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THE EFFECT OF HYPOXIA ON THE PRODUCTION OF INTERLEUKIN-10 (IL-10) IN HUMAN MONONUCLEAR CELLS

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STATEMENT OF ORIGINALITY

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(Please print)

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Abstract

Macrophages play an important role in both innate and adaptive immunity by engulfing intruding pathogens and damaged or infected cells, antigen presentation, and by secretion of immune mediators. Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine mainly produced by macrophages in response to cell activation, which serves to suppress immune reactions and inflammation.

Hypoxia refers to oxygen deprivation. It is a common condition found in pathological tissues. The relationship between hypoxia and the production of IL-10 by macrophage is not yet thoroughly understood. In previous unpublished work by Staples and Burke *et. al.*, it was found that both basal and LPS-induced IL-10 mRNA and protein are reduced in hypoxia. In the present study, it was shown that the transcription factor Hypoxia Inducible Factor 1 (HIF-1) appears to be involved in this reduction of IL-10 production in hypoxia. Although the regulatory elements on the IL-10 promoter responsible for this blockage effect were not definitively identified, our results indicated that the activity of a -4kb IL-10 luciferase reporter adenovirus was significantly reduced in cells treated with the HIF-1 inducing agents, cobalt chloride and desferrioxamine. The activity of a -195bp IL-10 luciferase reporter adenovirus was also decreased in the HIF-1inducing agent treated samples. These data imply that either by indirect interaction or physically binding to the IL-10 promoter or gene, HIF-1 does play a role in blocking IL-10 expression in hypoxia. Sequence and transcription factor analysis indicated the presence of a HIF-1 consensus sequence hypoxia responsive element (HRE) located in the -2,171bp to -2,187bp position

on the IL-10 promoter. This result suggests that HIF-1 may affect IL-10 production, at least in part, by physically binding to its promoter.

Lastly, we showed that the effect of hypoxia on IL-10 expression is observed with a range of different toll-like receptor ligands, and is not limited to induction by LPS.

1. Introduction

1.1 Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood mononuclear cells (PBMC) are mononuclear cells consisting of lymphocytes, monocytes and macrophages. These blood cells are a critical component in both innate and adaptive immune system. About 75% of the lymphocyte population are T cells (CD4+ and CD8+), and 25% of the lymphocytes in PBMC are B cells and natural killer cells (Delves *et. al.*, 2006).

PBMCs are widely used in research and clinical uses every day. The primary IL-10 producers – macrophages and regulatory T cells will be introduced hereafter.

1.1.1 Macrophage

Macrophages belong to the mononuclear phagocyte system, which is sometimes referred as the reticuloendothelial system. The Mononuclear phagocyte system is a family of cells comprising bone marrow progenitors, blood monocytes and tissue macrophages. They reside in most of the tissues in the body, and their numbers increase in infection, inflammation, or tumour growth (Hume D. A., 2006). Mononuclear phagocytes are derived from progenitor cells produced in the bone marrow. After being produced in bone marrow, these progenitor cells differentiate to form blood monocytes, which circulate in the blood. A minor portion of the monocytes remain in the blood circulation, whereas most of them enter body tissues, where they develop into phagocytic cells, macrophages. The macrophages differ in appearance and are classified and named differently according to the tissues they reside in. For instance, alveolar macrophages line the air spaces of the lungs,

red pulp macrophages are in spleen, Kupffer cells reside in the liver, histiocytes occur in skin and connective tissues, reticulum cells are found in the sinuses of spleen and lymph nodes, microglia are in nervous tissues, and Langerhans cells locate in skin tissues. The majority of the tissue macrophages remain as stationary cells within tissue, where they act as the first line of defence in the immune system by engulfing pathogens and harmful foreign particles that enter the body (Burke and Lewis, 2002). Despite the fact that macrophages are believed to be differentiated from monocytes and do not proliferate, some believe that not all tissue macrophages are replaced by blood monocytes in the steady state and that local proliferation makes a significant contribution (Hume *et al.*, 2002).

As the front line defender, macrophages play an important role in the innate immunity against protozoa, bacteria, fungi, and viruses by active engulfment. They are able to ingest dead or abnormal cells and migrate to tumour sites and destroy tumour cells. As an important member participating in adaptive immunity, macrophages are capable of secreting both proinflammatory cytokines such as TNF, IL-1 and IL-6 and anti-inflammatory cytokines like IL-10 involved in wound healing, tissue regeneration, angiogenesis and activation of lymphocytes. Besides, they also act as antigen presenting cells that present antigens (fragments of pathogen or foreign particles) on the major histocompatibility complex (MHC) class II molecules to CD4+ T cells.

It has been reported that macrophages have strong influence with the development of atherosclerosis in blood vessels, neoplastic cell control, and autoimmunity. Due to their

prominent role of migrating to infection and tumour sites, attempts on investigations of employing macrophages in gene therapies have often been made (Burke and Lewis, 2002).

1.1.2 Regulatory T Cells (Treg)

Regulatory T cells (Treg) are an essential component of the immune system. They play an important role in the immune response towards pathogens while establishing tolerance for harmless antigens (Wieczorek *et. al.*, 2009). There are different subsets of regulatory T cells including CD8+ (Treg that express the CD8 transmembrane glycoprotein), CD4+CD25+ (Treg that express CD4, CD25 and Foxp3), and other T cell types that have suppressive function. These cells are involved in switching off the immune responses after they have successfully confined the infection caused by invading organisms, and also in regulating autoimmunity (Delves *et. al.*, 2006, Wieczorek et. al., 2009).

Regulatory T cells occur naturally are called natural T regulatory cells (nTregs). They are selected in the thymus and move to the periphery. nTreg are self antigen specific CD4+ T cells that express CD25 in high levels and Foxp3. In addition, nTregs' phenotype is also characterized by the expression of CD62 ligand, CD103, glucocorticoid induced tumor necrosis factor receptor (GITR), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), CD152, neurophilin and CD45RO (Nandakumar *et. al.*, 2009). The CD4+ helper T cells in the periphery that are later induced to become regulatory T cells are called induced or adaptive T regulatory cells (iTreg). iTreg arise as a result of activation of mature T cells in the absence of optimal antigen exposure or, costimulation or in the presence of certain inhibitory cytokines. The induced Tregs include the type 1 regulatory T cells (Tr1) and Th3 cells. Tr1 cells have both Th1 and Th2 phenotypic markers (chemokine receptors) like the

CXCR3, CCR5, CCR3, CCR4 and CCR8. They express surface molecules including CD40L, CD69, CD28, CTLA-4, IL-2R-α, IL-15Rα and HLA-DR upon activation. In addition, Tr1 cells are characterized by an elevated production of IL-10, TGF-β and IL-5 (Romagnani *et. al.*, Nandakumar *et. al.*, 2009). Th3 cells are induced by oral antigen administration. The suppressive activity of Th3 is based on its ability of producting of TGFβ (Weiner *et. al.*, 2007, Nandakumar *et. al.*, 2009).

1.2 Hypoxia

Oxygen homeostasis is a crucial component in all biological systems. In mammals, oxygen level is controlled through respiratory and cardiac responses. The atmospheric oxygen level at sea level is ~20%, and the partial pressure is 160mmHg. The oxygen partial pressures in alveoli and arteries are both 100mmHg, in normal tissues is ranged from 24 to 66 mmHg (Vaupel *et. al.*, 1989). In addition, oxygen level was shown to influence the development and effector functions of murine T lymphocytes (Krieger *et. al.*, 1996).

Hypoxia in biological systems refers to low oxygen tension in tissues. It usually occurs in pathological tissues including tumours, cancers, wounds, ischemic tissues, psoriasis, chronic obstructive pulmonary disease, diabetes, atherosclerotic plaques (Brahimi-Horn & Pouyssegur, 2006; Bjornheden *et. al.*, 1999), and arthritic joints (Stevens *et. al.*, 1991). The oxygen partial pressure in these pathological tissues ranged from 0 to 15mmHg (Vaupel *et. al.*, 2006). However, hypoxia is also found in healthy tissues such as spleen (oxygen level as low as 0.5%) (Caldwell *et. al.*, 2001), and it is a condition encountered in embryogenesis in which hypoxic signalling is considered necessary for normal development (Brahimi-Horn & Pouyssegur, 2006).

In cancer sites, rapid growth and abnormal angiogenesis surrounding the neoplastic cells cause insufficient blood supply, which lead to depletion of oxygen. This eventually results in the formation of necrotic and hypoxic regions in the inner parts of the tumour (Griffiths *et. al.*, 2000). It has been demonstrated that the hypoxic region of the tumours showed increased resistance to ionising radiotherapy due to the non-proliferating property of the hypoxic cells (Brizel *et. al.*, 1999; Tannock, 1998). The inaccessibility of the drug to the interior regions of the tumour due to the disorganized blood vessels also makes it difficult for chemotherapy to efficiently destroy the hypoxic core of the tumour (Durand, 1994; Tannock, 1998). Hypoxia has also been shown to be related to increased mutation rates (Yuan & Glazer, 1998), tumour invasion (Pennacchietti *et. al.*, 2003) and metastasis (Subarsky & Hill, 2003).

Griffiths *et. al.* (2000) showed that macrophages tend to accumulate in hypoxic regions. And it has also been suggested that chemoattractants such as colony stimulating factor 1 (CSF), vascular endothelial growth factor (VEGF), endothelial monocyte activating polypeptide II and endothelin 1 recruit peripheral monocytes to tumour regions and differentiate into tumour associated macrophages (TAMs). In hypoxic regions, the hypoxia inducible factor 1 (HIF-1) is activated, and in macrophages it induces the up-regulation of other transcription factors and genes such as VEGF, glucose transporter 1 (GLUT-1), and matrix metalloproteinase 7 (MMP7) whose products promote tumour growth, angiogenesis and metastasis (Murdoch & Lewis, 2005; Burke *et. al.*, 2003; Murdoch *et. al.*, 2005; Leek & Harris, 2002). Because of these properties, macrophages have been proposed to be used as vehicles that deliver gene therapy to targeting pathological tissues and tumour sites (Griffiths*et. al.*, 2000).

From unpublished data by Staples, Burke *et. al.*, it was found that when macrophages were incubated in hypoxia (0.2% O_2 and 5% CO_2) for longer than 24 hours, the cell surface expression of mannose receptor (CD206) and CD40 showed significant reductions. The phagocytic ability for macrophages was reduced 2.7-fold when incubated in hypoxia for five days. Moreover, the mRNA levels of VEGF and versican were significantly increased, whereas both interleukin 10 (IL-10) mRNA and protein production in activated macrophages were diminished when incubated in hypoxia for greater than 24 hours (Figure 1.1B,D; Bernard Burke, personal communication,).

1.3 Hypoxia Inducible Factor (HIF)

1.3.1HIF

In hypoxia, a variety of signalling pathways are activated. Of which, the activation of a transcription factor, hypoxia-inducible factor (HIF) is a key element responsible for embryogenesis and the up-regulation of numerous hypoxia inducible genes (Sutter *et. al.*, 2000). HIF knockout mice are embryonic lethal and the embryos show cardiac and vascular abnormalities. In pathophysiological conditions, cancers or when cellular oxygen homeostasis is not reached, HIF is responsible for driving the cellular response by activating or repressing genes possessing various functions to alleviate the stress (Brahimi-Horn & Pouyssegur, 2006).

In general, there are two main types of HIF, HIF-1 and HIF-2. Both HIFs are heterodimeric molecules composed of an α subunit and a β subunit. The β subunit is oxygen independent whereas the α subunit is constitutively produced but is subjected to rapid degradation in

the presence of oxygen(half-life less than five minutes) but is stable and active in the absence of oxygen (i.e. hypoxia). In normoxia, the O_2-dependent degradation of the HIF-α subunit is triggered by the binding of the von Hippel–Lindau tumoursuppressor protein (VHL). In the cytoplasm, VHL interacts with the protein Elongin C and recruits an E3 ubiquitin–protein ligase complex that ubiquitinates HIF-α and targets it for degradation by the 26S proteasome (Semenza, 2007; Maxwell *et. al.*, 1999).

The binding of VHL with HIF-α subunit is mediated by the hydroxylation of the prolyl residues in HIF-α located in the oxygen-dependent degradation domain (ODD) by prolyl hydroxylase PHD (Jaakkola *et. al.*, 2001), and the hydroxylation of the asparagine 803 residue of HIF-1α by factor inhibiting HIF (FIH) (Hewitson, 2002). PHD is a family composed of prolyl 4-hydroxylases (PHD1, PHD2, *etc.*). PHDs require Fe^{2+} (Fe (II)), 2-oxoglutarate, O_2 and ascorbate as substrates; its activity is reduced in hypoxia due to the limitation in O_2 (Epstein *et. al.*, 2001; Ivan *et. al.*, 2001). In addition, PHD activity reduces in hypoxia due to the inhibition of the catalytic centre which contains Fe (II) by reactive oxygen species (ROS) generated at complex III of the mitochondrial respiratory chain and increase in response to hyperoxia (excess oxygen level; Kivirikko & Myllyharju, 1998; Guzy & Schumacker, 2006; Semenza, 2007). In the second oxygen-dependent regulatory mechanism, FIH blocks the interaction between HIF-α and p300 coactivator protein by hydroxylating asparagine residues of HIF-α (Hewitson *et. al.*, 2002; Peet & Linke, 2006).

In hypoxia, the PHD activity decreases and enables rapid accumulation of HIF-α (Sutter *et. al.*, 2000; Jewell *et. al.*, 2001). The decrease in oxygen availability further impairs FIH, and this results in a decrease in HIF-α C-terminal hydroxylation. The decrease in the C-terminal HIF-α hydroxylation incurs increased recruitment of the p300 coactivator protein and CREB binding protein (p300/CBP) transcriptional coactivators, which eventually leads to the enhanced transcriptional activation of HIF target genes (Wenger *et. al.*, 2005; Lando *et. al.*, 2002a, b; Mahon *et. al.*, 2001).

In hypoxia, a high level of HIF-α is able to translocate into nucleus and bind the HIF-β subunit. After the binding of the α and β subunits, HIF binds to the hypoxia responsive elements (HREs, see section 1.3.2) on the promoter of about 200 HIF target genes (among which around 70 genes have been confirmed) and initiate transcriptions by recruiting transcriptional coactivators such as p300/CBP (Wenger *et. al.*, 2005).

1.3.2 Hypoxia Responsive Elements (HREs)

A HRE is the consensus sequence located in the promoter regions in HIF target genes, and HIF binds to HREs in hypoxia after activation. The minimal cis-regulatory element required for hypoxic induction of gene transcription was identified by Semenza *et. al.* (1994), and they further indicated that a single core HRE consensus sequence is required but not sufficient for effective gene activation in response to hypoxia. A fully functional HRE requires neighboring DNA binding sites for additional transcription factors or co-activators, which may act to amplify the hypoxia response (Wenger *et. al.*, 2005).

Wenger *et. al.* (2005) further identified the consensus core HRE sequence CGTG, and the required neighboring nucleotides occurred with nonrandom frequency in the adjacent positions, especially in the 5′ flanking bases.

1.3.3Hypoxia Mimetic agents and their Relationship with HIF

Cobalt chloride ($CoCl_2$) and desferrioxamine (DFO) are chemical reagents that are commonly used to induce HIF-1 protein in normoxia. Maxwell *et al.* (1999) demonstrated that cobalt and DFO are capable of inducing HIF by stabilizing HIF-1α using mechanisms different than draining cellular oxygen. Although both are able to upregulate HIF, cobalt and DFO induce the pathway with different mechanisms. In this project, the correlation between HIF and our gene of interest, IL-10, was examined by inducing HIF in normoxia using treatment with $CoCl_2$ and DFO.

1.3.3aCobalt Chloride ($CoCl_2$)

As mentioned in section 1.3.1, the hydroxylation of the prolyl residues which reside in the oxygen-dependent degradation domain (ODD) in HIF-1α by prolyl hydroxylase (PHD) is one of the key mechanisms that mediates the binding of VHL with HIF-1α which eventually leads to proteasomal degradation of HIF-1α. Epstein *et. al.* (2001) suggested that the iron is critical to the activity of these hydroxylases as these enzymes have an iron-binding centre. Besides, the iron ion in the iron-binding centre can be removed by chelators. They further suggested that cobalt can act as a competitor that competes and replaces iron from its binding site in PHD. Hence, cobalt ions inactivate the hydroxylase activity by replacing the

iron ions, which in turn promotes the activation of HIF-1 by stabilizing the HIF-1α subunit (Epstein *et. al.*, 2001). Yuan *et. al.* (2003) showed a second mechanism in which HIF-α is stabilized by cobalt. In this mechanism, cobalt stabilizes HIF-1α protein by direct binding to the ODD in HIF-1α, thereby prevent the interaction between HIF-1αand VHL protein. They further demonstrated that cobalt can bind to HIF-1α regardless of the hydroxylation state of the prolyl residue in the ODD.

In short, $CoCl_2$ is a common hypoxia mimetic reagent used to mimic hypoxia by inhibiting PHD, hindering its interaction with the prolyl residue in ODD and induce HIF activity in normoxia.

1.3.3b Desferrioxamine (DFO)

Whereas cobalt inhibits the hydroxylation of the prolyl hydroxylase by replacing the iron in the iron-binding centre, Yuan *et. al.* (2003) suggested that DFO inhibits the hydroxylase by acting as an iron chelator that depletes the iron required for the enzymatic activity. As mentioned earlier, iron is required for the hydroxylase activity in PHD, its removal consequently causes the inactivation of PHD, and this allows the interaction between HIF α and β subunits in normoxic condition. Therefore, DFO is another common hypoxia mimetic reagent used to induce HIF.

1.4 Interleukin 10 (IL-10)

Interleukin 10 (IL-10), first described as a cytokine synthesis inhibitory factor (CSIF) produced in murine T_h2 cells by Fiorentino *et. al.* in 1989, is a pleiotropic cytokine with immunosuppressive and anti-angiogenic functions that plays pivotal roles in immunity. It is produced by a variety of cells including B-cells, T_h1 and T_h2 cells, dendritic cells, eosinophils, keratinocytes, mast cells, monocytes and macrophages (Enk & Kaz, 1992; Ding *et. al.*, 1993; Thompson-Snipes *et. al.*, 1991; Gollnick *et. al.*, 2001; Go et. al,. 1990; Yssel *et. al.*, 1992; Lamkhioued *et. al.*, 1996; Fiorentino *et. al.*, 1989; A. Schaefer PhD Thesis, University of Leicester, 2005, personal communication). IL-10 was also found to be produced by several types of tumour cells (Lu *et al.*, 1995; A. Schaefer, 2005). The major function of IL-10 is to suppress multiple immune responses through actions on T cells, B cells, antigen presenting cells and other cell types (Delves *et. al.*, 1998; Im *et. al.*, 2004). It has been shown that IL-10 inhibits the production of several pro-inflammatory cytokines including tumour necrosis factor (TNF), IL-1α, IL-1β, IL-6, IL-12, and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) produced in activated monocytes/macrophages (Fiorentino *et. al.*, 1991; Nicod *et. al.*, 1995). Also, IL-10 was able to inhibit NF-κB, an important transcription factor involved in the production of various inflammatory cytokines, and a broad range of activated functions such as monokine synthesis, nitric oxide production, CD80/CD86 co-stimulatory molecule expression in monocytes/macrophages, MHC class II molecule expression on dendritic cells, monocytes, Langerhans cells, and chemokine production and proliferation in CD4+ T-cells (de Waal Malefyt *et. al.*, 1999a, b; Conti *et. al.*, 2003; Ding *et. al.*, 1992, 1993; Fiorentino *et. al.*, 1999). de Waal Malefyt *et. al.* (1991a) pointed out that the production of IL-10 was also reduced

by IL-10 itself, probably through a negative autocrine feedback loop mechanism. Yet, the function of IL-10 in the natural environment is more complex. Cells in the immune system are exposed to a number of various cytokines and other stimuli simultaneously, and the target cells may act differently in response to combinations of different stimuli. Regardless of its immunosuppressive role, IL-10 was shown to be a chemoattractant (Jinquan *et. al.*, 1993) that induces proliferation (Rowbottom *et. al.*, 1999) and cytotoxicity of CD8+ T-cells (when administered in combination with IL-2; Santin *et. al.*, 2000). As well, IL-10 was found to be able to induce the proliferation/differentiation (Rousset *et. al.*, 1992) and production of immunoglobulin IgA (Defrance *et. al.*, 1992), and IgD (Levan-Peit *et. al.*, 1999; Nonoyama *et. al.*, 1993), IgG (Briere *et. al.*, 1994) by B-cells. Staples *et. al.* (2007) later demonstrated IL-10 is able to induce the production of IL-10 itself via Stat3 transcription factor.

