, none of these clones produced an inhibitory effect on HIF-1 $\alpha$  mRNA transcript levels (Fig 6.15).

There are several reasons which could explain the failure of this viral inhibition approach. First, the access to the cell by binding to the plasma membrane might be not efficient. Second, the activity of the viral promoter inside cells may be very weak which is unable to express shRNA. Third, the housekeeping gene used in RT-PCR was  $\beta$ -2m which is invariable under hypoxia, however this viral infection may need to use other endogenous control such as GAPDH. In fact, the manufacturer which provided these viruses clones offered another set of lentiviral particles to HIF-1 $\alpha$  to try with macrophages, however this beyond this project due to the time constraint.

Small interfering RNA (siRNA) is another approach of HIFs knockdown attempted in this project. The MM6 cell line was used for transfection in this experiment instead of primary macrophages. Therefore, firstly the expression of DDIT4 mRNA was investigated in two cell lines, MM6 and U937. As figure 6.5 shows, DDIT4 mRNA was sharply induced by hypoxia in both cell types, U937 and MM6. In addition, the data showed hypoxia plus LPS up-regulated DDIT4 mRNA level. MM6 cells were treated with siRNA specific to the HIF-1 $\alpha$  and HIF-2 $\alpha$  transcripts in order to analyse their inhibition capability. The transcription level of HIF in normoxia and hypoxia is unchanged because HIF regulation by hypoxia occurs at the post-translational level. Therefore, this preliminary experiment aimed to test the suppression effect of siRNA on HIF-1 $\alpha$ , HIF-2 $\alpha$ , and the downstream gene DDIT4. As shown in figure 6.16, neither siRNA to HIF-1 $\alpha$  nor HIF-2 $\alpha$  had a down-regulatory effect on HIF-1 $\alpha$  or HIF-2 $\alpha$  or DDIT4 mRNA levels.

In this chapter, siRNA to HIF-1 $\alpha$  was also delivered into primary MDMs. As shown in figure 6.17, HIF-1 $\alpha$  mRNA appeared to be moderately inhibited by 50 % in respect to

untreated samples when cells were transfected with 50 nM of siRNA. However, we should treat this data with caution because only one experiment was performed.

The data presented in this chapter showed for the first time that DDIT4 can be regulated by LPS at mRNA and protein levels in primary HMDMs. Furthermore, combined treatment of the cells by hypoxia plus LPS synergistically up-regulated DDIT4 expression. In order to understand DDIT4 regulation in HMDMs under hypoxia plus LPS, many approaches have been tested and analysed in this chapter. LPS-dose response experiments showed that macrophages can be activated with very low concentration of LPS to induce DDIT4. DDIT4 promoter activity under hypoxia and LPS in primary human macrophages was investigated. A synergistic effect between hypoxia and another TLR ligand (Pam 3 Cys) was also observed to induce DDIT4 mRNA expression. In addition, the expression of DDIT4 mRNA by hypoxia and LPS in primary HMDMs was shown to be linked to activation of PI3-Kinase.

## CHAPTER 7: General Discussion

Macrophages are a major component of the innate immune system. Macrophages accumulate in poorly vascularised tissues characterised by hypoxia (low oxygen tension) including human tumours, myocardial infarctions, atherosclerotic plaques, rheumatoid arthritis, and sites with bacterial infection (Lewis *et al*, 1999; Vaupel *et al*, 2001). In deep injury, hypoxia is predominantly present due to the blood supply damage and high consumption of oxygen by microorganisms and the high number of leukocytes present.

Macrophages can be strongly activated by lipopolysaccharide (LPS) and lead to up-regulate many genes involved in a wide variety of cellular immune responses, including the production of cytokines and chemokines, release of arachidonic acid metabolites (prostaglandin E<sub>2</sub>), and generation of reactive oxygen and nitrogen intermediates that have essential roles in infection and inflammation reactions (Bone *et al*, 1997). These genes regulated by several transcription factors including NF- $\kappa$ B (Muller *et al*, 1993), REL, ETV3, IRF4 and IRF8 (Roach *et al*, 2007), serum response factor (SRF) (Chai & Tarnawski, 2002), SP1 (Hirata *et al*, 2008) and as well as hypoxia-inducible factors (HIFs) (Cramer *et al*, 2003; Imtiyaz & Simon, 2010).

In hypoxia, macrophages rely heavily on HIFs for transcriptional activation of many essential genes needed for energy generation and activity (Semenza, 1999; Burke *et al*, 2002). HIF-1 $\alpha$  is a master regulator of transcriptional induction of most hypoxia inducible genes (Semenza, 1998; Semenza, 2000b). In addition to hypoxia, HIF-1 $\alpha$  can be induced in macrophages by a variety of non-hypoxic stimuli such as LPS under normal oxygen tension. Several studies have shown that LPS-treated macrophages up-

regulate many hypoxia-inducible genes such as VEGF, Glut-1, and iNOS which are known to be regulated by HIF-1 (Blouin *et al*, 2004; Frede *et al*, 2006).

Hypoxia also induces another transcription factor, nuclear factor- $\kappa$ B (NF- $\kappa$ B) (van Uden *et al*, 2008). The transcription of many proinflammatory genes, such as tumour necrosis factor (TNF), interleukin 1 (IL-1) and IL-8 is regulated by NF- $\kappa$ B activation; its activity has been correlated with apoptosis and the activation of apoptosis-associated genes, such as Fas ligand (Wang *et al*, 1998). It has also been documented that the activation of NF- $\kappa$ B plays a central role in inflammatory responses such as in deep tissue injuries characterized by hypoxia (Barnes & Karin, 1997). Fong and colleagues have shown that NF- $\kappa$ B DNA binding was increased after 1h of hypoxia exposure in RAW264.7 cells, suggesting that NF- $\kappa$ B plays an important role under hypoxia in regulation of many inflammatory cytokines (Fong *et al*, 2007).