In addition to its role in anti-inflammatory responses, few studies using animal models revealed that IL-10 can induce NK cell activation and thereby facilitates anti-tumour responses which leads to tumour cell destruction (Zheng *et. al.*, 1998; Kundu *et. al.*, 1998). Several studies using mouse models have demonstrated an anti-tumour role for IL-10. In particular, Adris *et. al.* (1999) showed reductions in tumour malignancy and inductions of T_h2-mediated tumour rejection response to IL-10-transfected tumour cells in studies using a colon carcinoma mouse model. Moreover, systemic administration of IL-10 was demonstrated to be able to inhibit tumour metastasis in various murine models (Zheng *et. al.*, 1996; Berman *et. al.*, 1996).

On the contrary, other researchers showed that IL-10 levels may be associated with suppression of anti-tumour immune responses (Kufe *et. al.*, 2005). It was suggested that IL-10 may also act as a tumour growth factor (Yue *et. al.*, 1997). Several studies indicated the elevation of IL-10 expression in both the serum and/or tumour lesions in patients with particular cancers, including lung (De Vita *et. al.*, 2000) and renal (Wittke *et. al.*, 1999) carcinomas.

1.4.1Physical Properties of IL-10

Human IL-10 is a 37kDa acid-sensitive protein that lacks detectable carbohydrate moieties. It is a member of the four alpha-helical bundle cytokine superfamily. Bioactive IL-10 appears to function as a homodimer composed of two non-covalently associated 18.5kDa subunits consist of 160 amino acids (Moore *et. al.*, 1993). Different from human IL-10, mouse IL-10 is a 35kDa homodimeric cytokine that is glycosylated at the N-terminus. Both mouse and human IL-10 contain intra-chain disulphide bonds that are essential to the biological function of IL-10 (Delves *et. al.*, 1998; Vieira *et. al.*, 1991; Zdanov *et. al.*, 1995).

Located on human chromosome 1q31 to 1q32, the human IL-10 gene contains five exons with size of 4.9kb; transcription of the human IL-10 gene produces mRNA of 2kb. Located on chromosome 1 and with 81% homology at nucleotide level, its murine homologue consists of five exons with size of 5.1kb (Kim *et. al.*, 1992), and transcriptions yield an 1.4kb mRNA construct. In addition, IL-10 viral homologues include the BCRF-1 gene of Epstein-Barr virus (EBV) with 84% homology in the protein level (Moore *et. al.*, 1990, 1991; Hsu *et. al.*, 1990), human and simian cytomegalovirus (CMV) (Kotenko *et. al.*, 2000; Lockridge *et. al.*, 2000), equine herpes virus type 2 (EHV2) (Rode *et. al.*, 1993) genomes.

This viral IL-10 homologue in EBV has been demonstrated to suppress antiviral immune responses by specifically inhibiting the synthesis of pro-inflammatory and other cytokines that may lead to the killing of the host cell (de Waal Malefyt *et. al.,* 1991b; Hsu *et. al.* 1990). This suggests that EBV may have acquired IL-10 functions during evolution to gain survival advantage by inhibiting the anti-viral response. Likewise, viral IL-10 homologues found in other viruses may serve similar functions that allow survival of the virus within the host cells.

1.4.2 IL-10 Expression

IL-10 is constitutively expressed at basal level in Th2 cells and macrophages, but its induction is tightly regulated by several signalling pathways (Brightbill *et. al.*, 2000). Lipopolysaccharide (LPS), a major cell wall component in gram negative bacteria, is a potent inducer that induces the expression of IL-10 in human monocytes and macrophages (Frankenberger *et. al.*, 1995). In the LPS-activated pathway, LPS in body fluid firstly binds with a plasma protein, LPS-binding protein (LBP). The LBP:LPS complex then transfers LPS to cell-bound CD14 receptor on monocytes/macrophages. After binding with LPS, CD14 interacts with the toll-like receptor 4 (TLR4) and activates Nuclear factor kappa B (NFκB), which leads to the activation of the cell (Wright *et. al.*, 1989). Other than LPS, IFNα (Ziegler-Heitbrock *et. al.*, 2003), IFNβ (Porrin *et. al.*, 1995), TNFα (Platzer *et. al.*, 1995), CpG and zymosan A (Saraiva *et. al.*, 2005) are also able to induce IL-10 expression. However, the exact mechanisms by which IL-10 expression is induced were not fully understood.

The 4kb sequence of the human IL-10 promoter upstream of the IL-10 gene was first reported by Kube *et. al.* (1995). To date, the human genome project has been completed, and the complete IL-10 promoter sequence has been identified and is available on the NCBI website. Yet, most of the identified IL-10 transcription factor binding sites locate within the 1kb region upstream of the IL-10 gene (*i.e.* within the -1kb IL-10 gene region). Upon activation, transcription factors involved in IL-10 gene expression bind to the IL-10 promoter region and initiate transcription. Several transcription factors were found to participate in the expression of IL-10. The identified transcription factors include activator protein (AP-1) (Gollnick *et. al.*, 2001), activating transcription factor (ATF-1), CAAT/enhancer binding protein (C/EBP) (Brenner *et. al.*, 2003), cellular-musculoaponeurotic fibrosarcoma (c-Maf) (Cao *et. al.*, 2005), cAMP response element binding protein 1 (CREB-1) (Platzer *et. al.*, 1999), interferon regulatory factor 1 (IRF-1), signal transducer and activator of transcription 3 (STAT3) (Ziegler-Heitbrock *et. al.*, 2003; Benkhart *et. al.*, 2000), nuclear factor of activated T cells (NFAT1) (Im & Rao, 2004) and specificity proteins 1 and 3 (Sp1/Sp3) (Brightbill *et. al.*, 2000; Tone *et. al.*, 2000).

In addition to the transcription factor binding site, the regions in which the protein-bound regulatory elements are located are fundamental to the regulation of the given gene. These regions could function as enhancers, silencers, locus control regions, matrix attachment regions or insulator/boundaries. At the chromatin level, these regions usually form nucleosome-free hypersensitive sites which are sensitive to DNase due to the lack of protection from nucleosomes (Elgin, 1988; Gross *et. al.*, 1988). The identified DNase hypersensitive sites locate in both the coding and none-coding regions in the IL-10 gene. In the coding region, the DNase hypersensitive site were found in intron 3 and 4, and are

present only in murine Th1, but not Th2 cells (Im & Rao, 2004) and two sites in the 3' flanking region downstream of exon 5 identified in murine Th1 and Th2 cells (Im *et. al.*, 2004; Wang *et. al.*, 2005). The DNase hypersensitive sites in non-coding sites contain the 5'-untranslated (5' UTR) region of IL-10 gene, which is within 1kb upstream of the transcription starting site in naïve Th2 cells (Im *et. al.*; 2004) and the HSS-4.5 site (hypersensitive site 4.5) in the IL-10 promoter identified in bone marrow macrophages (Saraiva *et. al.*, 2005).

1.4.3 Hypoxia & IL-10 Expression Associated Transcription Factors

Other than HIF-1, several transcription factors were suggested to be activated in hypoxia. The hypoxia inducible transcription factors that were suggested to participate in IL-10 expression include CREB (Beitner-Johnson & Millhorn, 1998) and Sp1/Sp3 (Kaluz *et. al.*, 2003). Besides, several other proteins such as NFκB, (Shi e. al., 1999), HIF-1 and GATA binding protein 2 (GATA-2) (Yamashita *et. al.*, 2001) have been identified to interact with AP-1, which has been thought to participate in IL-10 transcription in hypoxia. Most of the identified IL-10 transcription factor binding sites are located within the -1kb region of the IL-10 gene (Brightbill *et. al.*, 2000; Brenner et. al, 2003; Ma et. al, 2001).

1.5 Toll-Like Receptors

Toll-like receptors (TLR) are transmembrane receptors characterised by extracellular domains containing leucine-rich-repeat motifs and a cytoplasmic signalling domain homologous to the interleukin 1 receptor (IL-1R), known as the Toll/IL-1R homology (TIR) domain (Bowie & O'Neill, 2000). A variety of TLR homologues have been identified in

humans, and 12 members of the TLR family have been identified in mammals (Akira *et. al.*, 2001; Akira *et. al.*, 2006).

Acting as pattern recognition receptors for pathogen-associated molecular patterns (PAMP) such as LPS, the TLRs are capable of recognizing the invasion of micro-organisms and play a crucial role in innate nonspecific immune responses. TLR was initially identified to be involved in the development of embryonic dorso-ventral polarity in *Drosophila*. It was later shown to play a critical role in the antifungal response of these flies (Lemaitre et al., 1996).

The expression of TLRs is altered in response to pathogens, cytokines, and environmental stresses (Akira *et. al.*, 2006). They are prominently expressed in antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages, which ingest, degrade pathogens and present the degraded pathogen components including nucleic acids, sugars or peptides to T cells. Beside APCs, TLRs are also expressed in other immune cells like B cells and T cells as well as non-immune cells such as fibroblasts and epithelial cells (Akira *et. al.*, 2006).

TLRs play key roles in both innate and adaptive immunity; the innate immune system uses them to detect the presence of invading microbes and initiate host defence (Aliprantis *et. al.* 2000). On the other hand, the role of TLRs extends to adaptive immunity through the coupling of innate and adaptive immunity (Medzhitov 2001, Akira *et. al.*, 2001). In this instance, immature DCs express a full set of TLRs and are located in potential pathogen-entry sites. Upon ligation of the TLR ligands the DCs maturate, express high levels of MHC and co-stimulatory molecules (CD80 and CD86), migrate to draining lymph nodes and present the pathogen-derived antigens to naive T cells (Banchereau *et. al.*, 1998). Besides, TLRs also induce the expression of various cytokines by DCs which direct the

differentiation of naïve T cells into Th1 effector cells (Akira *et. al.*, 2001). Furthermore, Alexopoulou *et. al.* (2002) showed that certain TLRs are required for the formation of specific antibodies, and lack of certain TLRs may result in hyporesponsiveness to vaccination.

Engagement of the TLRs activates NF-κB and JNK (c-Jun N-terminal kinase), which are the key transcription factors responsible for the transcription of genes encoding pro-inflammatory cytokines and chemokines. Two predominant intracellular pathways have been identified in the TLR signalling. The first pathway is MyD88 (myeloid differentiation factor 88) dependent pathway or D pathway, in which the adaptor molecule MyD88 is activated, and this leads to early activation of NF-κB and production of cytokines such as TNF (Akira & Takeda, 2004). The MyD88 protein is not activated in the second pathway (MyD88-independent or I pathway); the signals are transmitted through the TRIF (Toll-IL-1 domain-containing adaptor-inducing IFN-β), IRF3 (interferon regulatory factor 3), and finally lead to the transcription of IFN-β (Yamamoto *et. al.*, 2002; Oshiumi *et. al.*, 2003). Alternatively, the I pathway also activates NF-κB, leading to production of TNF and other inflammatory cytokines in a delayed fashion (Bagchi *et. al.*, 2007).

Except TLR3, TLR4 and TLR8, other TLRs have been described to signal through the D pathway exclusively. TLR3 induces inflammatory cytokines such as TNF and IL-6 exclusively through the I pathway (Hoebe *et. al.*, 2003), whereas TLR4 (Kawai *et. al.*, 1999) and TLR8 (Alexopoulou *et. al.*, 2001) have been characterized to have an alternative MyD88 independent pathway after stimulation with LPS and Poly (I:C) respectively.

TLR pathways are activated by the different components of microbes, and the interaction between the TLR signalling pathways may have important effects on host inflammatory responses and outcomes. TLRs are expressed extra- or intracellularly depending on the TLR type. For instance, while TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface (Iwasaki & Medzhitov, 2004), TLRs 3, 7, 8, and 9 are almost exclusively found in intracellular compartments such as endosomes (Akira et. al, 2006). Nucleic acids are the major ligands for intracellular TLRs, and they need to be internalized to the endosome before being recognized by the receptors.

The TLR family can be subdivided into subfamilies base on the ligands. While lipids are the primary ligands for TLR1 and TLR6, nucleic acids are the major ligands for TLR3, TLR7, TLR8, and. TLR4 is capable of recognizing a divergent collection of ligands such as LPS, the plant diterpene paclitaxel, the fusion protein of respiratory syncytial virus (RSV), fibronectin, and heat-shock proteins (Vabulas *et. al.*, 2002), all of which have different structures (Akira *et. al.*, 2006).

1.5.1 TLR1

Designated as CD281 (cluster of differentiation 281), TLR1 recognizes peptidoglycan and (triacyl) lipoproteins from gram-positive bacteria as a heterodimer in concert with TLR2 (Rock *et. al.*, 1998; Jin *et. al.*, 2007). In leukocytes such as macrophages and neutrophils, TLR1 is ubiquitously expressed, and it is expressed at higher levels than the other TLR genes (Rock *et. al.*, 1998).

1.5.2 TLR2

Also designated as CD282 or TIL-4 (Toll/interleukin-1 receptor-like-4), TLR2s is highly expressed on peripheral blood leukocytes and other tissues (Chaudhary *et. al.*, 1998). It has been shown to be involved in the recognition of a broad range of microbial products including peptidoglycan, glycosylphosphatidylinositol lipid and yeast cell walls due to their ability to couple with other TLRs (Medzhitov, 2001). TLR2 is found to form heterodimers with TLR1 (Alexopoulou *et. al.*, 2002), TLR4 (Hirschfeld *et. al.*, 2001) and TLR6 (Ozinsky *et. al.*, 2000). TLR2 is capable of recognizing lipoproteins and lipoteichoic acid (LTA) from gram positive bacteria (Medzhitov, 2001), macrophage synthetic lipopeptide 2 (MALP-2) and LPS from *P. gingivalis.* In this case, induction using *P. gingivalis LPS* resulted in different gene expression from that normally induced in murine macrophages when induced by E. Coli LPS, signifying that the induction was via TLR2 (Hirschfeld *et. al.*, 2001).

1.5.3 TLR3

Also known as CD283, TLR3 is expressed only in dendritic cells but not in other leukocytes, including precursors of monocytes. Other than double stranded RNA (dsRNA) (Alexopoulou *et. al.*, 2001) Kariko *et. al.* (2004) showed that TLR-3 can also recognize heterologous mRNA released from necrotic cells. Moreover, activation via TLR-3 induces the expression of IRF1, TNF and IFN-α in human dendritic cells.

1.5.4 TLR4

Of the identified TLRs, TLR4 is one of the most thoroughly studied members. Also known as CD284, TLR4 is a type 1 transmembrane protein with an intracellular domain homologous to that of the human IL-1 receptor. It is expressed on a variety of cell types,

most predominantly in the cells of the immune system, including macrophages and DCs (Medzhitov et al, 1997).

TLR4 is known to be highly inducible by LPS. As mentioned earlier in section 3.4, in recognition of LPS, LPS is first bound to LBP (LPS-binding protein), a serum protein, which functions to transfer the LPS monomers to CD14 (Wright *et. al.*, 1989). MD-2 is another component involved in the LPS receptor complex (Medzhitov *et. al.*, 2001). Nevertheless, there is evidence suggesting that TLR4 may interact with LPS directly without the aid of CD14 (Lien *et. al.*, 2000; Poltorak *et. al.*, 2000).

1.5.5 TLR5

TLR5 is also called CD285 or TIL-3 (Toll-interleukin-1 receptor-like-3), and like TLR-2 and TLR-4, TLR-5 is expressed in myelomonocytic cells, but at lower levels (Chaudhary et al, 1998; Rock et al, 1998). TLR5 is involved in recognition of Flagellin, a protein that forms bacterial flagella (Hayashi *et. al.*, 2001).

1.5.6 TLR6

TLR-6 (CD286) protein is 69 % identical to human TLR-1. In co-operation with TLR2, TLR6 plays a major role in the recognition of the 2kDa mycoplasmal macrophage-activating lipopeptide (MALP-2). Yet, in the TLR2/TLR6 heterodimer, TLR6 plays the major role of discriminating between the diacylated mycoplasmal lipoprotein MALP-2 and the bacterial lipoproteins, which are triacylated at the amino-terminal cysteine residue (Takeuchi et al, 2000, Medzhitov, 2001).

1.5.7 TLR7 & TLR8

TLR7 (CD287) and TLR8 (CD288) genes show high homology to each other. They both respond to synthetic antiviral imidazoquinoline components including resiquimod (R848), and Imiquimod and uridine-rich or uridine/guanosine-rich single strand RNA (ssRNA) (Akira *et. al.*, 2006). As the endosomal receptors locating in endosomal membranes, TLR7 and TLR8 are activated by the ssRNA released from enveloped viruses trafficking into the cytosol through the endosomal compartment or virus-infected cells that have been engulfed by the phagocytes (Akira *et. al.*, 2006).

1.5.8 TLR9

To date, the endosomal receptor TLR9 (CD289) still remains poorly understood. It is an endosomal receptor whose ligands are bacterial oligodeoxynucleotides which contain unmethylated deoxycytosine – deoxyguanosine (ODN CpG) motifs, and the signalling requires the internalization of the CpG DNA into late endosomal or lysosomal compartments (Hacker et. al, 1998).

1.5.9 TLR10

Human TLR10 was reported to be expressed in a highly restricted fashion as a highly N-glycosylated protein in B cells from peripheral blood and plasmacytoid dendritic cells from tonsils. Also, it is able to homodimerize with TLRs 1 and 2 and transmits an activation signal via the D pathway (Hasan *et. al.*, 2005).

1.5.10 TLR11

Murine TLR11 responds to uropathogenic strains of *E.coli*. However, human TLR11 is non-functional because of the presence of a stop codon in the gene (Akira *et. al.*, 2006).

1.6 Project Aims

In previous work by Staples and Burke *et. al.* (unpublished data; personal communication with B. Burke), hypoxia blocked LPS-induced IL-10 production (Figure 1.1). They also showed that the half-life of the IL-10 mRNA, with or without LPS treatment, was not affected by hypoxia (data not shown), indicating that changes in the transcription of the gene were responsible for the observed blockade of IL-10 expression. In this project, it was aimed to identify the mechanisms and the IL-10 promoter region responsible for the blockade of LPS-induced IL-10 expression in hypoxia.

Knowing that HIF-1 is one of the pivotal transcription factors in hypoxia, whether it has any relationship with IL-10 expression and whether the blockage of IL-10 expression in hypoxia is mediated by HIF-1 are of interest.

Finally, the expression of IL-10 in monocytes/macrophages stimulated with different TLR ligands were studied, Whether the induction with these TLR ligands were influenced by hypoxia were also observed.