The definition of “synergy” is two factors interacting together, so that their combined effect is greater than the sum of their individual effects. In this study, a hypothesis was raised that a combination of hypoxia and LPS might cooperate with each other to up-regulate genes not induced by only one factor. Therefore, microarray experiments were conducted to elucidate the transcriptome expressed by primary human macrophages when cultured under normoxic (20.9% O<sub>2</sub>) and hypoxia (0.2% O<sub>2</sub>) conditions in the presence or absence of 100 ng/ml LPS. This facilitates the subsequent selection of synergy genes for further analysis by real-time RT-PCR. Microarray experiments were performed using primary human macrophages from two different donors whose response to hypoxia was confirmed by up-regulation of some known hypoxia-inducible genes such as VEGF, Glut-1, VCAN, and MMP-7 (figure 4.3), and the accumulation of HIF-1 $\alpha$  protein (figure 4.14). Modulated genes were selected from

microarray data if they exhibited  $\geq 2$  fold increase in gene expression, or if their expression exhibited  $\leq 0.5$  fold (which is equal  $\leq 2$  fold down regulated) decrease compared to normoxic cell cultures.

As seen in figure 4.8, my data revealed that small numbers of genes were modulated by hypoxia or/and LPS in both array when compared with the total number of transcripts in human gene chip that used in microarray experiment, suggesting that human macrophages have a selective gene expression when exposed to hypoxia or/and LPS. Only genes that were significantly differentially up- or down-regulated in both duplicate array experiments (as found in the overlapping areas of the two-circle Venn diagrams in figure 4.9) were selected for further analysis in this project. Furthermore, it should be noted that not all modulated genes have intrinsic induction, but the up-regulation of some genes could be attributed to mRNA stability. In hypoxia, mRNA expression may be enhanced by the effects of oxygen tension on mRNA stability. Some mRNA remain active for only a few minutes, however, turnover of other mRNA transcripts may take longer time such as for globulin gene (Shih & Claffey, 1998). Stability time is determined by mRNA binding proteins that bind either the 5` prime cap-structure or the 3` prime poly (A) tail of mRNA, preventing mRNA breakdown by exonucleases attack at AU-rich regions (Shih & Claffey, 1998). Hypoxia also was found to enhance the mRNA stability of well known hypoxia-inducible gene, VEGF and this involves AU-rich elements in the 3`-UTR region, which bind the protein HuR, stabilizing the transcript (Shima *et al*, 1995). Further studies involving analysis of steady-state of mRNA of selected genes in my data are attractive area of further investigation.

My data shows that 55 genes were up-regulated by hypoxia, 277 by LPS, and 384 by co-treatment with hypoxia plus LPS (figure 4.9). Thus the total number of genes up-regulated by hypoxia+LPS (384) is higher than the sum of the genes up-regulated by hypoxia and LPS used separately ( $55+277=332$ ), which means there are 52 extra up-regulated genes obtained when hypoxia and LPS are used together. This clearly suggests that there might be cooperation (ie synergy) between hypoxia and LPS which leads to up-regulation of extra genes which were not up-regulated when only one stimulus was applied.

As seen in figure 4.9, only 36 up-regulated genes were found shared between hypoxia and hypoxia plus LPS treatments. In contrast to the synergy observed between hypoxia and LPS, some genes were induced by hypoxia but when LPS was added, these genes were suppressed. For example, genes such as sulfatase 2 (sulf2), matrix metalloproteinase 12 (MMP12), angiogenin (ANG), and lactate dehydrogenase A (LDHA) were found up-regulated by hypoxia, but when LPS was added to hypoxia, they disappeared from the hypoxia+LPS list in figure 4.9. Sulf2, MMP12, and ANG were found suppressed by LPS alone in both arrays, however LDHA was found unchanged by LPS. This data showed consistency between the two arrays which indicated the suppression by LPS was a reproducible effect. A possible explanation for this is that LPS may decrease DNA binding activity of some transcription factors essential for induction by hypoxia.

In addition, array data has shown that 12, 174, and 330 genes were down-regulated by hypoxia, LPS, and hypoxia plus LPS respectively (figure 4.9). Again, the same effect of blockade (suppression) was found in LPS up-regulated genes when hypoxia was added. The number of genes up-regulated by LPS was decreased with 43 genes lesser when

cells were co-treated with hypoxia. This indicates that some LPS-inducible genes were blocked when hypoxia was added to LPS. Some of these are genes involved in macrophage immune responses such as CCL7 (chemokine (C-C motif) ligand 7), C1S (complement component 1), IRAK2 (interleukin-1 receptor-associated kinase 2), and PTGES (prostaglandin E synthase). IRAK2 contributes to multiple signalling pathways leading to downstream activation of NF $\kappa$ B and MAP kinases (Flannery & Bowie, 2010). The explanation for the suppression by hypoxia of LPS-induced genes could be that hypoxia decreases the mRNA stability of some genes by activating the mRNA-destabilizing protein, tristetraprolin (TTP) which can bind to RNA at 3'-untranslated region (as seen with the pro-inflammatory gene TNF- $\alpha$ ) and increase its degradation (Werno *et al*, 2010).

My data has shown that some genes up-regulated by hypoxia for 18h were the same as those up-regulated by hypoxia in macrophages and related myeloid cell type such as monocytes. These genes include Glut-1, VEGF, ADORA2A, ALDOC, and ADM (Fang *et al*, 2009; Burke *et al*, 2003; White *et al*, 2004; Bosco *et al*, 2006).

In addition, data in this project showed that LPS up-regulate a cluster of inflammatory cytokines such as IL1 $\alpha$ , CCL20, PTGS2, IL-1 $\beta$  and IL6. These genes were found to require LPS co-stimulation to be activated in macrophages and MDM (Lewis *et al*, 1999), these findings support the validity of my data on LPS regulated genes. In contrast, another study showed that these five genes are up-regulated in response to hypoxia alone (Bosco *et al*, 2006). Furthermore, genes like IL-1 $\beta$ , IL-8, NCF, IL-6, ADORA2A, SERPINB2, IL-1 $\alpha$ , and CCR7 were up-regulated by LPS in the current study (Table 4.6), however these genes were found up-regulated by hypoxia in the study by Fang (Fang *et al*, 2009). The differences in gene expression between the Bosco, and Fang studies and

the current study could be due to variation in the severity or/and duration of hypoxia applied to cells. For example, Bosco, Fang, and the current study have used human macrophages exposed to 1% O<sub>2</sub> hypoxia for 16 hour, 0.5% O<sub>2</sub> hypoxia for 18 hours, and 0.2% O<sub>2</sub> hypoxia for 18 respectively. My data also have shown up-regulation of some chemoattractants induced by LPS such as CCL1, CCL8, and CCL5 which are chemotactic factors that attract monocytes, lymphocytes, basophils, T cells, eosinophils, and neutrophils to the inflamed tissues, (Table 4.6). This emphasises that LPS is a potent stimulator for macrophages in response to inflammatory reactions in which macrophages enhance the accumulation of other leukocytes at the infected sites. LPS induces some transcription factors that involved in regulation of many genes, including signal transducer and activator of transcription 4 (STAT4) and NF- $\kappa$ B (Table 4.6).

Combined treatment with hypoxia plus LPS showed up-regulation of 384 genes. Among these genes a few show significant synergy between hypoxia and LPS. These synergy genes (Table 4.3) were selected based on low or moderate induction under hypoxia or LPS, but high up-regulation (at least  $\geq 2$  fold change) when both stimuli were applied. These data showed a unique transcriptional profile in primary HMDMs when co-treated with hypoxia plus LPS. Interestingly, some synergy genes induced in human macrophages have transcription regulatory activity and/or encode important components of the HIF-1 pathway. For example, the cloning studies of the 5'-flanking region of the promoter of metallothionein, CP, and DDIT4 genes which contain several consensus hypoxia-responsive elements (HREs) have shown to drive a luciferase reporters in transfected cells under hypoxia, this findings indicate that these genes rely on HIF on their transcription when exposed to hypoxia (Kojima *et al*, 2009; Mukhopadhyay *et al*, 2000; Shoshani *et al*, 2002). Metallothionein protein was found

to stabilize HIF-1 $\alpha$  and increases the binding activity of HIF to HRE and subsequently activates downstream target genes such as VEGF and Glut-1 (Kojima *et al*, 2009). Furthermore, a study by Murphy and colleagues found that metal-responsive transcription factor-1 (MTF-1) participates in metallothionein transcription and metallothionein protein contribute to the stabilization and nuclear accumulation of the HIF-1 $\alpha$  protein, suggesting that metallothionein may work in an autocrine manner for macrophages (Murphy *et al*, 2008). Hypoxia in combination with LPS also increased the expression of P4HA2 and PLOD2. A study by Hofbauer *et al*. has shown that hypoxia and hypoxia mimicked agents (the iron-chelator DFO and CoCl<sub>2</sub>) induced PLOD2 transcription in rat vascular smooth muscle cell line A7r5, mouse juxtglomerular As4.1 cell line, and mouse hepatoma cell line Hepa1, which indicate that the expression of the PLOD2 enzyme during hypoxia is regulated by HIF-1 (Hofbauer *et al*, 2003). P4HA2 synergy gene is a member of the prolyl-hydroxylase family which mediates HIF- $\alpha$  hydroxylation in well-oxygenated cells targeting the protein for proteasomal degradation by the von Hippel Lindau tumour suppressor protein (pVHL) (Wenger *et al*, 2005). So, hypoxia increases the level of P4HA2 gene to be ready for hydroxylation of HIF-1 $\alpha$  protein when oxygen levels increase. This may explain the rapid degradation of HIF-1 $\alpha$  protein in normoxia which was estimated less than 5 minutes (Wang *et al*, 1995; Jewell *et al*, 2001).

My microarray data showed three synergy genes up-regulated by hypoxia or LPS in HMDMs: ceruloplasmin, VEGF, and CA12 (Table 4.3). These genes were found to be regulated by the HIF-1 pathway under hypoxia in many cell types including macrophages (Mukhopadhyay *et al*, 2000; Chiche *et al*, 2009; Forsythe *et al*, 1996).

As mentioned above, most synergy genes identified here have been shown in previous studies to be regulated through the HIF-1 pathway under hypoxia, and LPS up-regulates many hypoxia-inducible genes such as VEGF, Glut-1, and iNOS which are known to be regulated by HIF-1 (Blouin *et al*, 2004; Frede *et al*, 2006). HIF-1 accumulation was stimulated by LPS through increased transcription via the NF- $\kappa$ B site in the promoter of the HIF-1 $\alpha$  gene (Blouin *et al*, 2004; van Uden *et al*, 2008). Another study attributed this increase of HIF-1 $\alpha$  mRNA by LPS to the increasing of MAPK signalling pathway, as seen in THP-1 cells (Frede *et al*, 2006). HIF-1 $\alpha$  protein is present at low levels in most cells, including primary macrophages and MM6 (Burke *et al*, 2002) under normal oxygen tension. This finding was repeated in primary human macrophages prepared under the conditions used in this project (figure 4.14 panel A). HIF-1 $\alpha$  protein induction by LPS in normoxia was observed, which is consistent with previous studies (Blouin *et al*, 2004; Frede *et al*, 2006; Mi *et al*, 2008; Frede *et al*, 2009; Brooks *et al*, 2010). Interestingly, cells treated with combined hypoxia plus LPS show more HIF-1 $\alpha$  protein than with hypoxia alone, suggesting a co-operative effect of hypoxia and LPS in these cells. The effect of hypoxia and LPS treatments on HIF-1 $\alpha$  mRNA levels were also analyzed in hMDMs by RT-PCR. As figure 4.15 shows, the HIF-1 $\alpha$  mRNA levels in both normoxia and hypoxia are similar, which indicates, as previously reported, that HIF-1 $\alpha$  regulation by hypoxia predominately occurs at the protein level (Semenza, 2009). However, LPS increased HIF-1 $\alpha$  mRNA levels in normoxic and hypoxic samples, consistent with previous reports in primary mouse bone MDMs, rat alveolar macrophage-derived cell line cells (Blouin *et al*, 2004) and in the mouse macrophage cell line ANA-1 (Mi *et al*, 2008).