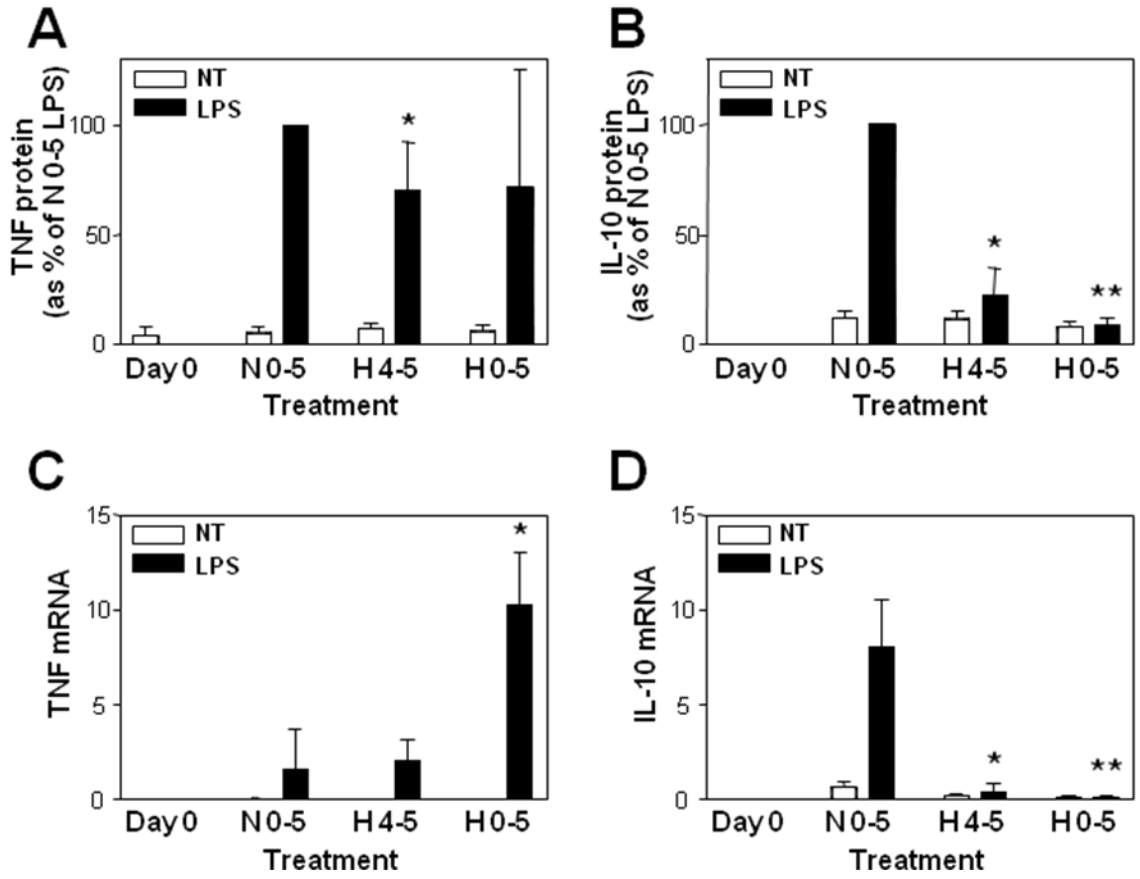


Figure 1.1: Hypoxia blocks LPS-induced IL-10 production (Staples, Burke *et. al.*, unpublished data).
Human PBMC were incubated in normoxia for 5 days (N 0-5); in normoxia for 4 days and then transferred to hypoxia for another 24hr(H 4-5); or incubated in hypoxia from d_1 to d_5(H 0-5). After incubation under the conditions indicated, cells were treated with or without *S. abortus equi* LPS (100 ng/ml) and incubated under normoxia or hypoxia as appropriate for a further (**A**&**B**) 24 h or (**C**&**D**) 4 h. (**A**&**B**) After 24 h supernatants were harvested and (**A**) TNF protein or (**B**) IL-10 protein was measured by ELISA. Data from 4 individual donors are expressed as means ± SD. Data were further analysed using a (**A**) one-tailed or (**B**) two-tailed, paired *t*-test. ** $p < 0.01$, * $p < 0.05$ when compared to N 0-5, LPS treated sample. (**C**&**D**) After 4 h, cells were harvested and RNA isolated and reverse transcribed. Day 0 cells were not LPS stimulated and only basal expression levels of protein and mRNA were determined. (**C**) TNF, (**D**) IL-10 and β_2M steady state mRNA levels were measured using real-time PCR. TNF and IL-10 were normalised toβ_2M levels. Data from 4 individual donors are expressed as means ± SD (except Day 0 where n=3). Data were further analysed using a (**C**) one-tailed or (**D**) two-tailed, paired *t*-test. ** $p < 0.01$, * $p < 0.05$ when compared to N 0-5, LPS treated sample.

2. Material and Methods

2.1Bacterial Culture & Cloning

2.1.1 Media

Table 2.1: LB Media.

Medium	Ingredient	Concentration
LB Medium (with carbenicillin)	LB (Luria broth; Sigma-Aldrich, Cat# L-3522)	25g/l
	dH2O	-
	Carbenicillin (Sigma-Aldrich, Cat# C9231)	100µg/ml
	*LB medium was sterilized by autoclaving and stored in room temperature until usage. Carbenicillin was added when the medium was cooled down.	
LB Agar & plates (with carbenicillin)	Agar	15g/l
	LB (Luria broth; Sigma-Aldrich, Cat# L-3522)	25g/l
	dH_2O	-
	Carbenicillin (Sigma-Aldrich, Cat# C9231)	100µg/ml
	*The agar was sterilized by autoclaving and stored in room temperature. The agar was heated using microwave and cooled down to 56℃ in hot water bath before adding carbenicillin and pouring into plates.	

2.1.2PCR Amplification

Polymerase chain reaction (PCR) is a powerful genetic amplification technique that allows exponential amplification of short DNA sequences within double stranded DNA molecules with larger sizes. PCR reactions are initiated from two primers which are short pieces (usually around 20 bas pair) of synthetic DNA, and the primer set is complimentary to opposing ends of a targeted sequence. Each reaction can be divided into three segments: denaturation of the template DNA, primer annealing, and primer extension. Amplification of the targeting sequence on the template is achieved by cycles of reaction that elongate the primers according to the targeting sequence by DNA polymerase. Each cycle doubles the number of DNA copy, therefore the target sequence is amplified exponentially through the

cycles. However, the specificity of the reaction depends on the uniqueness of the priming sequence within the template DNA used in the reaction, and the control of the stringency of the condition which the primers are allowed to interact with target rather than non-target sequences (Saunders *et. al.*, 1999). Below are the primer sets chosen and reaction set-up to amplify different regions of the IL-10 promoter.

Table 2.2: PCR Cloning Primers.

5' -7kb IL-10	5'aatt ggcctaactggcc—agcagagtca aaggaatgag aa 3'
5' -4kb IL-10	5'aatt ggcctaactggcc—atttgcataa gcacacacac ac 3'
3' IL-10	5'aatt ggccgccgaggcc—ggttttgcaa gagcaagccc 3'
5' -622bp IL-10	5'aatt ggcctaactggcc—gga acacatcctg tgacc 3'
5' -860bp IL-10	5'aatt ggcctaactggcc—agttggcact ggtgtacc 3'
3' +150bp IL-10	5'aatt ggccgccgaggcc—gttaggc aggttgcctggg 3'

The sequence "ggcctaactggcc" is the *Sfi*I restriction site. The oligonucleotides were synthesized by Eurofins MWG|Operon. The sequence "aatt" is a random sequence added to both ends of the clones in order to facilitate restriction activity.

Table 2.3: PCR Reaction Setup for Cloning.

-4kb ~ -1bp IL-10		
Reagent	Volume	Concentration
REDAccuTaq® LA 10× Buffer	5.0µl	1×
dNTP (10mM)	2.5µl	0.5mM
Genomic DNA (50pg/µl)	1.0µl	50pg
5' -4kb IL-10 primer (20mM)	1.0µl	0.4mM
3' IL-10 primer (20mM)	1.0µl	0.4mM
Sterile dH_2O	38.5µl	-
REDAccuTaq® LA DNA Polymerase	1.0µl	0.02U/µl
Final Volume	50.0µl	-
-7kb ~ -1bp IL-10		
Reagent	Volume	Concentration
REDAccuTaq® LA 10× Buffer	5.0µl	1×
dNTP (10mM)	2.5µl	0.5mM
Genomic DNA (50pg/µl)	2.0µl	50pg
5' -7kb IL-10 primer	1.0µl	0.4mM
3' IL-10 primer	1.0µl	0.4mM
Sterile dH_2O	36.0µl	-
REDAccuTaq® LA DNA Polymerase	2.5µl	0.05U/µl
Final Volume	50.0µl	-

-622bp ~ +150bp IL-10		
Reagent	Volume	Concentration
REDAccuTaq® LA 10× Buffer	5.0µl	1×
dNTP (10mM)	2.5µl	0.5mM
Genomic DNA (50pg/µl)	1µl	50pg
5' -622bp IL-10 primer	1µl	0.4mM
3' +150bp IL-10 primer	1µl	0.4mM
Sterile dH_2O	37.0µl	-
REDAccuTaq® LA DNA Polymerase	2.5µl	0.05U/µl
Final Volume	50.0µl	-
-860bp ~ +150bp IL-10		
Reagent	Volume	Concentration
REDAccuTaq® LA 10× Buffer	5.0µl	1×
dNTP (01mM)	2.5µl	0.5mM
Genomic DNA (50pg/µl)	1µl	50pg
5' -860bp IL-10 primer	1µl	0.4mM
3' +150bp IL-10 primer	1µl	0.4mM
Sterile dH_2O	37.0µl	-
REDAccuTaq® LA DNA Polymerase	2.5µl	0.05U/µl
Final Volume	50.0µl	-

The reaction mix was prepared on ice. REDAccuTaq® was manufactured by Sigma-Aldrich (Cat# D4812-250UN); dNTP mix was prepared by mixing dATP (Promega, Cat# U120D), dCTP (Promega, Cat# U122D), dGTP (Promega, Cat# U121D), and dTTP (Promega, Cat# U123D) and diluted to appropriate concentration.

Table 2.4: PCR Machine (Progene, Techne, Cambridge) Setup for Amplification of IL-10 Promoter Regions Prior to Cloning

Cloning of -4kb ~ -1bp human IL-10 promoter			
Segment	Temperature	Time	Cycles
Initial denaturation	96℃	30sec	1
Denaturation	94℃	12sec	35
Annealing	62℃	30sec	
Extension	68℃	6min	
Final extension	68℃	20min	1
Hold	4℃	-	-
Cloning of -4kb ~ -1bp human IL-10 promoter			
Segment	Temperature	Time	Cycle
Initial denaturation	96℃	30sec	1
Denaturation	95℃	12sec	45
Annealing	61℃	30sec	
Extension	68℃	12min	
Final extension	68℃	25min	1
Hold	4℃	-	-

Cloning of -622bp ~ +150bp human IL-10 promoter			
Segment	Temperature	Time	Cycles
Initial denaturation	96℃	30sec	1
Denaturation	94℃	12sec	45
Annealing	60℃	30sec	
Extension	68℃	3min	
Final extension	68℃	10min	1
Hold	4℃	-	-
Cloning of -860bp ~ +150bp human IL-10 promoter			
Segment	Temperature	Time	Cycles
Initial denaturation	96°C	30sec	1
Denaturation	94℃	12sec	45
Annealing	60℃	30sec	
Extension	68℃	3min	
Final extension	68℃	10min	1
Hold	4℃	-	-

The IL-10 promoter regions from human genomic DNA were PCR amplified according to Table 2.4. The restriction site chosen was *Sfi*I, which can be recognized and cleaved by the *Sfi*I restriction enzyme isolated from an *E.Coli* strain that carries the *SfiI* gene from *Streptomyces fimbriatus* (New England BioLabs®;

http://www.neb.com/nebecomm/products_intl/productR0123.asp).

As shown in Figure 2.1B, Acc65I, KpnI, EcoICRI, SacI, NheI, XhoI, EcoRV, BglII, and HindIII restriction sites are added in the multiple cloning region in pGL4 Vector. Other than SfiI sites, most of other restriction sites can be found in the IL-10 promoter sequences. Besides, SfiI site allows single restriction in which opposite end will not self ligate to each other, and the orientation of the insert is promised.

5'...GGCCN NNN NGGCC...3'
3'...CCGGN NNN NCCGG...5'

As illustrated above, the GGCCNNNNNGGCC palindrome is recognized by *Sfi*I and cleavage occurs between the fourth and fifth N. The amplified promoter constructs were separated using gel electrophoresis (0.6% agarose gel; Sigma-Aldrich) in 1× TAE buffer; the agarose gel containing the required DNA fragment was excised and purified using the QIAquick Gel Extraction Kit (QIAGEN, Cat# 28704) according to the manufacturer's protocol.

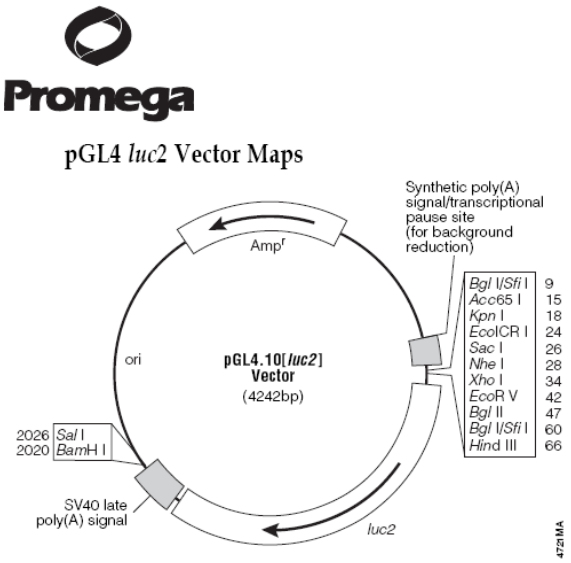
The protocol can be accessed from:

http://www1.qiagen.com/HB/QIAquickGelExtractionKit_EN

2.1.3 Cloning plasmid: pGL4.10(luc2) (pGL4 basic; Promega, Cat# E6651)

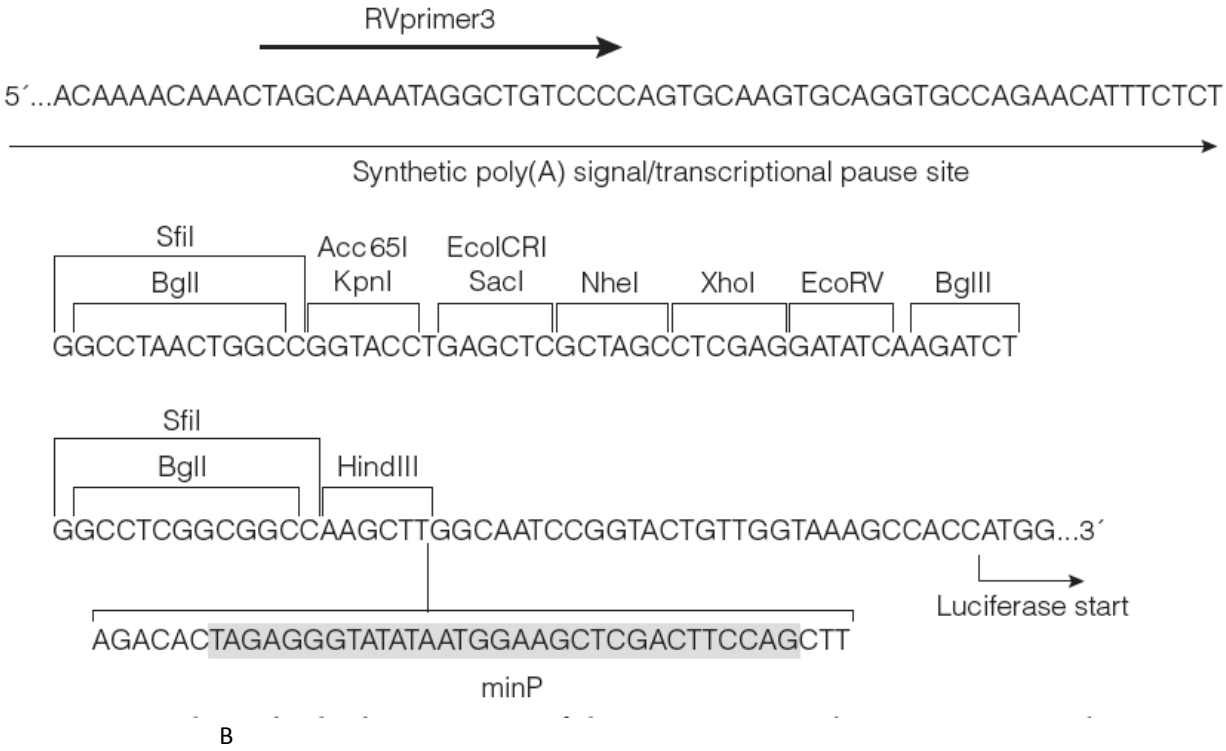
-1kb IL-10-pGL3 and a -4kb IL-10-pGL3 plasmids were kindly provided by Ziegler-Heitbrock. The -4kb IL-10-pGL3 construct was reported to contain mutations.

The major cloning vector chosen for this piece of research is pGL4, which is synthesized by Promega. In addition to the restriction sites found in the conventional reporter gene vector pGL3, BglI, SfiI and EcoRV restriction sites have been added to the pGL4 Vector multiple cloning region. This increases the number of choice in choosing restriction sites during cloning. The two BglI/SfiI restriction sites in pGL4 permit the moving the DNA of interest such as response elements, enhancers and promoters among the vector. Additionally, transfers between pGL4 Vectors by using either the BglI or SfiI restriction enzymes retains the desired orientation of the DNA of interest due to the unique DNA recognition properties of BglI and SfiI restriction sites (Promega).



Multiple cloning region	1–70
luc2 reporter gene	100–1752
SV40 late poly(A) signal	1787–2008
Reporter Vector primer 4 binding region	2076–2095
*Col*EI-derived plasmid replication origin	2333
Synthetic β-lactamase (Amp^r) coding region	3124–3984
Synthetic poly(A) signal/transcriptional pause site	4089–4242
Reporter Vector primer 3 binding region	4191–4210

A



B

Figure 2.1: A, pGL4.10 [luc2] Vector Map; B, pGL4 multiple restriction region.
Information on the vector can be obtained from Promega: http://www.promega.com/tbs/tm259/tm259.pdf

2.1.4Digestion & Ligation

The restriction sites in the pGL4 vector and IL-10 promoter clones were digested using *Sfi*I restriction enzyme (New England BioLabs®, Cat# R0123S) according to the manufacturer's instructions (Table 2.5) in 50℃ for 2 hours in a PCR machine with heated lid on to prevent changes in reaction volume caused by evaporation. Concentrations of the amplified IL-10 promoter PCR products were determined by comparing to known concentrations of DNA standards in ethidium bromide-stained agarose gels. The estimated DNA concentration was adjusted to 300ng/µl for each IL-10 promoter construct.

Table 2.5: *Sfi*I Digestion Reaction Setup

Reagent	DNA inserts				vector
	-4kb IL-10	-7kb IL-10	-622bp IL-10	-860bp IL-10	pGL4.10 (2.6µg/µl)
*Sfi*I Restriction Enzyme	0.75µl	0.75 µl	0.75 µl	0.75 µl	0.75 µl
DNA	25.95µl	25.95 µl	25.95 µl	25.95 µl	3.0 µl
10×*Sfi*I Buffer	3.0 µl	3.0 µl	3.0 µl	3.0 µl	3.0 µl
100× BSA	0.3 µl	0.3 µl	0.3 µl	0.3 µl	0.3 µl
Sterile dH_2O	-	-	-	-	22.95 µl
Total Volume	30.0 µl	30.0 µl	30.0 µl	30.0 µl	30.0 µl

Table 2.6: Ligation Reaction Setup

Reagent	Construct			
	-4kb IL-10	-7kb IL-10	-622bp IL-10	-860bp IL-10
T4 DNA Ligase	3.0 µl	4.0 µl	3.0 µl	3.0 µl
10× Reaction buffer	3.0 µl	4.0 µl	3.0 µl	3.0 µl
ATP	3.0 µl	4.0 µl	3.0 µl	3.0 µl
Digested pGL4	1.0 µl	1.0 µl	3.0 µl	3.0 µl
Digested IL-10 promoter	20.0 µl	27.0 µl	18.0 µl	18.0 µl
Total Volume	30.0 µl	30.0 µl	30.0 µl	30.0 µl

The digested DNA was run in 0.6% agarose gel and extracted with QIAquick Gel Extraction kit, and the concentration estimated by comparison with known standards in ethidium bromide-stained agarose gels. Purified DNA was cloned into pGL4.10 (luc2) vector using T4 DNA ligase (Stratagene, Cat# 600011; see Table 2.6for reaction mix), the reaction was incubated in 4°*C* overnight, and the DNA clones were stored in -20 *°C*.