It was possible in chapter 4 to confirm the induction of some synergy genes listed in table 4.3 at the mRNA level by real-time RT-PCR (figures 4.11, 4.12, and 4.12). Only two of the six selected genes, TRAIL and DDIT4, showed statistically significant up-regulation by hypoxia plus LPS when compared with hypoxia alone and LPS alone. The variation in results between my array and RT-PCR data could possibly have been due to the inherent genetic differences between individuals, although this was disproved because with some donors used during this project, when the same donors were re-tested by RT-PCR, different results were obtained (data not shown). Genetic variation could however be one of the reasons for some of the variations in fold change between the 1<sup>st</sup> and 2<sup>nd</sup> arrays, since each represents only one donor. It is likely that these discrepancies between the two microarrays and real-time RT-PCR data might have been reduced if it had been possible to increase the number of donors used in microarray experiments. Some of the variation could also be due to the different methodologies used to examine gene expression (microarray versus real-time RT-PCR). Discrepancies between array and RT-PCR data in some genes were also found in a previous study (Vengellur *et al*, 2005). For example, INHBN and MCT3 were shown as up-regulated genes by microarray, whereas by RT-PCR showed them to be down-regulated. In addition, 15PGDH was shown down-regulated by microarray, but RT-PCR shows up-regulation. This indicates that there are some marked anomalies in their data, however overall there is correlation between microarray and RT-PCR in other genes (Vengellur *et al*, 2005). Using microarray chips with a large number of genes represented (28,000 transcripts), it is a possibility to get some aberrant false synergy in some genes by chance, in both array experiments, especially when doing only two array experiments. However, there is consistency between the data in both arrays, as

shown in figure 4.8; greater than 50% genes in common between both arrays in each treatment. This also indicates that the data is in general quite reproducible and reliable.

In this project, I tried to shed particular light on two synergy genes up-regulated by hypoxia plus LPS to understand their regulation at the molecular level. These two genes were TRAIL (TNF-related apoptosis-inducing ligand) and DDIT4 (DNA-damage-inducible transcript 4). The current study has shown for the first time that TRAIL mRNA expression is up-regulated by hypoxia plus LPS in primary HMDMs. The product of this gene plays crucial role in host defence mechanisms by activating a signal that induce apoptosis cell death in several types of transformed cells without affecting normal cells *in vivo* (Wiley *et al*, 1995).

In addition, various studies have shown that macrophages up-regulate expression of TRAIL at the mRNA and protein levels in response to LPS, interferon-alpha (IFN-alpha), -beta and -gamma (Halaas *et al*, 2004; Zheng *et al*, 2005; Genc *et al*, 2003).

Hypoxia-inducible factor 1 (HIF-1) is the main transcription factor controlling transcription in response to hypoxia, however hypoxia has also been shown to activate a number of transcription factors such as activating protein-1 (AP-1) and Sp1 (Cummins & Taylor, 2005). For example, VEGF, a well known hypoxia-inducible gene regulated by HIF-1, also has found to be partially regulated by AP-1 (Salnikow *et al*, 2002) and Sp1 (Lee *et al*, 2004) in response to hypoxia. The TRAIL promoter has previously been identified to have binding sites for AP-1 and Sp1 (Wang *et al*, 2000), suggesting that this gene is also regulated partially by hypoxia and this might be the cause of slight

induction under hypoxia in primary human MDMs. Up to date, there is no report showing that TRAIL could be regulated by HIFs.

Regarding regulation of TRAIL by LPS, computer analysis of the TRAIL promoter has shown one binding site for NF- $\kappa$ B located between -254 and -263bp (Fig 5.1). The role of NF- $\kappa$ B-signalling in TRAIL regulation has been studied and showed that NF- $\kappa$ B plays an important role (Wurzer *et al*, 2004). Several reports have shown that TRAIL expression is increased by LPS stimulation in many cell types including macrophages, suggesting this activation of TRAIL might be mainly through NF- $\kappa$ B pathway (Zheng *et al*, 2005; Halaas *et al*, 2000). Hypoxia also stimulates NF- $\kappa$ B activation and subsequently the transcription of other TNF family member, TNF- $\alpha$  (Koong *et al*, 1994). Taken together, it is plausible that TRAIL might be regulated principally by NF- $\kappa$ B in response to hypoxia and LPS at least in HMDMs. To further investigate the regulation of TRAIL, I cloned a sequence of a 685bp DNA fragment of the TRAIL promoter (-585/+100) into the pGL4.10 [luc2] vector. This sequence was chosen for analysis because it has several putative binding sites for important transcription factors sensitive to hypoxia or LPS (figure 5.1). This construct was transfected into human MDMs and exposed to hypoxia and LPS, and the luciferase activity was measured after 6h and 18h. As seen in figure 5.7 A (left panel), the TRAIL promoter (-585/+100) reporter construct shows little activity fold induction by hypoxia, LPS, or by combined treatment with LPS and hypoxia for 6 hrs.

In further experiments, I decided to use a longer TRAIL promoter construct (Wang *et al*, 2000) to transfect human MDMs. As seen in figure 5.8 A, the TRAIL-1523bp reporter construct did not show induced induction by any treatment at 6 hrs, however, when the transfection incubation period was extended to 18 hrs, the TRAIL-1523bp reporter

construct had low induction of luciferase activity under hypoxia and hypoxia plus LPS compared with the normoxic sample (figure 5.8 B). Despite the low inducibility of this construct (around 2 fold up-regulation), these data indicate that the TRAIL-1523bp reporter construct is partially functioning under those conditions.