2.1.5 Transformation

To transform the IL-10 clones, The DNA ligation was diluted 5× in 10mM Tris-HCl (pH7.5) and 1mM EDTA. Library Efficiency® DH5α™ competent cells (Invitrogen, Cat# 18263-012) were thawed on ice, and 100µl of the cells were aliquoted into 17mm tubes (Falcon® 2059). 1µL (1~10ng) of the diluted DNA and 5µl (50pg) of the control pUC19 DNA (Invitrogen, Cat# 18263-012) were added into each tube and gently shaken to mix. The cells were incubated on ice for 30 minutes. After 30 minutes incubation on ice, the cells were heat shocked at 42*°C* in water bath for 45 seconds, followed by 2-minute incubation on ice. 0.9ml room temperature S.O.C medium (Invitrogen, Cat# 18263-012) was added to each transformation, and the cells were incubated in 37*°C* with shaking at 225rpm for 1 hour. 100µl, 200µl, and 600µl of each transformation was spread to different LB agar plates containing 100µg/ml carbenicillin and incubated overnight at 37*°C*.

2.1.6 Small & Large Scale Plasmid Preparation

After transformation, bacterial colonies were picked and grew in 3.0ml LB medium containing 100µg/ml carbenicillin overnight. The cells were harvested, and plasmid DNA was extracted using QIAprep Spin miniprep kit (QIAGEN, Cat# 27104) according to manufacturer's protocol (available at: http://www1.qiagen.com/HB/QIAprepMiniprep

Kit EN). Random purified DNA samples from each clone were quantified with absorption spectroscopy (see section 2.1.7 for details) and confirmed by sequencing. Sequencing confirmed clones were amplified and purified using the EndoFree™ Plasmid Maxi Kit (QIAGEN, Cat# 12362) according to the manufacturer's protocol (http://www1.qiagen.com/HB/EndoFreeKit_EN). The extracted plasmid DNA is low in endotoxin contamination (<0.1 EU/µg plasmid DNA) and is suitable for transfection.

2.1.7 DNA Quantification and Purity

The concentration of deoxyribonucleic acid (DNA) is determined using absorption spectroscopy. Nucleic acids 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 the given solution.

Generally, DNA concentration can be determined with the equation: $c = OD_{260} \times \varepsilon \times d$

c = concentration of the nucleic acid in µg/ml; OD_{260} = absorbance reading at 260nm; ε = extinction coefficient, ε = 50µg/ml for dsDNA, ε = 40µg/ml for ssDNA and ε = 33µg/ml for RNA; d = dilution factor

DNA samples are sometimes contaminated with trace of protein. Proteins have a UV absorbance spectrum that is easily distinguished from DNA. They have a maximum absorption at 280nm. Protein contamination in the given sample can be determined by the ratio of the absorbance reading at 260nm (OD_{260}) and 280nm (OD_{280}). An uncontaminated sample of DNA will have a ratio of 1.8 to 2.0, and contamination with protein results in lower values (Saunders *et. al.,* 1999).

2.2Cell Culture and Treatment

Table 2.7: Media Used in Cell Cultures

Medium	Reagent	Concentration
Iscove's Dulbecco's Modified Medium	Iscove's Dulbecco's Modified Medium	-
	L-Glutamine	2mM
	Penicillin	200U/ml
	Streptomycin	200µg/ml
	Human AB Serum	2.5%
COMPLETE RPMI 1640 medium (MonoMac 6 Medium)	RPMI 1640 medium	-
	L-Glutamine	2mM
	Penicillin	200U/ml
	Streptomycin	200µg/ml
	Oxalacetate	1mM
	Pyruvate	1mM
	Insulin	9µg/ml
	Non-essential amino acids	1×
	Foetal Calf Serum	10%

2.2.1Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood mononuclear cells were isolated from blood obtained from healthy donors . Consent form with brief descriptions of the project was signed by all of the donors prior to the blood taking procedure. Blood samples (with 2µl of heparin (Natrium-25000-ratiopharm®) per 1ml added) was diluted 1:1 with Hank's Balanced Salt Solution (SIGMA-ALDRICH, Cat No. H6648). 30ml of the diluted blood was carefully layered into sterile 50ml Falcon tubes containing 15ml Ficoll-Paque™ Plus (GE healthcare, Cat# 17-1440-03), followed by 30 minutes centrifugation (Sorvall Legend™, 20057916) at 400g (400rcf) at room temperature. The PBMC layer was carefully transferred to a new tube using 3ml sterile Pasteur pipettes and washed twice with Hank's Balanced Salt Solution and a final wash with filtered Iscove's Modified Dulbecco's medium (SIGMA-ALDRICH, Cat# 13390) and spun down in room temperature at 400g for 5 minutes. Isolated cells were re-suspended in Iscove's Dulbecco's medium containing 2.5% human AB serum (SIGMA-

ALDRICH®, Cat# H4522, Lot 017K0443), Glutamine (SIGMA-ALDRICH®), and Penicillin/Streptomycin (Invitrogen, Cat# 15140-122; see Table 2.7 for detailed concentration).

Note: In some experiments such as transductions, filtered Complete RPMI1640 (see Table 2.7 for the ingredients and respective concentrations) containing 10% Fetal Bovine Serum (FCS; Biochrom AG, Cat# S0115) was used for the final wash, re-suspension, and cell culture. In the filtration (Schaefer *et. al.*, 2008), the medium was passed through a Gambro U-2000 ultrafiltration column (Gambro Medizintechnik GmbH, Planegg-Martinsried, Germany). The medium was filtered to eliminate potential contaminants such as endotoxin and other bacterial components that may activate the cells prior to our experiments. After filtration 10% (v/v) low endotoxin foetal calf serum was added (Biochrom; confirmed to contain <0.1EU/µl).

2.2.1a Determination of cell concentration

An aliquot of the cells was diluted 1:10 (*i.e*. dilution factor = 10) in the given medium. 10µl of the diluted cells was transferred to an improved Neubauer hemocytometer (cell counting chamber) with cover-slip, and 5 random squares in the 25 central squares (Figure 2.2, shaded squares with thick outline) were counted and multiplied by 5 (If cell number in the 25 squares was less than 50, all of the cells in the 25 squares were counted, and multiplication by 5 will not be necessary). The number of cells counted from the slide was then converted to the concentration of the cell. The calculation used is as follows:

$$X \text{ cells/ml} = n \times 5 \times 10^4 \times \text{dilution factor}$$

Where x is the concentration of the cell; n = cell count from the 5 random squares; 10^4 represents the conversion factor of volume of counting chamber into ml; the dilution factor in our cell preparation is 10

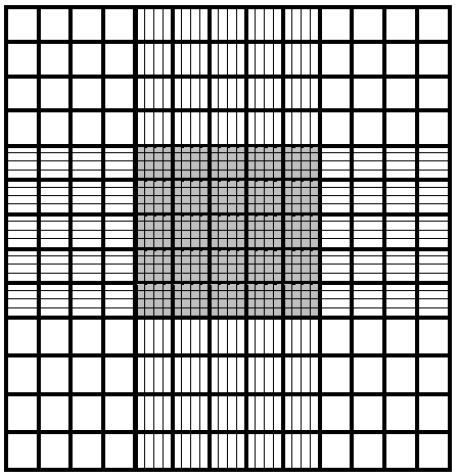


Figure 2.2: Neubauer hemocytometer.
Cells in 5 Random (within the 25 shaded squares in the centre) were counted and calculated to obtain the cell concentration according to the equation listed above.

2.2.1bPreparation of Total Peripheral Blood Mononuclear Cells for Further Cell Treatment

With final concentration 5×10^6 cells/ml, 0.5ml (or 0.25ml depending on the experiment performed) cells were seeded into 24-well Corning® Ultra Low Attachment Plates (Corning Life Science, Cat# 3473) and returned to 37℃ cell culture incubator equilibrated with 5% CO_2 gas (Kendro Laboratory, Hera Cell 150, Model 51013568) for either 3 days (for transduction), or 4 days (for transfection or endogenous gene experiments). Cell viability was observed through microscope.

2.2.1c Preparation of Adherent Monocytes Cell Culture

With final concentration 1 · 10^6 cells/ml, 2ml cells were seeded into 6-well plates (2 · 10^6 cells/well) and returned to 37℃. After 2 hours incubation, the media containing the

non-adherent cells were removed and replaced with 2ml of fresh media. The cells were then returned to 37℃ for 4 days before adenovirus transduction (see section 2.5).

2.2.2THP-1 Cells

2.2.2a Thawing cells

75cm^2 tissue culture flask and 50ml Falcon tube each containing 9ml Complete RPMI1640 medium containing 10% FCS (at room temperature) were prepared. The THP-1 cell stock was taken out from the liquid nitrogen and thawed slowly with gloved hand until completely thawed. The cells were carefully transferred to the falcon tube with 5ml pipette. The cryotube containing the cell stock was gently washed with the medium from the Falcon tube several times to take most of the cells out. The cells were centrifuged in room temperature for 5 minutes at 400g, and the supernatant was discarded. The cell pellet was resuspended with 1ml Complete RPMI1640 medium containing 10% FBS, transferred to a 75cm^2 tissue culture flask with vented cap containing 9ml medium, and incubated at 37℃ with 5% CO_2 for 4 days before being split.

2.2.2b Maintaining THP-1 Cell Culture

THP-1 cells are human acute monocytic leukemia cell line which tend to adhere loosely on the surface culture plate or flask (Tsuchiya *et al.*, 1980). They were cultured in 75cm^2 tissue culture flasks and were split once every 3-4 days to keep the cells healthy. When splitting the THP-1 cells, the flask was gently tapped to resuspend the cells. A small aliquot of the cells were taken out to determine the cell concentration. Adequate number of cells were transferred to fresh 10ml Complete RPMI1640 medium with 10% FCS in the 75cm^2

flask to make concentration 2.0×10^5 cells/ml. The flask was labeled with the date split and the final concentration and was returned to 37℃ incubator with 5% CO_2.

2.2.2c Splitting THP-1 cells for transfection:

THP-1 stock was taken out from the 37℃ incubator, observed under a microscope to make sure no contaminants such as bacteria were observed, resuspended by gentle taps, transferred to a 50ml Falcon tube, and centrifuge in room temperature for 5 minutes at 400g. The supernatant after centrifugation was discarded; the cells were re-suspended with 1ml Complete RPMI1640 medium containing 10% FCS, and the cell concentration was determined. The cells were diluted with adequate Complete RPMI1640 medium with 10% FCS to make concentration 10^6 cells/ml. 2ml of the diluted THP-1 cells were seeded into each well in normal 6 well plates.

2.2.3 Inductions

For endogenous IL-10 stimulation, the cells were prepared as in section 2.2.1 or 2.2.2, treated with either nothing or HIF-1 inducer (as in section 2.2.3c) before normoxia or hypoxia incubation as in section 2.3 and were treated with the given stimuli for 4 hours in either normoxia or hypoxia.

For inductions on cells transfected or transduced with DNA constructs, the cells were stimulated with the given stimuli for a further 6 hours (for transduction)or 24 hours (for transfection) in either hypoxia or normoxia after transfection or transduction before being harvested for further analysis.

2.2.3a Lipopolysaccharide (LPS)

LPS is one of the most commonly used stimuli to activate monocytes. The LPS primarily used for our cell stimulation was isolated and purified from *Salmonella abortus equi* (SAE LPS, ALEXIS Biochemicals). Laboratory SAE LPS stock was prepared by diluting the manufacturer stock with RPMI1640 mediums to a final concentration of 10ng/μl. In LPS treatments, purified LPS was added to the cell culture. The cells were returned to the desired condition (normoxia or hypoxia, as described in section 2.3) for 4 hours (for endogenous gene experiments), 6 hours (for adenovirus transductions) or 24 hours (for transfections) before harvested.

2.2.3b Toll-like Receptor (TLR) Ligands

Pam_3CSK_4 triacylated lipopeptide (ALEXIS® Biochemicals, Cat# APO-54N-030-KI01) was used to stimulate the cells via the TLR1/2 heterodimer. In addition to SAE LPS, we also used purified *E. Coli* LPS (ALEXIS® Biochemicals, Cat# APO-54N-030-KI01) to activate the cells via TLR4. Flagellin isolated and purified from *S. Typhimurim* was the TLR5 ligand used for cell stimulation. MALP-2 (Macrophage stimulatory lipopeptide 2; ALEXIS® Biochemicals, Cat# APO-54N-030-KI01) was used to stimulate via TLR6. The TLR7 ligand used was imiquimod (R-837;ALEXIS® Biochemicals, Cat# APO-54N-030-KI01), an imidazoquinoline molecule. Resiquimod (R-848; ALEXIS® Biochemicals, Cat# APO-54N-030-KI01) was used to stimulate via the TLR7/8heterodimer.

Optimization of activation of TLR3 (with polyinosinic-polycytidylic acid; ALEXIS® Biochemicals, Cat# APO-54N-030-KI01) and TLR9 (with oligodeoxynucleotides CpG; ALEXIS® Biochemicals, Cat# APO-54N-030-KI01) was attempted using human primary macrophages, but the ligands failed to activate the cell (TNF level did not increase after

treatment). Both the suggested and optimized quantity of each TLR ligands added for stimulation is listed in Table 3.1 (personal communication, Kounnis, University of Leicester, MSc thesis 2008)

TLR stimulation experiments were aiming at stimulation of endogenous genes. Adequate ligands were added to the cell culture, and the cells were returned to the destined condition (normoxia or hypoxia, as in section 2.3)

2.2.3c HIF-1 inducers

Cobalt chloride ($CoCl_2$)

The manufacturer's$CoCl_2$ (SIGMA-ALDRICH®) stock was diluted in sterile 1×PBS(137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 2mM KH_2PO_4) to make laboratory stock with concentration of 30mM.To produce a final concentration of 300μM $CoCl_2$, the 30mM lab stock was diluted 1:100 (e.g. 5μl 30mM $CoCl_2$ stock in 0.5ml cell culture medium).

Desferrioxamine (DFO)

The manufacturer's DFO (SIGMA-ALDRICH®) stock was diluted in 1×PBS to generate laboratory stock with a concentration of 20mM. In 200μM DFO treatment, the DFO lab stock was diluted 1:100 (e.g. 5μl 20mM DFO stock in 0.5ml cell culture).

2.3Normoxia & Hypoxia Incubation

Cells were either stayed in the normoxia (normal) incubator (37℃, 5% CO_2) or transferred to hypoxia incubator equilibrated with 5.0% CO_2 and 0.2% O_2 (nitrogen gas was used to eliminate excessive O_2 until the incubator reached equilibrium; RS Biotech, Galaxy CO_2 Incubator) on either day 3 or day 4 for 24 hours depending on the experiment carried out.

2.4Transfection

2.4.1Transfecting PBMC with JetPEI™

The protocol in this section was derived from the manufacturer's protocol which can be accessed from:

http://www.polyplus-transfection.com/sysmodules/RBS_fichier/admin/download.php?fileid=1381

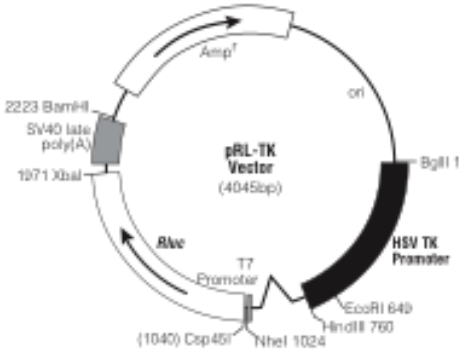


Figure 2.3: pRL-TK Vector (Promega).
The vector contains Renilla gene which encodes Renilla luciferase that can be used to normalize the firefly luciferase activity from the targeting constructs.

The ratio we used was 1µl DNA:8µl JetPEI™. PBMC was seeded in 6-well Corning® Ultra Low Attachment plates and incubated in normoxia, 37℃ with 5% CO_2 for 4 days before transfections. For each transfection, two eppendorfs were prepared. 100µl of 150mM NaCl (PolyPlus Transfection), 3.0µg IL-10 or control plasmids and 150ng pRL-TK (Promega) Renilla plasmid was added to tube A and mixed by vortex for 15 seconds. Another 100µl of 150mM NaCl and 9.6µL JetPEI™ (PolyPlus Transfection, Cat# 101-40N) were added to tube B and mixed by vortex for 15 seconds. The NaCl-JetPEI™ mixture from tube B was transferred to tube A and was mixed thoroughly by vortex. The NaCl-plasmid DNA-JetPEI™

mixture was incubated in room temperature for 30 minutes, and 200µl of the mixture was added dropwise to cells in each well followed by gentle swirl. The cells were returned to 37℃ with 5% CO_2 for 1 hour and were either transferred to hypoxia or stayed in normoxia for 24 hours as described in section 2.3. After 24-hour incubation in either normoxia or hypoxia, SAE LPS was added to the designate cell sample for further 24-hour stimulation followed by harvest for dual luciferase assay (see section 2.6.1b).

2.4.2Transfecting THP-1 cells with FuGENE®6 Transfection:

The protocol in this section was derived from the manufacturer's protocol with some modifications which can be accessed from:

https://www.roche-applied-science.com/pack-insert/1815091a.pdf

The DNA:FuGENE®6 (Roche, Cat# 11 814 443 001) ratio used in the experiments was 1:3. To prepare the transfection, 6µl FuGENE®6 was added to each eppendorf containing 194µl serum free medium (SFM) without contacting the wall of the eppendorf. The SFM-FuGENE®6 was mixed by vortex for 15 seconds, and incubated in room temperature for 5 minutes. 2µg plasmid DNA and 150ng pRL-TK (Promega) Renilla plasmid were added into each tube and vortex for 15 seconds. The SFM-FuGENE®6-Plasmid DNA mixtures were incubated in room temperature for 20 minutes. Each mixture was added to the cells, and the cells were returned to 37℃ with 5% CO_2 for 1 hour. After 1 hour incubation in normoxia, the cells were transferred to designated condition, either returned to normoxia or transferred to hypoxia for 24-hour incubation as described in section 2.3. 20µl of LPS was added to each well (final concentration = 100ng/ml) for 24 hours before harvesting for luciferase assay (see section 2.6.1b).

2.5 Adenovirus Transduction

Each adenovirus stock was prepared in 1×PBS with concentration of 10 plaque forming unit per 1µl (10pfu/µl). PBMC were prepared in Complete RPMI1640 containing 10% FCS as described in section 2.2.1; 2ml cells with concentration 10^6 cells/ml were seeded into 6-well Corning® Ultra Low Attachment Plates (LA plates) and incubated in 37℃, 5% CO_2. After 3 days incubation, the cells were resuspended by pipetting vigorously (some monocytes did loosely adhere to low attachment plates) and transferred to 15ml Falcon tubes. Cells were span down at 400g for 5 minutes, and supernatant was discarded. The cells were washed with 5ml Complete RPMI1640 medium (serum free), span down, and resuspended with 1ml Complete RPMI1640 medium for counting. The cells were diluted with Complete RPMI1640 medium (serum free) to final density 5×10^5 cells/ml and 1ml of the diluted cells (5×10^5 cells) were reseeded into new 6-well LA plates; each well is adequate for one transduction. Sufficient adenovirus was added to the cells (e.g. for multiplicity of infection (moi) = 10, 5µl of the AdV stock, or 5×10^7 pfu was added to each well containing 5×10^5 cells) carefully, and the cells were swirled to allow the virus to spread evenly to the cells and were returned to 37℃ with 5% CO_2. After 2 hours incubation, 55µl of FCS was added to each well of cells. The cells from each well were then equally split into two wells in a new 24-well Costar® Ultra Low Attachment Plate (24-well LA plate), and were either returned to normoxia or transferred into hypoxia as appropriate for 24 hours. 100ng/ml SAE LPS was added to appropriate wells for stimulation. After 6 hours incubation with or without SAE LPS, the cells were transferred to eppendorfs, span down in 400g for 5 minutes. The supernatant was removed, and the cells were gently washed

with 1ml of filtered 1× cell culture grade PBS and span down. Supernatant was removed, and cells were harvested for luciferase assay.

2.5.1 Transduction & HIF-1 Induction

To see the effect of the HIF-1 inducer on the expression of IL-10 AdV constructs, the cells were prepared and transduced as described in section 2.5, treated with either nothing, $CoCl_2$, or DFO (as in section 2.2.3c) before transferring into hypoxia for 24-hour incubation. After 24-hour incubation in hypoxia, the cells were stimulated with SAE LPS for an additional 24 hours in either normoxia or hypoxia. The cells were harvested for luciferase assay (see section 2.6.1a).