In this project, further investigation was carried out to elucidate the effect of different LPS concentrations on TRAIL mRNA expression under hypoxia in HMDMs. Figure 5.10 shows that LPS-treated cells up-regulated TRAIL mRNA at all different LPS concentrations.

In addition, the higher LPS dose group showed evidence of the synergy effect from combined treatment (hypoxia plus LPS), although this synergy is not statistically significant. Despite the non-significance, there is clear evidence that both LPS and hypoxia are able to regulate TRAIL expression, and signs of an upward trend when the two are combined. This, and the high degree of variability inherent in experiments such as these and the array experiments, using primary human cells from different donors, is a possible explanation for the cooperative effect between LPS and hypoxia on TRAIL expression not reaching statistical significance.

A second gene shown by my microarray data to be synergistically up-regulated by hypoxia and LPS was DDIT4 (Table 4.3). DDIT4 mRNA up-regulation was confirmed by real-time PCR (figure 4.12 A). Different studies have shown that DDIT4 mRNA is constitutively expressed at very low levels in most cell types, but under hypoxia its transcription is sharply increased both *in vitro* and *in vivo* (Shoshani *et al*, 2002; Jin *et al*, 2007). The current study has shown for the first time that DDIT4 expression is also induced by LPS at the mRNA and protein levels. The synergistic up-regulation of DDIT4 by hypoxia plus LPS was achieved even with low doses of LPS, as seen in figure 6.1. As

seen in figures 4.12 (mRNA level) and 6.7 (protein level), the synergistic effect of hypoxia plus LPS is statistically significant. This indicates that LPS is an important co-activating factor in increasing up-regulation of DDIT4 under hypoxia. In order to confirm the synergy effect of hypoxia plus LPS on DDIT4 expression, mRNA levels of two known hypoxia-inducible genes were quantified, VEGF and Glut-1. VEGF was chosen because this gene also found up-regulated by LPS in macrophages (Botero *et al*, 2003; Itaya *et al*, 2001) as well as in other cell types (Kim *et al*, 2008), and this up-regulation was shown to be through the NFkB pathway (Kiriakidis *et al*, 2003). In addition, VEGF is one of the synergy genes elucidated by my microarray data, therefore it was chosen in this approach to allow comparison with the DDIT4 expression pattern (Table 4.3). As figure 6.2 showed, VEGF mRNA is increased synergistically by hypoxia plus LPS. Similar to DDIT4 expression, the data shows low doses of LPS, 0.2, 0.4 and 10 ng/ml and 100ng/ml in combination with hypoxia can up-regulate VEGF mRNA, and this synergy induction is statistically significant when compared with hypoxia or LPS samples. This finding by RT-PCR supports my array data, obtained using 100 ng/ml of LPS. The RT-PCR data on the synergy genes DDIT4 and VEGF were also compared with another known hypoxia-inducible gene, Glut-1. Glut-1 is known to be induced by hypoxia in range of cell types, including macrophages (Chen *et al*, 2001; Cramer *et al*, 2003). Furthermore, Glut-1 was also found to be induced by LPS in the rat alveolar macrophage-derived cell line, NR8383 (Blouin *et al*, 2004). However, my microarray data have shown no effect of LPS on Glut-1 mRNA level in primary macrophages. As indicated in figure 6.3, there is no difference in fold induction between hypoxia treated samples and hypoxia plus LPS samples, indicating that LPS cause no further up-regulation of Glut-1 mRNA in HMDMs, in contrast with

VEGF and DDIT4. This raises the question that if HIF-1 is the main mediator of the synergy effect in DDIT4 and VEGF, why was Glut-1 not also affected? With the Glut-1 data (on the same samples) in mind, these data suggest that HIF-1 may not be the only, or even the major, mediator of the LPS/hypoxia synergy effect observed for DDIT4 and VEGF. An alternative possibility is that the LPS/hypoxia synergy effect may, via HIF-1, activate distinct downstream transcription factors which are essential for the synergistic up-regulation of DDIT4 and VEGF, but which do not affect Glut-1 transcription.

DDIT4 mRNA expression was also investigated in the monocyte cell lines MM6 and U937. As indicated in figure 6.5, the combined treatment of these cells with hypoxia plus LPS synergistically induced DDIT4 mRNA in both cell types, consistent with real-time RT-PCR conducted in primary HMDMs, as described in chapter 4. These data indicate that the synergy effect of hypoxia plus LPS on DDIT4 mRNA expression is not restricted in HMDMs, but also occurs in monocyte cell lines.

In addition to mRNA induction, we wanted to investigate DDIT4 protein expression in macrophages. As expected, I found that hypoxia can up-regulate DDIT4 protein (Fig 6.7), which is consistent with previously published findings on DDIT4 protein expression in other human tissues such as colon, testis, liver, kidney, lung, heart, and spleen (Shoshani *et al*, 2002). Furthermore, my data showed that LPS alone can slightly up-regulate DDIT4 protein expression in HMDMs (figure 6.7), and this data is in accordance with the RT-PCR in this project (figure 6.1). Moreover, the exposure of human macrophages to combined treatment of hypoxia plus LPS strongly induced the accumulation of DDIT4 protein in primary in a synergistic manner (figure 6.7).

Analysis of the DDIT4 promoter by MatInspector software shows a number of potential binding sites for transcription factors associated with induction by hypoxia or LPS. Therefore, I investigated the effect of hypoxia plus LPS in the transcriptional regulation of DDIT4 promoter activity. Four luciferase reporter promoter constructs of 495bp located at the 5'-flanking sequence from the ATG translation initiation codon were used in this project (Lee *et al*, 2004). The wild type DDIT4-495bp promoter construct had been mutated in two regions, the HRE and Sp-1 binding sites (see section 6.3.6). The luciferase activity of all DDIT4-495 constructs was investigated under two different incubation periods, 6 hrs and 18 hrs of hypoxia. As figures 6.11 and 6.12 show, no significant fold induction was obtained from DDIT4-495bp (WT) construct under hypoxia or/and LPS as well as the mutated reporter promoter constructs in of HIF and Sp-1 binding sites. Another two transfection experiments were performed with 18 hrs incubation (figure 6.13). However, firefly luciferase data could not be normalized by the preferred internal control (Renilla) due to the very low expression. So, luciferase values of firefly were normalized to the protein concentrations instead of Renilla values. As seen in figure 6.13, no significant fold induction of this wild type construct was observed in any of these conditions, hypoxia, LPS, or hypoxia plus LPS in comparison with pGI3-basic plasmid. These findings may suggest that the DDIT4-495 construct is not fully functional in this particular cell type, or the conditions used in these experiments need further optimization.