2.6 Quantification of the Reporter or Endogenous Gene

2.6.1a (Single) Luciferase Assay

Luciferase assay is widely used to detect promoter activity. In our experiment, IL-10 promoter regions were cloned into pGL4.10 reporter plasmid. After transfected into cells, the cellular machinery will be utilized to produce modified firefly luciferase protein according to the promoter (IL-10) activity. As shown in the following diagram, luciferase protein catalyzes the reaction that breaks beetle luciferin down and produce light that can be detected as relative light unit per second (RLU/s) with luminometer.

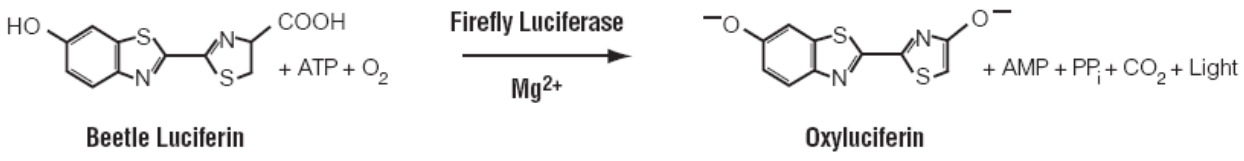


Figure 2.4: Firefly Luciferase Reaction (Promega).

The promoter activity is proportional to the number of copies of the luciferase protein (reporter), and the number of copies of the reporter protein will be reflected in the relative light units emitted in the luciferase assay.

After transduction (as described in section 2.5) and 24-hour stimulation with LPS, the cells were harvested with 1× Lysis buffer (5× Lysis buffer was diluted 1:5 with sterile dH_2O, Promega, Cat# 1500), and the cell lysates were incubated at -80°*C* for 30minutes followed by thawing in room temperature. Tubes containing 100µl luciferase assay reagent (LAR; Promega, Cat# 1500) were measured as background in the luminometer (Berthold Technologies, Lumat LB9501). The luminometer measured the emitted light units for 30 seconds and converted the collected light units into RLU/s. 20µl of the room-temperature cell lysates were added to LAR and mixed gently and followed by measurement in the luminometer. The protocol is modified from the manufacturer's protocol available online at: http://www.promega.com/tbs/tb281/tb281.pdf

2.6.1b Dual-Luciferase® Reporter Assay

IL-10 transfection was accompanied by co-transfection with Renilla plasmid (pRL-TK). While firefly luciferase is cloned and derived from the firefly(*Photinus pyralis*), Renilla luciferase is cloned from the sea pansy(*Renilla reniformis*). As described below, Renilla luciferase catalyzes the oxidative decarboxylation of coelenterazine to produce coelenteramide and light. In the Dual-luciferase® Reporter Assay System (Promega, Cat# E1910), Renilla luciferase serves as an internal control that can be used for normalization.

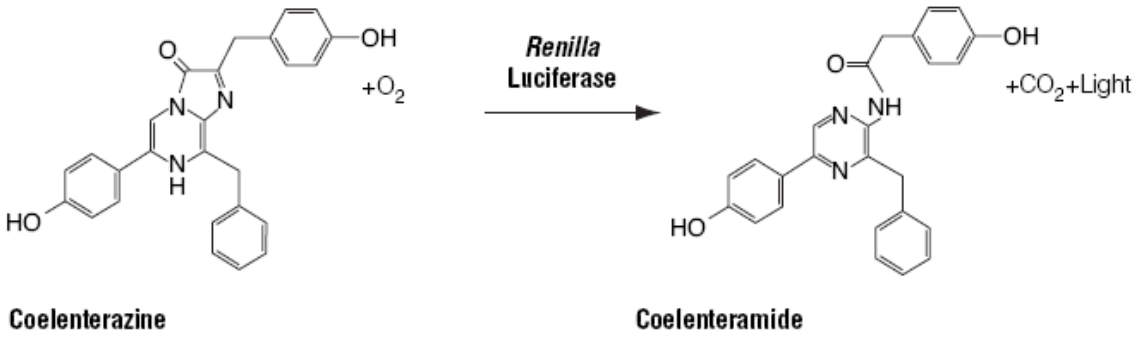


Figure 2.5: Renilla Luciferase Reaction (Promega).

Similar to single luciferase assay, cells were lysed in 1× Lysis buffer (Promega, Cat# E1910) after transfection as described in section 2.4.1, and after freezing and thawing, firefly luciferase reporter is measured and quantified first by adding 20µl of the cell lysates into100µl Luciferase Assay Reagent II (LAR II, Cat# E1910) to generate a "glow-type" luminescent signal. The reaction is later quenched, and the Renilla luciferase reaction is initiated simultaneously, by adding Stop & Glo® Reagent (Cat# E1910) to the same tube. The protocol is modified from the manufacturer's manual, accessible from: http://www.promega.com/tbs/tm040/tm040.pdf

2.6.2a Total RNA Isolation

One of the most common ways to determine the expression level of genes is to quantify the mRNA level of the gene of interest. To quantify the given gene, total RNA needs to be isolated. After 4-hour stimulation as described in section 2.2.3aor2.2.3b, the cells were transferred into a 0.6ml eppendorf, spun down in room temperature at 400g for 5 minutes, supernatant discarded, and lysed in 250µl TRI Reagent® (Sigma-Aldrich, Cat# T9424).

50µl chloroform (Sigma-Aldrich, Cat# C2432) was added to the lysates, thoroughly mixed by vortex, and centrifuged in 12000g at 4°*C* for 15minutes. Aqueous layer was transferred to fresh 0.6ml eppendorf, mixed with 125µl isopropanol, and the RNA pellet was achieved by centrifuge in 13,000g at 4°*C* for 5 minutes. Supernatant was removed, and the RNA pellet was washed with 500µl 70% ethanol and spun down at 12,000g in 4°*C* for 5 minutes. Ethanol was discarded and RNA pellet was air-dried and resuspended with 20µl diethylpyrocarbonate (DEPC)-treated water. DEPC-treated dH_2O was treated with 0.1% DEPC v/v and autoclaved prior to use. Purified RNA was stored at -20°*C* for later usage. The protocol was derived from the manufacturer's product guide.

2.6.2b Reverse Transcription

Isolated total RNA was converted to cDNA by reverse transcription.

Table 2.8: Reverse Transcription Reaction Mix

Reagent	Volume	Final Concentration
Hexanucleotides (0.2µg/µl)	1.0 µl	10ρg/µl
10mM dNTP mix	2.0 µl	1mM
DEPC-treated H_2O	2.4 µl	-
5× AMV Buffer	4.0 µl	1×
AMV Reverse Transcriptase (10U/µl)	0.8 µl	0.4U/µl
RNasin® Ribonuclease Inhibitor(40U/µl)	0.8 µl	1.6U/µl

The reaction mix was prepared on ice. Hexanucleotides was synthesized by Eurofins MWG|Operon; dNTP was prepared as described previously in Table 2.3 from section 2.1.2; AMV RT Enzyme was manufactured by Promega (Cat# M510A); RNasin® Ribonuclease Inhibitor was manufactured by Promega (Cat# N251B).

Isolated RNA was pre-heated in 70°*C* in PCR machine (Techne Cambridge, Progene PCR machine) for 5 minutes before the reaction mix was added. The reaction was incubated in 42°*C* for 60 minutes, and the reaction was heated up in 90°*C* for 4 minutes to inactivate the enzymes. The reaction was diluted 1:4 with sterile dH_2O and stored in -20°*C*.

2.6.2c Quantitative PCR (Real-time PCR)

The instrument used for the analysis was LightCycler® (Roche) with LightCycler® version 3.5 software, in which the samples were loaded in glass capillaries with large surface area when compared to the sample holders used in other real-time PCR instrumentation. The reaction mix and set up was prepared according to the following tables. SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, Cat# S1816) was used.

Table 2.9: Primers for Quantitative PCR

IL-10	5'Forward	5'gcctaacatgcttcgagatct3'
	3' Reverse	5'cggccttgctcttgttttcac3'
β_2M	5'Forward	5'ggctatccagcgtactccaaag3'
	3' Reverse	5'caacttcaatgtcggatggatg3'
TNF	5'Forward	5'cagagggaagagttccccag3'
	3' Reverse	5'ccttggtctggtaggagacg3'

Table 2.10: Quantitative PCR Reaction Mix.

Reagent	Volume Added	Final Concentration
2×SYBR® Green JumpStart™ Taq ReadyMix™	10µl	1×
5' Primer (5µM)	2µl	0.5µM
3' Primer (5µM)	2µl	0.5µM
cDNA	3µl	-
Sterile dH_2O	3µl	-

Table 2.11: Quantitative PCR Reaction Parameters.

Quantifying IL-10 mRNA			
Segment	Temperature	Time	Cycles
Initial denaturation	95℃	30sec	1
Denaturation	96℃	10sec	45
Annealing	62℃	10sec	
Extension	74℃	35sec	
Quantifying β2M mRNA			
Segment	Temperature	Time	Cycles
Initial denaturation	94℃	30sec	1
Denaturation	94℃	10	45
Annealing	60℃	10	
Extension	72℃	25sec	
Quantifying TNF mRNA			
Segment	Temperature	Time	Cycles
Initial denaturation	94℃	30sec	1
Denaturation	94℃	10	45
Annealing	60℃	10	
Extension	72℃	25sec	

Primers used for real-time PCR. The primers are synthesized by MWG Biotech|AG.

Beta-2 Microglobulin (β_2M) is a 12kDa polypeptide found in serum. It is associated with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells (Gussow *et. al.*, 1987). It was used as a housekeeping gene whose expression does not alter with the activation of the host cell and is commonly used as an internal control in a variety of experiments (Lee *et. al.*, 2007). β_2M is used to normalize the gene of interest in this project. As mentioned earlier, the SYBR® green system chosen was SYBR® Green JumpStart™ Taq ReadyMix™ which contains 20mM Tris-HCl, 100mM KCl,

7mM $MgCl_2$, 0.4mM for each dNTP, stabilizers, Taq DNA polymerase, JumpStart Taq antibody and SYBR® Green I with pH8.3. To obtain relative concentration, four standards with serial dilution of 1:5 were prepared and were run in parallel with the samples, and the standard curve were plotted by the software to calculate the relative concentration for each sample. The specificity and purity of the quantified samples was confirmed by examining the melting curve, with continuous acquisition of the fluorescent signal at intervals of less than $1°C$ from $65°C$ to $96°C$.

2.7 Software

2.7.1 Bioinformatics

The promoter, gene, and mRNA sequences were obtained from the NCBI (National Center for Biotechnology Information National Library of Medicine National Institutes of Health)website (available at: http://www.ncbi.nlm.nih.gov/sites/entrez; accession: NT_167186 REGION: 459760..463559 GPS_000125226).

The design of the primer was carried out with Primer3 web-based software (available at: http://frodo.wi.mit.edu/primer3/input.htm) and BLAST (Basic Local Alignment Search Tool:http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=blast2seq) with input of the desired promoter sequence fragment with other parameter unchanged.

Prediction and assessment of the transcription factors on IL-10 promoters were carried out using MatInspector (Genomatix Software GmbH, Germany).Detailed information can be retrieved from:

http://www.genomatix.de/online_help/help_matinspector/matrix_help.html?s=c95b08194b10ef495c6f23d0f70aa702#ci). Users are required to register the software for trials. The predicted binding site and the associated promoter binding proteins can be obtained by inputting the promoter sequence followed by selection of the correct species.

2.7.2 Statistics

Statistical analysis was done using the statistical software GraphPad Prism 5 (demo version; GraphPad software, available at: http://www.graphpad.com/demos/). Student's two-tailed, paired t-test was selected for the analysis after inputting the data. P-value was calculated for significance level. The results were considered statistically significant when $p<0.05$ (statistically significant refers to a result that is unlikely to have occurred by chance).

3. Results

3.1Hypoxia suppresses the induction of the endogenous IL-10 gene by LPS

Aiming at characterizing the effect of 24-h hypoxia on IL-10, we firstly confirmed Staples' previous results (Bernard Burke, personal communication) by repeating the experiment. 5 · 10^6 PBMC (peripheral blood mononuclear cells) were seeded into 1ml Iscove's Dulbecco's medium with 2.5% human AB serum in 6-well LA plates and were incubated in normoxia or hypoxia for 24 hours on day 4 post preparation followed by 4-hour LPS treatment. Total RNA was then extracted, reverse transcribed, and IL-10 mRNA quantified. With the given condition, however, we found it difficult to optimize the LPS induction after several trials. The result showed in Figure 3.1 is one of the results obtained in the optimizations trials.

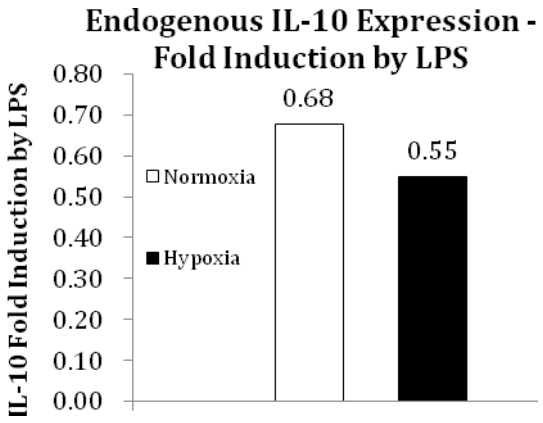


Figure 3.1: Endogenous IL-10 Expression in PBMC in Normoxia and Hypoxia.
The white bar is the normoxia sample whereas the black bar is the hypoxia sample. Fold induction is relative to the normoxic, untreated sample.

After several trials, the experiment was optimized with some modifications to the culture conditions (e.g. shifted to 24-well LA (low attachment) plates). 2.5×10^6 PBMC were incubated in 0.5ml Iscove's Dulbecco's medium with 2.5% AB serum in normoxic conditions ($O_2 \approx 20\%$) for four days before being transferred into hypoxic conditions for a further 24 hours. The cells were then treated with 100ng/ml LPS. In normoxic conditions, when PBMC were treated with 100ng/ml LPS (Figure 3.2A), the expression of endogenous IL-10 was increased by an average of 6.53-fold (n=8). With half the number of the cells (i.e. 250µl medium/well; 1.25×10^6 cells/well), stimulation with 200ng/ml LPS (Figure 3.2B), the IL-10 induction was increased to an average of 9.07-fold (n=5). Yet, when the cells were incubated in hypoxia ($O_2 = 0.2\%$) for 24 hours, the induction stimulated by LPS, was inhibited in both (LPS-treated and non LPS-treated) conditions. Moreover, not only the LPS induced IL-10 production was blocked, the basal mRNA level of IL-10 was also decreased in hypoxia.

Despite the finding that IL-10 was inducible in hypoxia, the induced level was much lower than in normoxic samples. 100ng/ml LPS treatment yielded 2.93fold IL-10 induction in hypoxia, whereas 200ng/ml LPS treatment yielded 3.25 fold IL-10 induction. In contrast, 100ng/ml LPS treatment stimulated 6.53FI of IL-10 expression, and 200ng/ml LPS treatment induced 9.07 fold IL-10 expression in normoxia. The results from both experiment sets confirmed the finding that hypoxia diminishes the IL-10 expression; IL-10 production with or without stimulation greatly reduced in hypoxia.

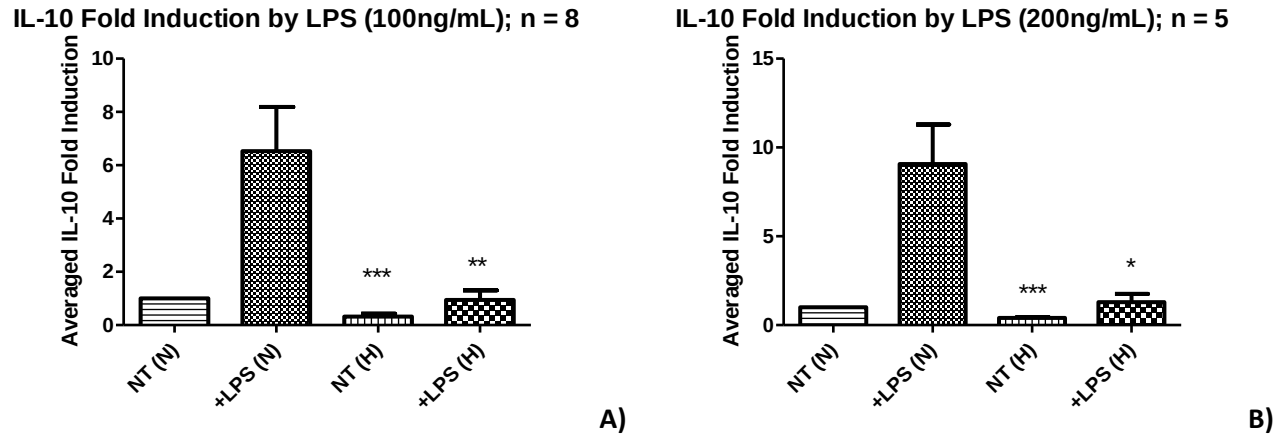


Figure 3.2: IL-10 Induction in PBMC Treated with LPS.

A, PBMCs were prepared, stimulated and harvested as described in sections 2.2.1, 2.2.3a and 2.6.2a. Total RNA was prepared as in 2.6.2b, and IL-10 and β2M mRNA levels were determined with real-time PCR as described in section 2.6.2c. The IL-10 level was normalized by β2M. Fold induction was calculated by dividing the normalized IL-10 level from each sample by the non-treated normoxia control (NT (N)). In normoxia, IL-10 expression increased 6.53 fold (+LPS (N)). In hypoxia, the non-treated NT (H) sample showed 0.32 fold induction of IL-10 relative to NT (N) (i.e. Decreased from FI=1 to FI=0.32 or a 68% reduction;), whereas LPS induction failed to induce IL-10 mRNA and gave 0.94 fold IL-10 expression. On the other hand, addition of 100ng/ml LPS induced 2.94fold IL-10 expression in hypoxia (when compare +LPS(H) with NT (H)). The figure summarized 8 independent experiments with means ± SEM (Standard error of the mean). The data were further analyzed using Student's two tailed, paired t-test; ***p value<0.001 when compared to the NT (N); **p-value<0.01 when compared to +LPS (N) sample. B, the volume and amount of PBMC were reduced to 0.25ml medium with 1.25 · 106 cells while the concentration of the cells stayed the same. An additional 100μL of fresh medium was added on day 4. The +LPS (N) and +LPS (H) samples were treated with LPS with final concentration of 200ng/ml for 4hr after 5-day incubations. Other conditions stayed the same as the experiments in A. In normoxia, IL-10 mRNA increased by 9.07 fold after LPS treatment (+LPS (N)). For the hypoxia sample (H), fold induction of the non-LPS treated sample is 0.40, and the LPS treated sample is 1.30 when compared with NT(N). The figure is the summary with mean ± SEM from 5 independent experiments. Data were further analyzed using Student's two-tailed, paired t-test; ***p value<0.001 when compared to the NT (N); **p-value<0.01 when compared to +LPS (N) sample.