DDIT4 mRNA expression has been reported to be regulated mainly through the HIF-1 pathway under hypoxia (Shoshani *et al*, 2002). To confirm that DDIT4 expression relies specifically on HIF but not on other factors due to the effect of hypoxia, in the HMDM model used in my work, I used cobalt chloride (CoCl<sub>2</sub>) and desferrioxamine (DFO). My

data showed that  $\text{CoCl}_2$  or DFO can up-regulate DDIT4 mRNA similarly to hypoxia (figure 6.14). This suggests that up-regulation of DDIT4 mRNA transcription by hypoxia is likely to depend, at least in part, on HIF-1. Furthermore,  $\text{CoCl}_2$  and DFO showed synergy with LPS in up-regulating DDIT4 mRNA expression in primary HMDMs as seen in figure 6.14. This indicates that the hypoxia part of the hypoxia / LPS synergistic induction of DDIT4 mRNA depends partially on HIF. Taking together these data and the previously discussed data on Glut-1 versus VEGF and DDIT4 synergistic regulation, the most plausible hypothesis is that synergy may be mediated by HIF-1 acting indirectly on VEGF and DDIT4 via a downstream transcription factor which does not enhance Glut-1 expression.

The important role of HIF-1 in induction of DDIT4 under hypoxia and LPS encouraged me to also investigate the signalling pathway of PI3-kinase. Several researchers have documented the key role of the PI3-K pathway in many human pathologies including inflammation, allergy, and cancer (Stein, 2001; Liu *et al*, 2006). An interesting mechanism was proposed by which the activation of PI3-K could increase the rate of HIF-1 $\alpha$  translation in normoxia (Page *et al*, 2002). A study by Jin and colleagues has demonstrated that PI3-K participates in the regulation of DDIT4 mRNA and protein expression by HIF-1 $\alpha$  and Sp1 (Jin *et al*, 2007). As seen in figure 6.6, the expression of DDIT4 mRNA by hypoxia appeared to be slightly down-regulated in cells treated with the two specific inhibitors of PI3-Kinase, 10  $\mu\text{M}$  LY294002 and 200 nM wortmannin (with 3-fold reduction by each inhibitor) although these data were not statistically significant due to high variability in hypoxic untreated sample fold induction among these different donors. Furthermore, the possible inhibitory effect of LY294002 and wortmannin was lost in hypoxic cells when LPS was added. As previously mentioned in

this project, LPS up-regulated DDIT4 mRNA expression in normoxia. Here my data have shown that both inhibitors have no effect on LPS-induced DDIT4 expression. Overall, the above data indicate that the expression of DDIT4 mRNA by hypoxia and LPS in primary HMDMs was shown to be linked to activation of PI3-Kinase.

Binding of LPS to TLR4 in macrophages resulting in production of inflammatory cytokines such as TNF- $\alpha$  through the activation of transcription factor, NF- $\kappa$ B (Muller *et al*, 1993). To investigate whether NF- $\kappa$ B might also be responsible for the effect of LPS on DDIT4 expression, I investigated the effect of using another TLR ligand, Pam 3 Cys (Heuking *et al*, 2009; Heuking *et al*, 2009), which is detected by its binding to TLR2. Figure 6.4 showed that the stimulation of human macrophages by LPS or Pam 3 Cys induced DDIT4 mRNA expression at similar levels and both ligands appeared to synergize with hypoxia to up-regulate DDIT4 mRNA expression.

Several experiments were attempted in order to further understand DDIT4 regulation. RNA interference (RNAi) delivered by Lentivirus transfection is a method has used as vector to intracellular transducing shRNA in to the cells (Sliva & Schnierle, 2010). I used five clones of shRNA with different targeting sequences to HIF-1 $\alpha$ . Unfortunately, none of these clones had inhibitory effects on HIF-1 $\alpha$  transcripts (Fig 6.15). The failure of this viral inhibition approach could be due to inefficient binding of virus to the plasma membrane of the cells or the activity of the viral promoter inside cells may be very weak, and unable to express shRNA.

As shown in figure 6.16, neither siRNA to HIF-1 $\alpha$  nor HIF-2 $\alpha$  had a down-regulatory effect on HIF-1 $\alpha$  or HIF-2 $\alpha$  or DDIT4 mRNA levels. In addition, siRNA against to HIF-1 $\alpha$  was also delivered into primary MDMs. As shown in figure 6.17, HIF-1 $\alpha$  mRNA appeared to be moderately inhibited by 50 % in respect to untreated samples when

cells were transfected with 50 nM of siRNA, but this data is not reliable because only one experiment was performed, and because the level of HIF-1a mRNA at 50nM was not markedly different from the mRNA level in cells treated with 5 nM of the scrambled nonsense siRNA.

Programmed cell death (apoptosis) is a fundamental mechanism for the maintenance of tissue integrity and homeostasis (Kerr *et al*, 1972; Prindull, 1995; Fumarola & Guidotti, 2004). During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to form the final shape (sculpturing) of organs and tissues (Meier *et al*, 2000). Apoptosis is highly regulated and failure of this regulation results in pathological conditions such as developmental defects or cancer (Thompson, 1995; Schattenberg *et al*, 2011). Apoptotic cells are characterized by shrinkage, chromatin condensation and DNA fragmentation, finally ending with the engulfment by macrophages, thereby avoiding an inflammatory response in surrounding tissues (Savill & Fadok, 2000). There are two major apoptotic pathways, namely the extrinsic pathway initiated by external signals (such as the binding of TRAIL to death receptor) or the intrinsic pathway (following intrinsic signals including DNA damage within a cell), and DDIT4 is involved in this mechanism (Green *et al*, 2004; Lin *et al*, 2005).

The host response to pathogenic insults involves complex inflammatory responses and cellular immune reactions (Sibille & Reynolds, 1990). These reactions function to successfully deal with pathogens before they spread to other areas. Despite their important to host defence and clearance of dangerous invaders, they are often associated with nonspecific injury to nearby tissue. These insulted cells should be killed (via apoptosis) by TRAIL and removed by macrophages to minimize inflammatory

reaction due to the released contents of these localized tissues to clear reactions (Vassina *et al*, 2005). Several studies have shown that hypoxia up-regulates TRAIL receptors (DR5), and this enhances the signalling effect of TRAIL on cancer cells (Mahajan *et al*, 2008; Cao *et al*, 2006). The important role of TRAIL in the regulation of inflammation along with apoptosis suggests that a better understanding of the intracellular signalling pathways downstream of TRAIL activation may lead to novel therapeutic strategies for many diseases such as cancer and aberrant inflammatory response resulted by bacterial infections.

Macrophages also up-regulate DDIT4 in response to LPS plus hypoxia in a synergistic manner which indicates a crucial role for macrophage to play in inflammatory diseases that characterized by low oxygen tension. In hypoxia, DDIT4 transcription increases rapidly and sharply, and this induction is through HIF-1 (Shoshani *et al*, 2002). Several reports have shown that LPS treated macrophages up-regulate genes such as VEGF and iNOS which are known to be regulated through HIF-1. In contrast to hypoxia, which was documented not to up-regulate HIF-1 $\alpha$  mRNA, LPS has been shown to stimulate HIF-1 $\alpha$  mRNA expression at transcription level in normoxia (Blouin *et al*, 2004). Taken together, this suggests that hypoxia plus LPS increases the expression and accumulation of HIF-1 $\alpha$  more than either factor alone, and subsequently this results in induction of DDIT4. In addition to HIF-1, the synergistic effect of hypoxia and LPS may activate a distinct array of transcription factors that might play roles in the synergistic up-regulation of DDIT4.

The adaptive response to hypoxia involves inhibition of energy-requiring processes including protein translation (Heerlein *et al*, 2005; Liu *et al*, 2006). Several studies have reported that hypoxia activates 4E-binding protein-1 (4E-BP1), a repressor of mRNA

translation following hypoxic inactivation of eukaryotic elongation factor-2 (eEF2) which is essential factor for protein synthesis (Tinton & Buc-Calderon, 1999; Arsham *et al*, 2003; Connolly *et al*, 2006). In addition, the inhibition effect is mediated in part through a decrease in the kinase activity of mammalian target of rapamycin complex 1 (mTORC1), a master regulator of protein translation. It was found that DDIT4 protein is essential for hypoxia regulation of mTORC1 activity in which DDIT4 represses mTORC1 activity under hypoxia (Ellisen, 2005). It has also been found that mTORC1 is highly active in many cancers and plays a role in tumourigenesis and in other diseases. Over-expression of HIF-1 $\alpha$  is also frequently seen in many human cancers (Zhong *et al*, 1999). Furthermore, other studies have shown that over-expression of DDIT4 inhibits the invasive activity of cancer cells such as H1299 through suppression of mTOR activity which suggests that DDIT4 expression is correlated with inhibition of cell invasion activity, thus controlling tumour spreading (Jin *et al*, 2011). In response to hypoxia, HIF-1 has dual roles, mediating hypoxia-induced cell death (Carmeliet *et al*, 1998), and also mediating anti-apoptotic responses by inducing angiogenic genes like VEGF (Zaman *et al*, 1999; Baek *et al*, 2000). As with HIF-1, DDIT4 also has dual roles in the response to hypoxia depending on the cellular context. Shoshani *et al* reported that over-expression of DDIT4 promotes the apoptotic death of differentiated neuronal PC12 and parenchymal cells. In contrast, over-expression of DDIT4 in dividing PC12 and MCF7 breast cancer cells enhance them to resist hypoxia-induced apoptosis (Shoshani *et al*, 2002) and this finding was supported by other study in which DDIT4 increase invasive PC-3 cell growth (Schwarzer *et al*, 2005). So, DDIT4 can be work as pro- or anti-apoptotic regulator depending on the cell situation.

MicroRNAs are thought to play critical roles in gene regulation in a variety of processes, including differentiation, proliferation, death and metabolism. MicroRNAs are partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to down-regulate gene expression by mRNA translational repression or mRNA degradation (Jackson & Standart, 2007; Bartel, 2004).

A study by Kulshreshtha R *et al.* has identified a set of hypoxia-regulated microRNAs (HRMs), providing an additional link between a tumour-specific stress factor and gene expression control. The HRM group includes: miR-21, 23a, 23b, 24, 26a, 26b, 27a, 30b, 93, 103, 103, 106a, 107, 125b, 181a, 181b, 181c, 192, 195, 210 and 213, which were induced in responses to hypoxia in breast and colon cancer cells (Kulshreshtha *et al.*, 2007). In addition to the microRNAs that are up-regulated by hypoxia, a set of microRNAs were down-regulated in hypoxic cells, including miR-15b, 16, 19a, 20a, 20b, 29b, 30b, 30e-5p, 101, 141, 122a, 186, 320 and 197. Other studies also reported the up-regulation of microRNAs that respond to low oxygen including miR-210, miR-30b, 93 and 181b (Hua *et al.*, 2006; Donker *et al.*, 2007). Kulshreshtha *et al.* confirmed an important regulatory role for HIF, at least for some hypoxia-induced microRNAs, such as miR-210, 26 and 181 (Kulshreshtha *et al.*, 2007). In the last few years, some studies have also shown that additional transcription factors which respond to hypoxia/anoxia, such as p53 and NF- $\kappa$ B, affect the expression of select microRNAs (He *et al.*, 2007; Kluiver *et al.*, 2007).