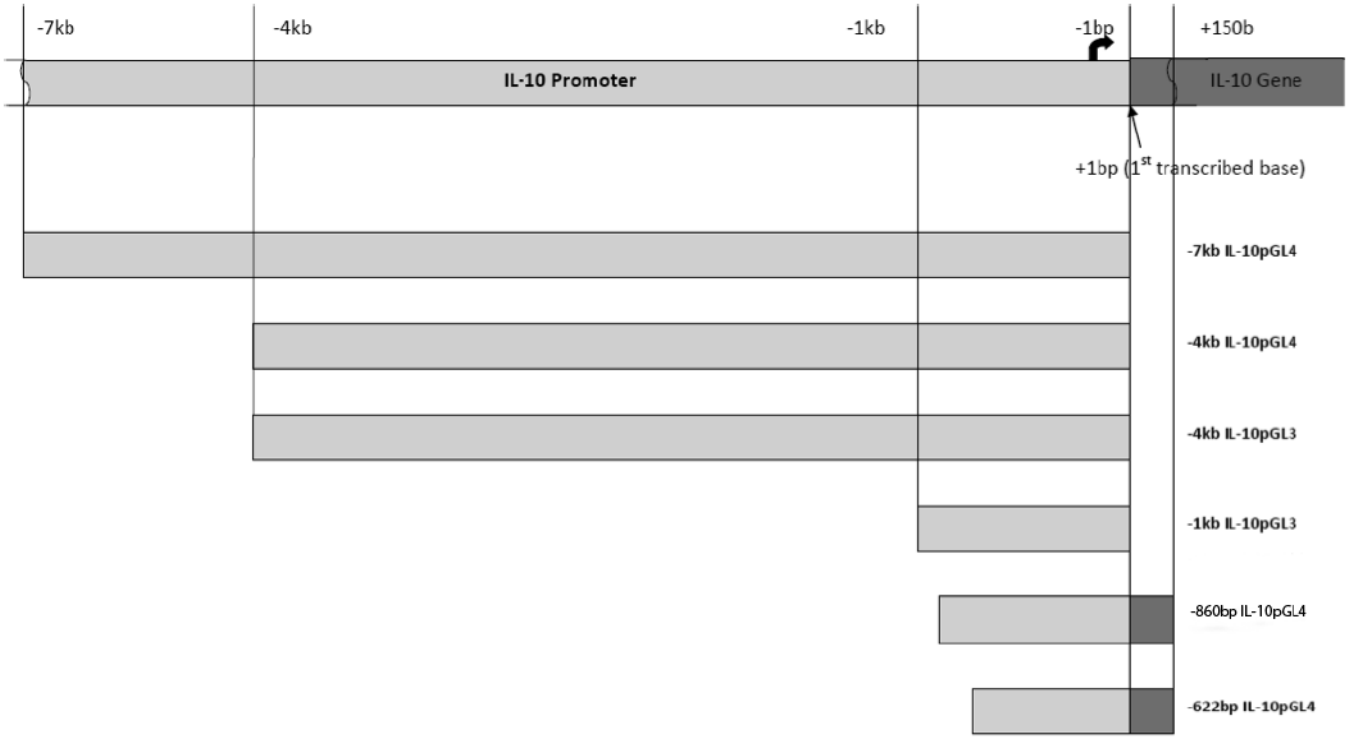
3.2Dissecting the human IL-10 promoter

IL-10 is regulated by several transcription factors, but the transcription elements responsible for the regulation of IL-10 in hypoxia is not known. For this reason, we aimed to characterize IL-10 promoter. By sequentially analysing segments of the IL-10 promoter,

it may be possible to deduce which part of the promoter sequence contains the hypoxia-sensitive element and what the element is.

3.2.1Cloning of IL-10 promoter segments

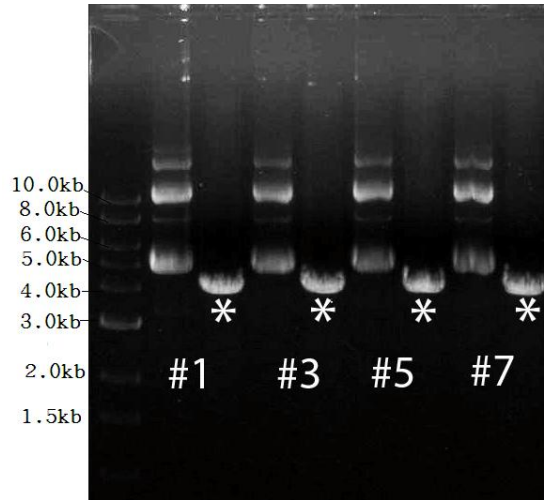
To determine which IL-10 promoter elements are required for the negative regulation in hypoxia, we wanted to test different segments of the IL-10 promoter region for their ability to drive a luciferase reporter construct.



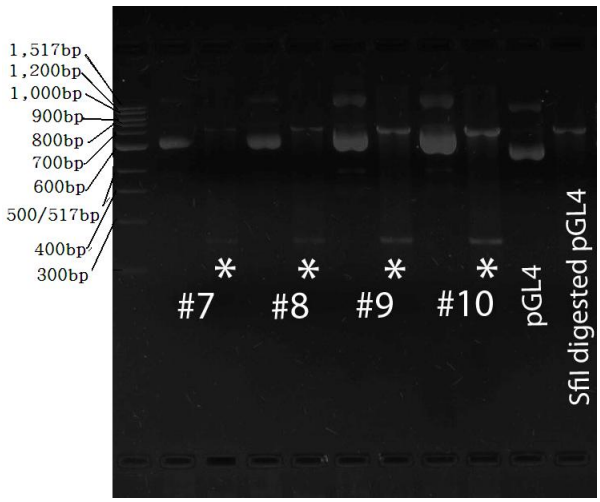
A

Figure 3.3A: IL-10 Constructs

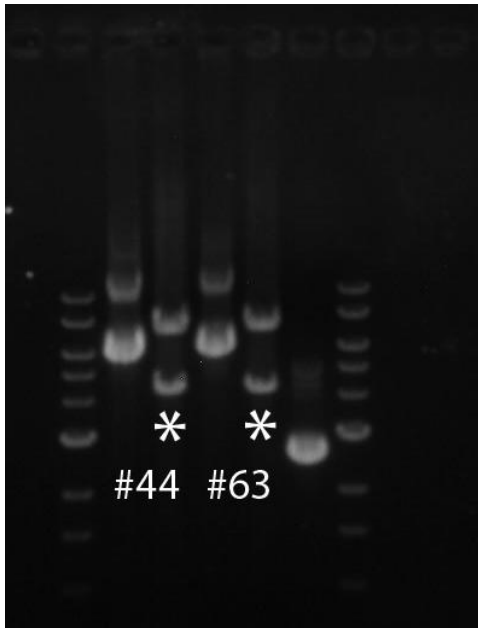
A, Relative sizes and positions of the IL-10 Constructs; the pale-gray portion of each construct signifies the IL-10 promoter region, and the dark-gray portion of the constructs refers to the IL-10 coding region. The -1kb IL-10-pGL3 and -4kb IL-10-pGL3 constructs were provided by Ziegler-Heitbrock *et. al.* The -622bp and -860bp IL-10pGL4 constructs contain 150 additional nucleotides. The 3' end of the rest of the constructs end at the -1base position.



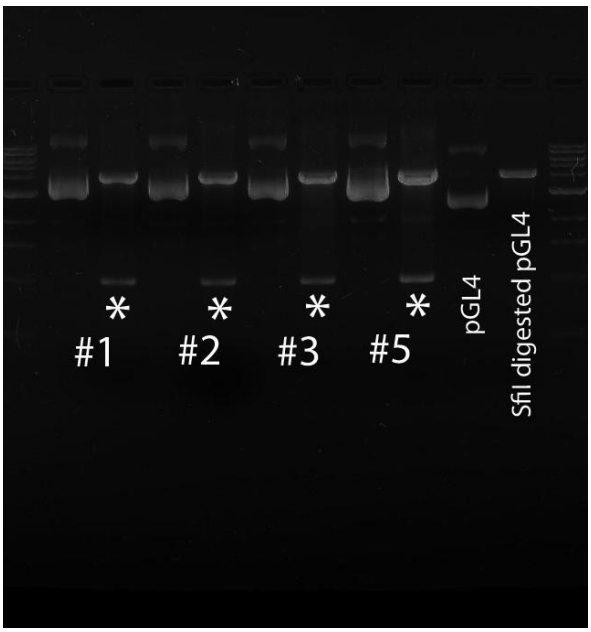
B



D



C



E

Figure 3.3 B ~ E

B to E, IL-10 clones, panels with asterisk are *SfiI* digested IL-10 recombinants. B, -4kb IL-10pGL4 clones; from left to right: 1kb DNA ladder (BioLabs, Cat#N3232S; from top to bottom: 10.0kb, 8.0kb, 6.0kb, 5.0kb, 4.0kb, 3.0kb, 2.0kb, 1.5kb, 1.0kb), undigested and *SfiI* digested -4kb IL-10pGL4 clones #1, #3, #5 and #7. C, -7kb IL-10pGL4 clones; from left to right: 1kb DNA ladder, undigested and *SfiI* digested -7kb IL-10pGL4 clones #44 and #63, undigested pGL4 plasmid (4.2kb), and -1kb IL-10 ladder. D, -622bp IL-10pGL4 clones; from left to right: 100bp DNA ladder (BioLabs, Cat#N3231S; from top to bottom 1,517bp, 1,200bp, 1,000bp, 900bp, 800bp, 700bp, 600bp, 500/517bp, 400bp, 300bp, 200bp, 100bp), undigested and *SfiI* digested -622bp IL-10pGL4 clones #7, #8, #9, #10, undigested pGL4, and *SfiI* digested pGL4. E, -860bp IL-10pGL4 clones; from left to right: 100bp DNA ladder, undigested and digested -622bp IL-10pGL4 clones #1, #2, #3, #5, undigested pGL4, *SfiI* digested pGL4, and 1kb DNA ladder.

We focused on several different IL-10 constructs shown in Figure 3.3A, including -1kb and -4kb IL-10 pGL3 constructs provided by Ziegler-Heitbrock *et. al.* However, several mutations were found in the -4kb IL-10-pGL3 construct. We therefore decided to make a new clone of the -4kb IL-10 construct, and also a longer -7Kb construct.

As mentioned earlier in the Material and Method section, the rest of the constructs were cloned into the pGL4 reporter vector via the *SfiI* restriction site. (i.e. -622bp IL-10pGL4, -830bp IL-10pGL4, -4kb IL-10pGL4, and -7kb IL-10pGL4). When cloning -4kb IL-10pGL4, 9 different clones (clones #1 ~ #9) were screened in 0.6% agarose gel for correct size. All of the clones showed positive results; clones #1, #3, #5, #7 were SfiI digested and run in 0.6% agarose gel to examine correct insert size (Figure 3.3B) followed by sequencing. Sixty three -7kb IL-10pGL4 clones (clones #1 ~ #63) were picked for screening on 0.6% agarose gel, only 3 clones showed correct size. -7kb IL-10pGL4 clone #26, #44 and #63 were then SfiI digested to examine the insert size (Figure 3.3C) followed by sequencing. Similarly, -622bp IL-10pGL4 clones #7 ~ #10, -830bp IL-10pGL4 clones #1 ~ #4 were screened for insert and sequenced for correct sequence.

The -4kb-pGL4 construct contains base pairs from -4kb to -1bp position, and the -7kb construct contains base pairs from -7kb to -1bp position (+1bp = first transcribed base) and constructs containing the first 150bp downstream of the transcription starting site). The -622bp construct containing base pairs from -622bp position to -1bp position and the -860bp construct containing base pairs from -860bp position to -1bp position were cloned according to a published work from Ma *et. al.* (2001) except a different reporter vector was

used (In Ma's research, human IL-10 promoter fragments were amplified, cloned into PCRII-TOPO vectors for sequencing, and subcloned into pGL3B for transient transfection).

A few clones from each construct were picked for sequencing to confirm the integrity of the constructs. Despite it was indicated in the manual stating that the Red AccuTaq has a 3' to 5' proofreading exonuclease (Sigma), mutations were identified in most of the clones. There was 1 mutation identified on the -622bp construct and more than 10 mutations were found spanning across both -4kb and -7kb clones. Transfections with these IL-10 constructs showed certain levels of luciferase activity whereas the empty reporter vector (*i.e.* pGL4) showed significantly lower activity (Figure 3.10). This suggested that despite the presence of the mutations identified, the constructs are functional.

3.2.2 Transfections, LPS inductions, and optimisation of luciferase assays

As a pilot experiment, we first studied the -1kb (-1kb–-1bp) and -4kb-pGL3 (-4kb–-1bp cloned into pGL3) constructs provided by Ziegler-Heitbrock *et. al.* To optimize the transfection, several trials of transfections were carried out. With aid of JetPEI™ DNA transfections reagent, 1.5μg of the constructs or pGK (a hypoxia-inducible positive control luciferase reporter construct including a trimer of the Hypoxia Responsive Element from the mouse phosphoglycerate kinase-1 gene; Ameri *et al.2002*) were used to transfect 2×10^6 PBMC cultured in 6-well plate with Iscove's Dulbecco's medium for four days in normoxia followed by 24-hour-incubation in hypoxia and then 24-hour stimulation (in either hypoxia or normoxia) with LPS. However, none of the constructs showed consistent induction by LPS after several trials (Figure 3.4).

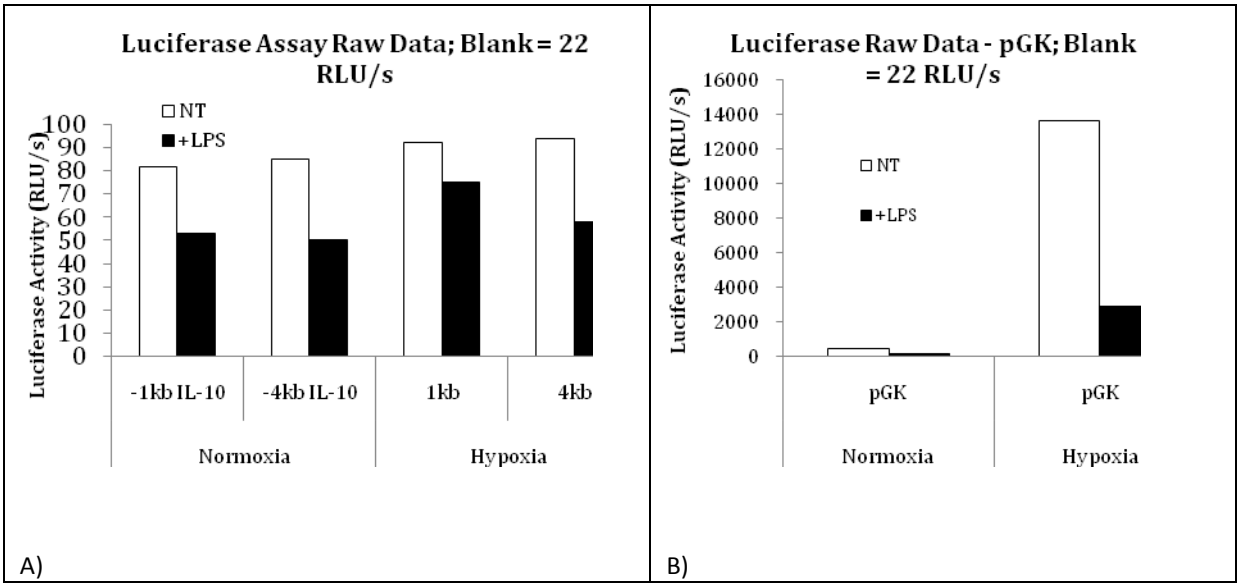
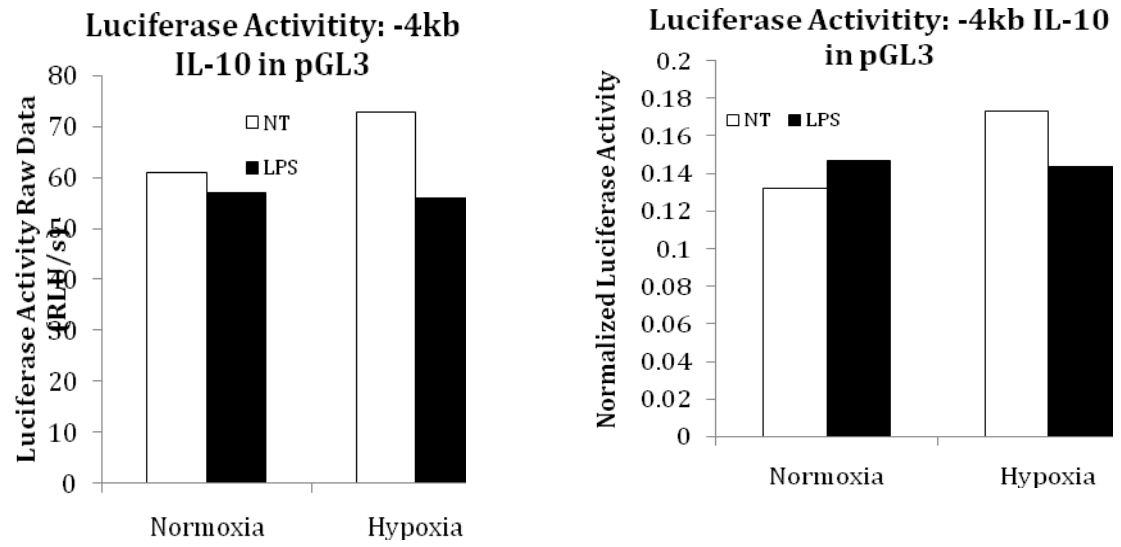


Figure 3.4: Promoter Activities of -1kb & -4kb IL-10-pGL3 Promoter Constructs.

A & B, white bars are the negative control (non-LPS-treated samples) and black bars are the LPS-treated samples. In experiments where 2.0ml of 1 × 106cells were seeded in each well of a 6-well culture plate, incubated in normoxia for 4days prior to transfection and further 24-hour incubation in either normoxia (control) or hypoxia, and induced with 100ng/ml LPS for 24 hours in either normoxia or hypoxia (as described in section 2.4.1, except each transfection was taken place in 2・ 106 cells, non-filtered Iscove's medium and in normal adherent plate). A, The promoter activities from each construct (-1kb IL-10 & -4kb IL-10) were weak and were not inducible by LPS (close to negative control; 22RLU/s), whereas B, the positive control transfected with equal amount of pGK constructs gave 128RLU/s in normoxia and 2884RLU/s in hypoxia or 22.5 fold induction by hypoxia.

Without an internal control, it is difficult to conclude the cause of the failure in the experiments. We decided to set up another experiment using the -4kb IL-10-pGL3 (Ziegler Heitbrock *et. al.*) construct with internal control. To set an internal control in each transfection, PBMC were co-transfected with pRL-TK plasmid (containing Renilla Luciferase, which serves as an internal control for normalization) and the -4kb IL-10-pGL3 construct. However, luciferase failed to give signals that could be distinguished from background (firefly luciferase gave signal very close to background, 52RLU/s) under such conditions (Figure 3.5). Since the produced light was weak, normalization would be meaningless. Further optimization was required.



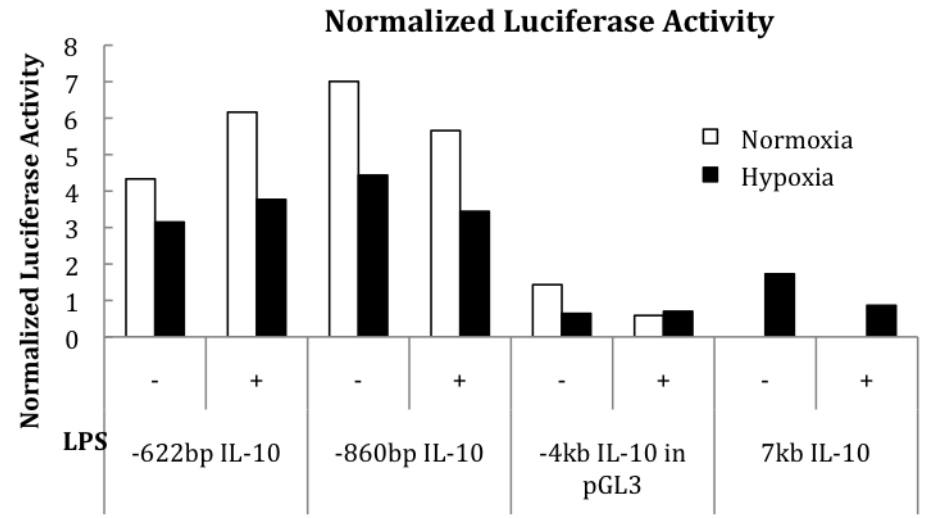
A) **B)**

Figure 3.5: Promoter Activity of -4kb IL-10-pGL3 Construct.

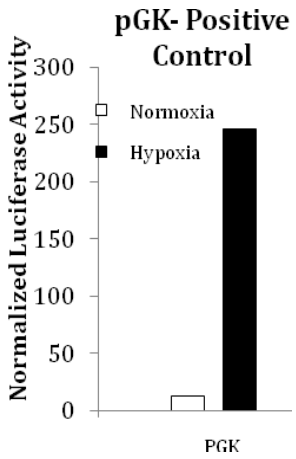
A & B white bars are non-LPS-treated samples whereas black bars are LPS-treated samples. Cells were prepared and transfected as in sections 2.2.1c and 2.4.1 with the -4kb IL-10-pGL3 construct except $2 \cdot 10^6$ cells and adherent plates were used. The Luciferase result signals were close to background: A, raw Luciferase data; blank reading = 52RLU/s; B, normalized data obtained by dividing the firefly luciferase reading by the Renilla luciferase reading.

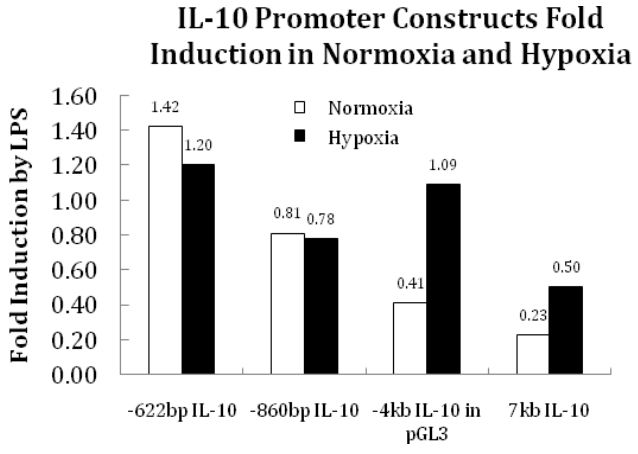
To further dissect the IL-10 promoter and determine which promoter elements are required for the negative regulation in hypoxia, we made a series of reporter constructs. -4kb (-4kb~-1bp) and -7kb (-7kb~-1bp) IL-10 promoter regions (+1bp = first transcribed base) and constructs containing the first 150bp downstream of the transcription starting site, -622bp (-622bp~+150bp) and -860bp (-860bp~+150bp) IL-10 promoter region (Figure 3.3A). The -622bp IL-10 pGL4 & -860bp IL-10 pGL4 were replicas of the constructs used by Ma*et. al.*, 2001), which we cloned into the pGL4 reporter vector via the *SfiI* restriction site.

In addition, we employed few new conditions by increasing the cell number, transfected plasmids, and using low adherent (LA) plate as described in section 2.2.1b and 2.4. Cells were transfected with -622bp IL-10, -860bp IL-10, -4kb IL-10 and -7kb IL-10 constructs as described in sections 2.2.1c and 2.4.1. Using LA plates, increased number of PBMC and transfected plasmids, luciferase signals were successfully optimized. However, we found that LPS failed to induce the promoter constructs (Figure 3.6).



A)





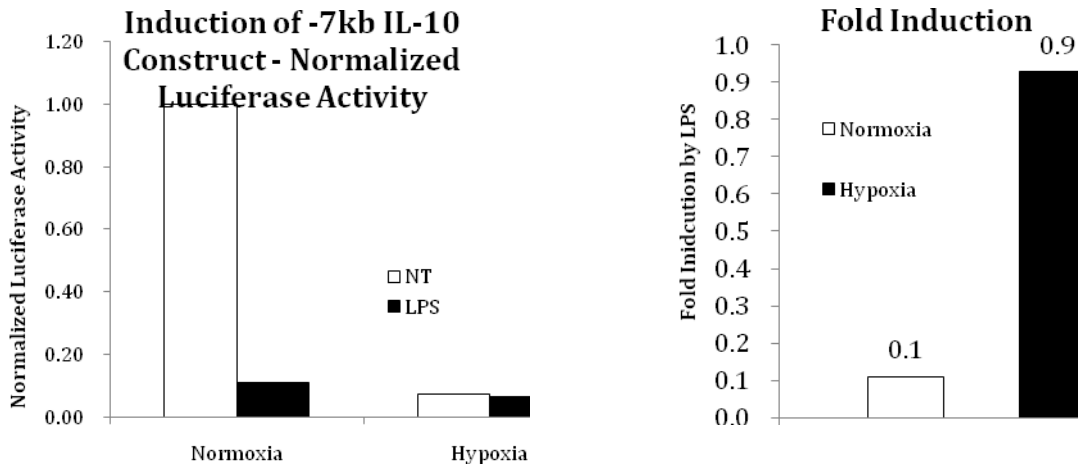
B) C)

Figure 3.6: IL-10 Promoter Activities of each Construct in Normoxia and Hypoxia.

A to C, white bars are the fold inductions in normoxia samples and black bars are the fold inductions in hypoxia samples. The cells were prepared, transfected with pGL4.10 basic (control), -622bp IL-10, -860bp IL-10, -4kb IL-10-pGL3 or -7kb IL-10 constructs and induced with LPS. A & B, each piece of data was normalized with Renilla luciferase. Left panels are the non-treated (-) samples and right panels are the LPS-stimulated samples (+). C, the result showed that none of the constructs were successfully induced by LPS (fold inductions less than 2 are generally considered weak) whereas the positive control, pGK transfected samples showed 19.5fold (not shown) induction by hypoxia.

The medium used for these experiments had not been filtered to remove endotoxin. Therefore, it is possible that the cells were already activated by the trace of endotoxins in the unfiltered medium before LPS treatment.

To address this problem, we used filtered medium for another experiment using the -7kb IL-10 construct. In this experiment setup, all the parameter (e.g. cell number, LA plates, incubation and stimulation time, as in sections 2.2.1c and 2.4.1) remains the same while the medium was filtered as described in section 2.2.1. Unfortunately, no notable induction was observed (Figure 3.7). We can not exclude the possibility that there are trace of endotoxin in the non-filtered medium, yet, the result inferred that the medium was not the major cause of the failed LPS inducibility of the constructs. To eliminate the potential problem that the presence of endotoxin may stimulate the cells before the experiment, we decided to use filtered medium to carry out the rest of the experiments (including characterization of endogenous IL-10 production and TLR ligand experiments).



A) **B)**

Figure 3.7: Luciferase Assay with Cells Prepared and Transfected in Filtered Medium.

A, white bars refer to the non-LPS-treated samples and black bars refers LPS-treated samples, the results were normalized by Renilla luciferase; B, the white bar is the normoxia sample, black bar is the 24-hour hypoxia sample. The only condition changed in this experiment setup is that filtered medium was used.

After another few experiments which failed to show induction, we hypothesised that it might be the transfection reagent that stimulated the activation of the cell prior to LPS induction, blocking further activation by LPS to produce more luciferase protein for detection. To solve this potential problem, an extra 24 hours of incubation in normoxia was included to allow the cells to be deactivated after transfections (Bernard Burke, personal communication) before another 24-hour incubation in normoxia or hypoxia. However, no consistent result was observed after repeated experiments when transfecting cells with -7kb IL-10 construct (Figure 3.8).

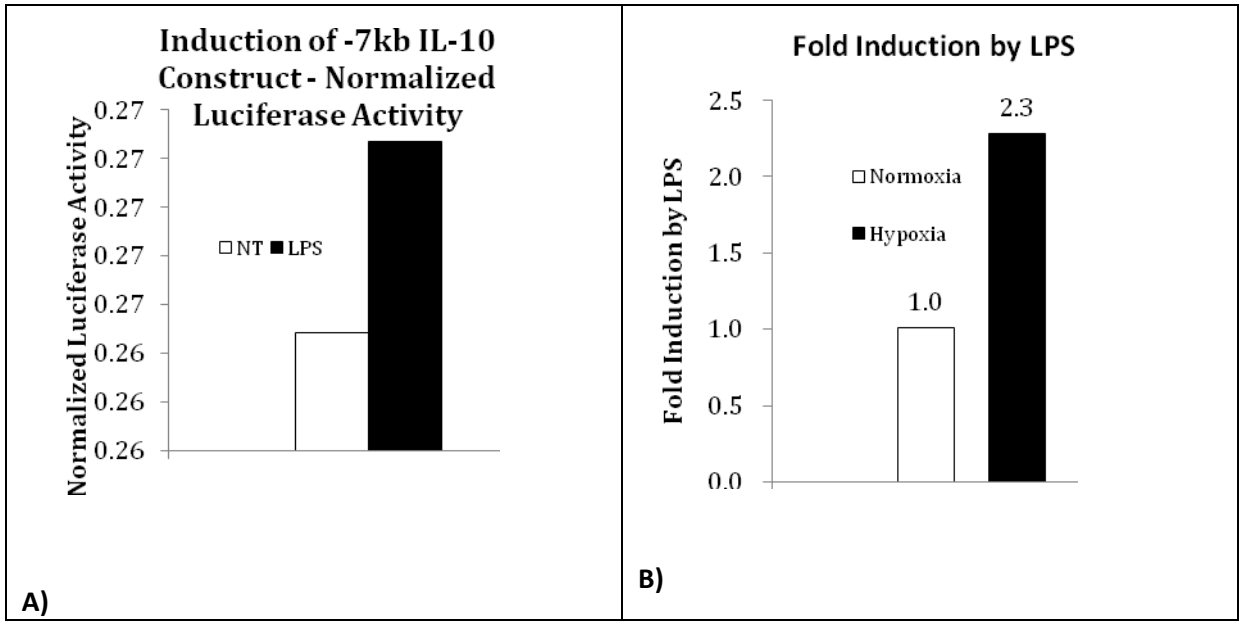
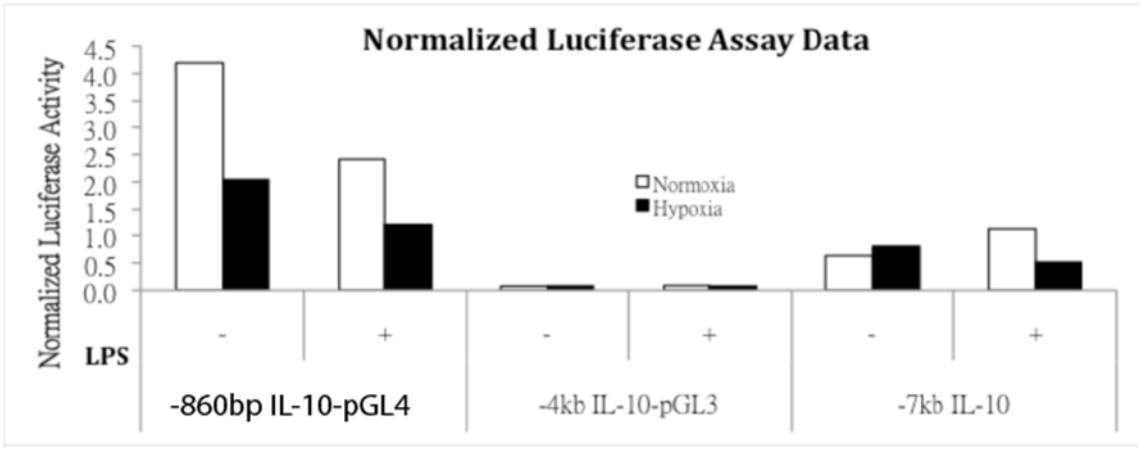


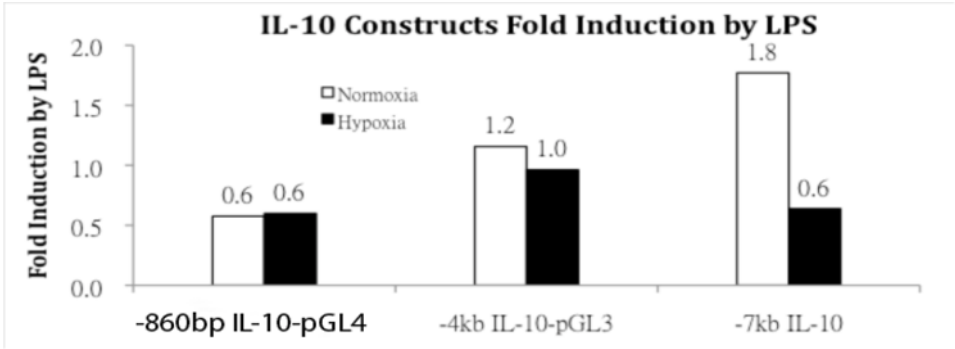
Figure 3.8: Luciferase Assay Result – The Cells Rested 24 hours after Transfection before Induction.
The experiments were carried out in both normoxia and hypoxia conditions as described in sections 2.2.1c and 2.4.1 except the cells were incubated for 24 hours after transfection. Cells were transfected with -7kb IL-10 constructs. A, white bar is the non-LPS treated samples and black bar is the LPS-stimulated samples, and the firefly luciferase activity was normalized by Renilla luciferase. B, the white bar is the normoxia fold induction of the LPS-treated sample in normoxia, and the black bar is the fold induction of the LPS-treated sample in hypoxia. As mentioned earlier, the increase of the incubation time (from 1 hour to 24 hours) is to allow the activated cells (by transfection reagent) to become deactivated before induction. B, the hypoxia sample showed 2.3 fold induction but the normoxia sample did not show induction. The experiment was repeated again and no induction was observed for either sample.

Up to this point, transfections carried out were performed in 6-well LA plates. In the endogenous IL-10 experiments, the conditions in 24-well LA plate showed better results. For this, we wanted to see whether such reaction setup would be suitable for the transfections. While all of the other conditions remained the same as described in section 2.2.1c and 2.4.1, half the number of cells ($2.5 \cdot 10^6$ cells in 0.5ml filtered Iscove's medium) were seeded into each well of the 24-well LA plates. PBMCs were transiently transfected

with -860bp IL-10, -4kb IL-10pGL3, and -7kb IL-10 constructs. However, we still could not successfully induce the IL-10 constructs with LPS (Figure 3.9).



A)



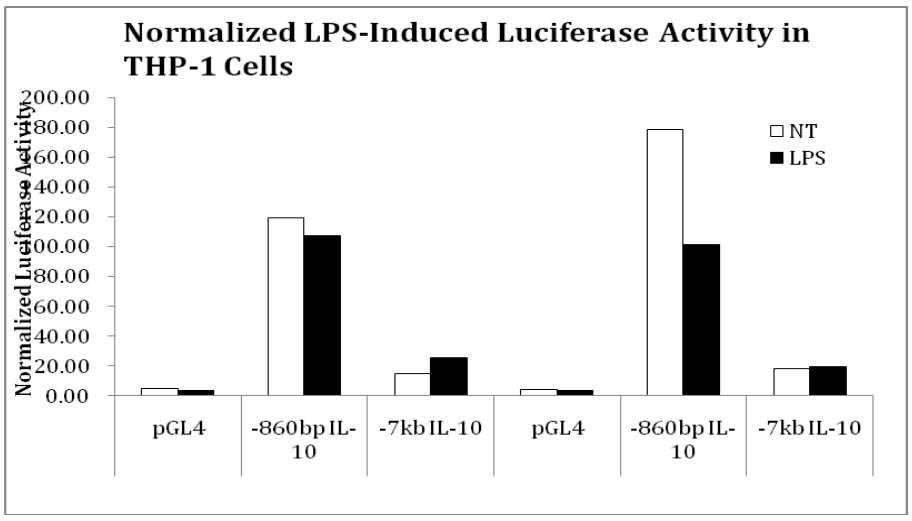
B)

Figure 3.9: Luciferase Activity of Cells Prepared and Transfected in 24-well LA Plates.
White bars are the normoxia samples and black bars are the hypoxia samples. The cells were prepared and transfected in 24-well LA plates. A, the firefly luciferase activity normalized with Renilla luciferase; left panels (-) of each sample are the non-treated sample, and the right panels (+) are the LPS treated samples. B, the IL-10 promoter construct fold induction by LPS. The result showed that LPS was not able to successfully induce the constructs in either normoxia or hypoxia.

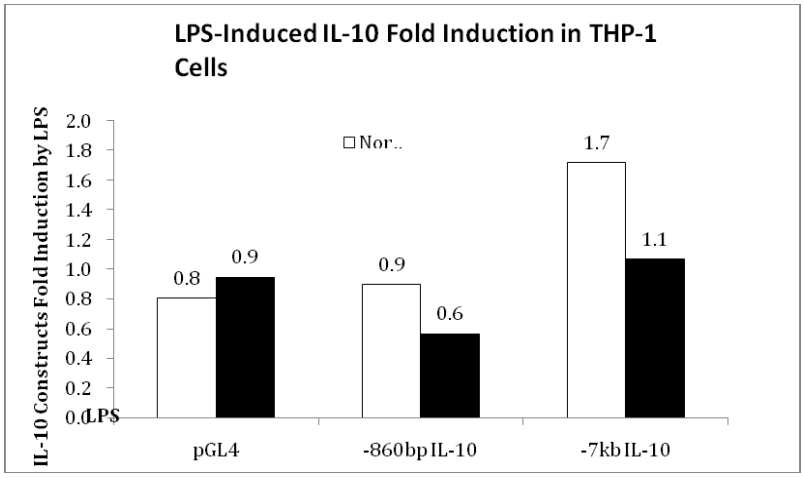
As mentioned earlier in section 3.2.1, mutations were identified on the -4kb IL-10-pGL3, -622bp, -4kb and -7kb IL-10-pGL4 constructs, but all of them showed basal promoter

activity. This implies that the promoter constructs are functional. Nevertheless, LPS failed to induce the constructs in all the tested conditions.

Ma *et. al.* (2001) demonstrated the participation of the transcription factorSp1 in IL-10 regulation using the -622bp (referred as the -652bp construct in Ma's published work) and -860bp (-890bp in Ma's work) IL-10 constructs. In his work, LPS successfully induced the constructs after transfection into THP-1 cells, a human monocytic cell line, using LipofectAMINE Reagent (Life Technologies Inc.). To confirm this, we shifted the host cell toTHP-1 cells, transfected using FuGENE 6® (Roche) transfection reagent, but the constructs again did not show any prominent increase in the promoter activity in response to LPS stimulation (Figure 3.10).



A)



B)

Figure 3.10: LPS-Induced IL-10 Promoter Activity in THP-1 Cells.
THP-1 cells were prepared, transfected and induced as described in sections 2.2.2 and 2.4.2. pGL4.10 empty vector was used as negative control. A, all the firefly luciferase activities were normalized with Renilla luciferase activity; white bars are the non-LPS-treated samples and the black bars are the LPS treated samples. B, the IL-10 promoters fold induction by LPS; white bars are the normoxia samples whereas the black bars are the hypoxia samples. Despite none of the constructs being inducible by LPS, the IL-10 constructs basal levels are much higher than the pGL4 luciferase level, indicating that the constructs are functional.

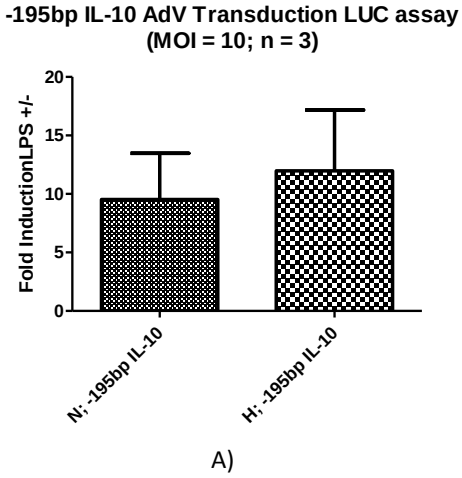
3.2.3The negative effect of hypoxia was not observed in LPS-stimulated -195bp and -4kb IL-10 promoter constructs when transduced into PBMC

After failures in the IL-10-pGL4 constructs, we shifted our approach to the use of IL-10 promoter luciferase reporter adenoviruses. Viral transduction is known to be much more

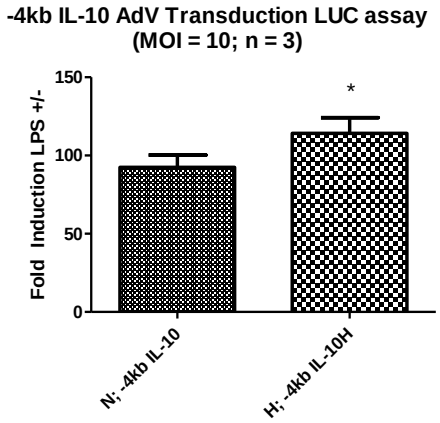
effective than conventional passive transfection. Isolated PBMC prepared in MonoMac 6 medium with 10% FCS (Foetal Calf Serum; Biochrom AG, cat. No. S0115, Lot. 477U) were incubated for three days before transduction with -195bp IL-10 or -4kb IL-10 adenovirus (multiplicity of infection = 10; Ziegler-Heitbrock). Transduced cells were then transferred to either normoxia or hypoxia for 24 hours prior to 6 hours of LPS stimulation.