Cell death regulation is an important process in a stressful environment, such as hypoxia. Several key genes of the apoptotic response were found to be potentially targeted by HRMs: PAR-4 (miR-26, 30, 181), PCDC10 (miR-103/107, miR-181), BID (miR-23), BIM (miR-24); CASP3 (miR-30), CASP 7 (miR-23), APAF1 (miR-27), BAK1 (miR-

26), Bnip3L (miR-23). The anti-apoptotic gene Bcl2 is an experimentally-confirmed target of miR15 and 16. These microRNAs were found to respond to hypoxia by their down-regulation (Hua *et al*, 2006; Cimmino *et al*, 2005). The angiogenic factor VEGF has a particular importance in tumour progression and control, and for this gene a group of candidate regulatory microRNAs have been identified which play role in its regulation: miR-16, miR-20, let-7b, miR-17-5p, miR-27, miR-106, miR-107, miR-193, miR-210, miR-320 and miR-361 (Hua *et al*, 2006).

HIF-1 $\alpha$  protein accumulates under low oxygen concentrations (Semenza, 2000a). Non-hypoxic stimuli, including LPS, can also induce HIF-1 via mechanisms distinct from hypoxic induction by increasing transcription rate of the  $\alpha$  subunit (Blouin *et al*, 2004). So, the synergistic effect of hypoxia plus LPS could be exerted through these different mechanisms on genes known to be HIF-1 targets such as CP, DDIT4, VEGF, CA12, MUC1, and P4HA2. Another possible synergy mechanism could be that cells stimulated with LPS generate and accumulate reactive oxygen species (ROS) (Wiesel *et al*, 2000) leading to activation of NF- $\kappa$ B (Maitra *et al*, 2009) in which in turn induces HIF transcription through NF- $\kappa$ B sites in its promoter (Blouin *et al*, 2004; Frede *et al*, 2006). In addition, hypoxia increases the accumulation of HIF-1 $\alpha$  and activates NF- $\kappa$ B, so both factors may work in synergy in order to induce genes. Several studies have shown that the activation of the NF- $\kappa$ B pathway in response to hypoxia (Ryan *et al*, 2005) in different cell types can induce transcription of the IL-6 gene, as seen in cardiac myocytes (Matsui *et al*, 1999), and also EPO was induced by short term hypoxia through the NF- $\kappa$ B pathway in the Hep3B human hepatocellular carcinoma cell line (Figuroa *et al*, 2002). Genes coding for macrophage inflammatory protein-2 (MIP-2) (Zampetaki *et al*, 2004), and IL-8 (Shi *et al*, 1999), in RAW264.7 and COLO357 human

pancreatic cancer cells, respectively, were also up-regulated through NF- $\kappa$ B activation. Battaglia and colleagues showed that NF- $\kappa$ B binding activity was increased when monocytes were exposed to short-term hypoxia (Battaglia *et al*, 2008). Taken together, hypoxia and LPS can activate and increase the accumulation of NF- $\kappa$ B and HIF-1, which could work cooperatively in synergistic induction gene expression.

A better understanding of how synergy genes are regulated by hypoxia plus LPS in human macrophages will be helpful for the development of future therapies for a range of different disease. As macrophages have been shown to accumulate in the areas of low oxygen tension where large numbers of genes are up-regulated, the knowledge of how the promoters of these genes are induced by hypoxia, in synergy with other stimuli, by elucidation of the hypoxia responsive elements would be an additional advantage for future tumour gene therapy whereby a therapeutic gene could be engineered to be regulated by the hypoxia responsive promoter.

## **Conclusion and future work**

I have studied the transcriptome of primary human MDMs and identified the genes regulated in response to reduced oxygen concentrations or/and LPS stimulation. The data presented in this project show that hypoxia and LPS work together to synergistically up-regulate several genes including CA12, wnt5a, TRAIL, VEGF, PLOD2, DDIT4, MT1M, and CP. Furthermore, this study provides insights into the molecular mechanisms of synergistic (hypoxia plus LPS) induction of two genes, TRAIL and DDIT4. These data will be useful in increasing understanding of the regulation of immune and inflammatory responses, leading to new perspectives on the activities of macrophages when exposed to hypoxia and LPS within pathological sites and identifying potential molecular targets for the development of rational and efficient therapeutic approaches in several diseases.

Further experiments are required to extend the findings presented in this project. mRNA decay analysis of TRAIL and DDIT4 is required in order to determine whether the observed up-regulation by hypoxia plus LPS in HMDMs is due to increased transcription or mRNA stability. Moreover, the analysis of promoter constructs of TRAIL and DDIT4 by Genomatix MatInspector software has shown binding sites for NF- $\kappa$ B and Sp-1, so knockdown of these two transcription factors by siRNA is required to determine whether they are essential for induction for TRAIL and DDIT4 expression under hypoxia plus LPS or not. Although this project focused mainly on *in vitro* experiments, it would be interesting in future to do *in vivo* experiments as well using animals to see whether similar data would be obtained. In addition, it would be

interesting to try using differently-structured LPS from other Gram-negative bacteria such as *Porphyromonas gingivalis*. As mentioned in chapter one, in some cases pathogens can invade the body via the intestinal mucosa, which is often characterized by low oxygen levels. To gain new insights into this mechanism of infection and the immune response elicited, whole bacteria (heat killed) or live bacteria such as *Salmonella typhimurium* could be used. Furthermore, it might be informative to try TLR ligands which are not structurally related to LPS, such as such unmethylated CpG DNA from bacteria, in combination with hypoxia or normoxia, to investigate the effect on expression of TRAIL and DDIT4.

Further work could also be carried out to investigate in more detail the effect of hypoxia and LPS on TRAIL protein synthesis in human primary macrophages using flow cytometry to quantify cell membrane bound TRAIL or by ELISA to quantify secreted TRAIL.

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