Cells transduced with -195bp IL-10 adenovirus showed 9.5 fold induction in normoxia and 12.0 fold induction in hypoxia (n=3; Figure 3.11A). The hypoxia samples did not show a significant difference (p value > 0.05) to the normoxia samples. This may imply the absence of significant hypoxia-sensitive regulatory elements within the -195bp region in the IL-10 promoter region. Interestingly, cells transduced with -4kb IL-10 adenovirus showed 92.4 fold promoter activity in normoxia and 114.2 fold induction in hypoxia (n=3; Figure 3.11). Contrary to our expected outcome, the result implies that there is significant increase of IL-10 promoter fold induction (p value < 0.05 in Student's two-tailed paired t-test) in hypoxic conditions. From both the -195bp and -4kb Adv constructs, we did not see any negative effect in the IL-10 promoters in hypoxia. Based on this result, it might appear that the hypoxia-associated negative regulatory element that down-regulates IL-10 expression locates further upstream of the -4kb IL-10 promoter region. However it must be borne in mind that these adenovirus experiments are not normalized as are the other transfection experiments. Besides, slight changes in the fold induction level against a huge induction background of greater than 100 fold (Figure 3.11) may appear to be small or easily ignored (i.e. considered insignificant) when the number of experiment trials is small. The hypoxia effect on the -4kb Adv IL-10 constructs was able to be observed in later experiment setup

when the transducing moi was reduced to 1 (Figure 3.13). Therefore, the conclusiveness of this experiment result should be reconsidered.



A)



B)

Figure 3.11: -195bp IL-10 & -4kb IL-10 adenovirus transduction.

PBMC were prepared, and transduction with a multiplicity of infection of 10 was carried out on day3. The cells were returned to normoxia for 2hr after transduction. Each cell sample was equally split and seeded into two new wells, transferred to either normoxia (N) or hypoxia (H) for a further 24hr. After 24hr incubation in designated condition (N/H), the cells were incubated for another 6hr either with or without LPS. After 6hr incubation with or without LPS, the cells were harvested for quantification by luciferase assay. Fold induction was calculated by dividing the LPS treated samples by the non-treated sample. A, -195bp IL-10 Adv transfected samples; LPS induced 9.53 fold luciferase activity in normoxia (N; -195bp IL-10; left bar) and 11.97 fold in hypoxia (H; -195bp IL-10; right bar). B, -4kb IL-10 Adv transduced samples; LPS induced 92.4 fold in normoxia (N; 14kb IL-10; left bar) and 114.2 fold in hypoxia (H; -4kb IL-10; right bar). Each figure summarizes data collected from 3 independent experiments with means ± SEM. The data were further analyzed with Student's two tailed, paired t-test; A, hypoxia sample failed to show significant difference from normoxia sample; B, *p value<0.5 showing hypoxia samples significantly differ from normoxia samples.

3.3 The effect of cobalt chloride ($CoCl_2$) and desferrioxamine (DFO) on IL-10 gene expression

3.3.1 Expression and characterization of endogenous IL-10 in PBMC in response to $CoCl_2$ and DFO treatment

Hypoxia-inducible factor 1 (HIF-1) is a hypoxia-induced transcription factor that plays important roles in the control of a vast variety of genes (Wenger, 2002). It is a heterodimer consists of a constitutively expressed HIF-1β subunit and an oxygen sensitive HIF-1α subunit that normally degrades in physiological oxygen level (*i.e*. normoxia; Semenza, 2007). Cobalt chloride ($CoCl_2$) and desferrioxamine (DFO) are hypoxia mimetic agents that are often used to induce the production of HIF-1 protein by stabilizing HIF-1α subunit in normoxia (Wang and Semenza, 1993; Hirsilä*et al.*, 2005). To study the involvement of HIF-1 in the regulation of endogenous IL-10, we treated PBMC with 200μM of DFO (Hirsilä*et al.*, 2005), and also with $CoCl_2$(300μM) for 24 hours followed by LPS stimulation for another four hours.

In Figure 3.12, the negative control (normoxia, $CoCl_2^-$, DFO^-, LPS^-) is used for normalization and for fold induction calculations (e.g., negative control in normoxia gives fold induction (FI) = 1). As expected, cells which were not treated with $CoCl_2$ or DFO gave high fold induction (FI=6.24) in response to LPS stimulation in normoxia. DFO treated sample showed 5 fold induction after stimulation with LPS in normoxia. $CoCl_2$ treated samples did not show induction (FI<1) after stimulation with LPS even in normoxia. On the other hand, all of the samples in hypoxia showed no induction (FI<1) in response to LPS stimulation.

We found that $CoCl_2$ in normoxic conditions down-regulated both the basal and LPS-induced IL-10 expression (Figure 3.12). DFO, on the other hand, down-regulated the basal

IL-10 transcription level ($p<0.05$), but the LPS-treated sample did not show significant decrease ($p>0.05$) when compared with the LPS-treated sample in normoxia (-ve control + LPS (N)).

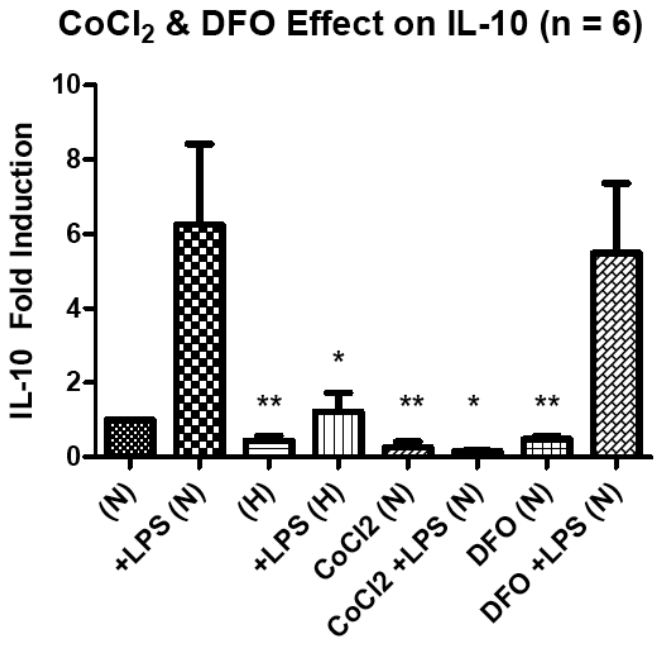


Figure 3.12: Effect of the HIF-1 inducing agents, $CoCl_2$& DFO on the expression of the endogenous IL-10 gene measured by RT-PCR. Fold induction was calculated by dividing the averaged normalized IL-10 mRNA level for each sample by the negative control (N). The fold inductions from left to right are: 1.0, 6.24, 0.44, 1.22, 0.56, 0.16, 0.49, 5.49. After incubation for 4 days in normoxia, the hypoxia controls (H) were transferred to hypoxia, whereas other samples were treated with nothing, $CoCl_2$ (300µM) or DFO (200µM) and returned to normoxia (N) for another 24hr. Further 4hr incubation was carried out in the same condition (N or H) either with or without LPS at concentration of 100ng/ml. $CoCl_2$ and DFO are hypoxia mimetic agents that mimic hypoxia and activates HIF-1, therefore hypoxia controls for $CoCl_2$ and DFO were not required. The cells were harvested for RNA extraction and reverse transcription. IL-10 and β_2M mRNA were quantified using real time PCR. IL-10 expression level was normalized with β_2M. The figure represents the mean ± SEM from 6 independent experiments. The data were further analyzed with Student's two-tailed, paired t-test; **p value<0.01, the none-LPS treated samples showed significant difference (-ve Control (H), $CoCl_2$ (300µM) (N), and DFO (200µM) (N)) when compared to the normoxia negative control (-ve Control (N)); *p value<0.5, LPS-treated negative control in hypoxia (-ve +LPS (H)) and LPS-treated $CoCl_2$ sample in hypoxia ($CoCl_2$ (300µM) (H)) were proved to be significantly different from the normoxia LPS treated control (-ve +LPS (N)). Hypoxia did not have significant effect (ns; $p>0.5$) on LPS treated DFO sample.

3.3.2 The effect of $CoCl_2$ and DFO on -195bp and -4kb IL-10 promoter adenoviral constructs

Despite the finding that the negative role of hypoxia in the regulation of IL-10 adenoviral constructs was not seen in hypoxia as discussed previously (section 3.2.3), the possibility of the presence of a potential IL-10 regulator that is controlled by HIF-1 in the given promoter regions cannot be completely ruled out. According Staples *et al.*(Bernard Burke,

personal communication*)* and the induction we obtained in previous experiments (Figure 3.2A, B) we expect to see 10 to 20 fold induction. However, LPS induced a 92-fold increase in luciferase activity in -4kb IL-10 Adv transduced cells (Figure 3.11), which is much higher than the expected level. We predict that the amplified luciferase activity was caused by excessive virus.

In the experiment shown in Figure 3.13, MOI was reduced to 1 while other conditions stayed the same in the following experiments. On day 3, $CoCl_2$ and DFO were added 2 hours after the transduction. LPS was added to a final concentration of 100ng/ml for 6-hour stimulation after 24 hours incubation in normoxia (with or without $CoCl_2$ or DFO) and hypoxia.

For -195bp IL-10 AdV transduction shown in Figure 3.13, from left to right, the control in normoxia ((N) -195bp IL-10; –ve) showed 7.56FI (fold induction) after LPS stimulation; $CoCl_2$ treated sample gave 1.55FI in normoxia after LPS treatment; DFO treated sample gave no induction; and the hypoxia control ((H) -195bp IL-10; –ve) gave 3.35FI. In-4kb Adv transduction, from left to right, the normoxia control ((N) -4kb IL-10; -ve) showed 31.1FI in response to LPS treatment; $CoCl_2$ treated sample gave 7.36FI after LPS treatment; DFO treated sample resulted in 4.21FI; finally, LPS induced the reporter protein in the hypoxia control ((H) -4kb IL-10; -ve) by 18.72 fold.

Treatment with both $CoCl_2$ and DFO had the same negative effect on the -195bp and -4kb IL-10 samples. The LPS induction decreased more than 5 fold in the -195bp sample and 20 fold in the -4kb sample after treatment with $CoCl_2$ and DFO. These results suggested that HIF-1 may be involved in the suppression/inhibition of IL-10 production after LPS

stimulation in hypoxia. However, only the decrease in $CoCl_2$ and DFO treated -4kb IL-10 samples are considered statistically significant ($P<0.05$). This implies the presence of the hypoxia-induced blockage element of IL-10 gene within the -4kb or even the -195bp region of the IL-10 promoter. Yet, more repeated experiments would be required to confirm the significance of the HIF-1 involvement in the -195bp Adv construct. To reinforce this finding that HIF-1 is involved in the hypoxia-blockade of IL-10 production, the effect of hypoxia on these IL-10 Adv constructs should also be verified.

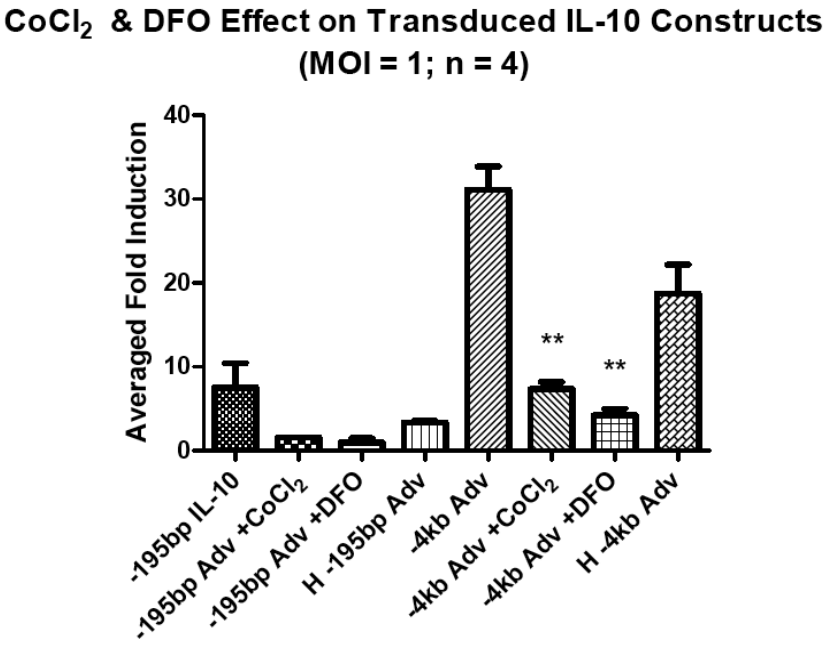


Figure 3.13: The effect of the HIF-1 inducers $CoCl_2$ and DFO on luciferase expression of -195bp and -4kb IL-10 reporter adenoviruses

After 3 days incubation in normoxia, cells were transduced with -195bp or -4kb IL-10 adenoviruses and returned to normoxia. After 2hr incubation in normoxia, each cell sample was equally split and seeded into 2 new wells, and transferred to hypoxia (H) or treated with 300µM $CoCl_2$ or 200µM DFO and returned to normoxia (N) for 24hr. After 24hr incubation in hypoxia or normoxia (with or without HIF-1 inducer treatment), the cells were incubated for a further 6hr with or without LPS (100ng/ml). Finally, the cells were harvested and reporter (luciferase) activities were quantified by luciferase assay. Fold induction was obtained by dividing the RLU/s (relative light units/second) obtained from LPS treated sample by RLU/s from the non-treated sample (not shown). The graph summarizes data collected from 4 independent experiments with mean ± SEM. The data was further analyzed using Student's two-tailed paired t-test. In the -4kb AdV transduced samples, **p value<0.01; $CoCl_2$ and DFO treatment showed significant decrease when compared to the normoxia control (-4kb Adv).

3.3.3Presence of HRE – IL-10 Promoter Sequence Analysis

Upon activation, HIF-1 binds to the HRE located on the oxygen-dependent genes. From the result shown in Figure 3.13 we may hypothesize that HIF-1 may be involved in the hypoxia –induced IL-10 inhibition, and the element that cause the blockage effect may be present within the -4kb region, and possibly within the -195bp region. We would want to see whether it is possible that HIF-1 might directly bind to the IL-10 promoter and cause the inhibition. To answer this question, we analysed the IL-10 promoter sequences using MatInspector, a sequence analyzing software that identifies the potential transcription factor that bind to the promoter sequence of a given gene. The result we obtained (Figure 3.14) indicated the presence of three HREs located in the -7kb IL-10 promoter region. The closest HRE to the IL-10 gene is the caaccctaCGTGgttat sequence located on the antisense strand between the -2,171bp and -2,187bp IL-10 promoter region; next to it is a second HRE sequence, taccccACGTcctcac, which locates on the antisense strand between the -6,843bp to -6,859bp to the IL-10 promoter. The third HRE consensus sequence is ggtgaggaCGTGggggt , which locates on the sense strand between the -6,842 to -6,858bp on the IL-10 promoter.

If HIF-1 blocks IL-10 expression by direct binding to the IL-10 promoter, the results obtained from MatInspector analysis and previous section (Figure 3.13) suggests that HIF-1 may physically bind on the HRE located on the -2,171bp to -2,187bp region on the antisense strand of the IL-10 promoter. However, MatInspector analysis suggested the absence of HRE in the -1bp to -195bp IL-10 promoter region. Together with the results obtained from the -195bp IL-10 Adv construct in Figure 3.13, this result may provide a

contradictory supposition that HIF-1 may not affect the IL-10 expression by direct interaction.

Several human IL-10 polymorphisms have been identified in the promoter region, including −1082 G>A, −819 C>T and −592 C>A (Smith & Humphries et. al., 2008). However, none of these allelic variations on IL-10 promoter was identified to locate on the HIF binding site. This may imply that the IL-10 suppression in hypoxia may not be affect by ethnicity.

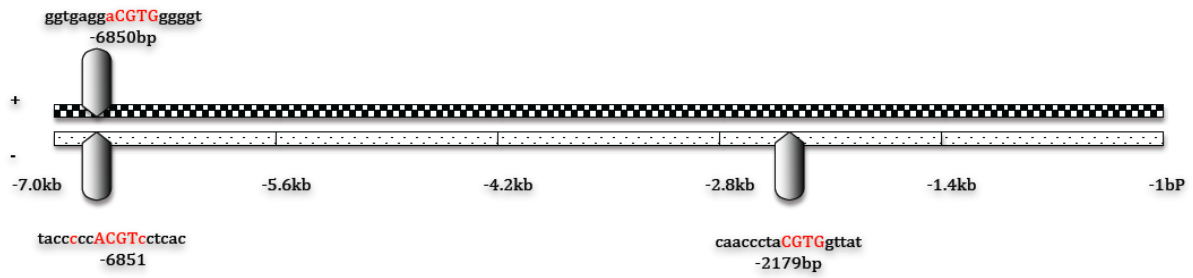


Figure 3.14: MatInspector Analysis; Locations of the HRE on the-7kb IL-10 promoter
The -7kb IL-10 promoter region is represented by the two strands. The upper strand labeled with "+" sign refers to the sense strand of the IL-10 promoter, whereas the lower "-"strand is the antisense strand of the IL-10 promoter. The relative positions of the promoter are shown below the "-" strand. The sequences showed are the identified HRE consensus sequences, and the midpoints of these HREs locate at the base-pairs labeled below the consensus sequence. MatInspector analysis indicated the presence of a HRE on the antisense strand between the -2,171bp and -2,187bp, a second HRE on the antisense strand between the -6,842bp and -6,858bp position, and a third HRE on the sense strand between the -6,843bp and -6,859bp on the IL-10 promoter.

3.4 Effect of Hypoxia on IL-10 production stimulated by other TLR (Toll like receptor) ligands

So far, the blockage of IL-10 expression was only observed in cells induced by SAE LPS. LPS activates the cell via TLR4, CD14, and LBP (LPS binding protein). To further characterize

the inhibitory role of hypoxia on IL-10 induction, we conducted the following experiments to test whether similar results can be observed when the cells are activated by other TLR ligands. The recommended dosage for each reagent from the manufacturer is shown in Table 3.1(Alexis® Biochemicals, Manual APO-54N-030 v3).

While most of the parameters remained the same as in section 2.2.1b, IL-10 induction was optimized by decreasing the cell number in each well (*i.e.* decrease the cell density to 2.5 · 10^6 cells/ml prevent depleting the required nutrient in the media, avoid excessive release of metabolic product that cause toxicity and cell death, and allow better expression; Bernard Burke, personal communication) and increasing the dosage for each TLR ligand to achieve final concentration listed in Table 3.1. An additional 100µl of fresh media with 2.5% AB serum was added on day 4 before transferring the cells to normoxia & hypoxia (Figure 3.15). However, Poly I:C and CpG ODN failed to induce IL-10 in any condition.

In Figure 3.15, treatment with Pam_3CSK_4, a TLR1/2 heterodimer stimulant, showed an average of 2.09 fold induction in normoxia and 0.62 in hypoxia. SAE LPS induced IL-10 production by 10.68 fold in normoxia and 1.46 fold in hypoxia. *E. Coli* LPS, also activating macrophages via TLR4, stimulated 7.65FI and 1.42FI in hypoxia.The TLR5 ligand, Flagellin, gave 4.77FI in normoxia and 0.39FI in hypoxia. TLR6/2 heterodimer stimulant – macrophage activating peptide 2 (MALP-2) induced 3.34FI in normoxia and 0.36FI in hypoxia. Imiquimod (R-837), which induces macrophage activation via TLR7, gave 0.633FI in normoxia and 0.335FI in hypoxia, whereas the TLR7/8 heterodimer ligand, resiquimod (R-848), induced 7.39 fold IL-10 expression in normoxia and 0.26 fold in hypoxia. In all of our experiments, we failed to induce the expression of IL-10 with Imiquimod even in

normoxia. Therefore, whether hypoxia has any effect on IL-10 expression in Imiquimod treated cells could not be determined.

Table 3.1: TLR Ligands, dosages recommended by Alexis® Biochemicals, and optimized dosage

TLR Ligand	TLR	Dosage	
		Lowest suggested	Optimized
Pam_3CSK_4	TLR 1/2 Heterodimer	10ng/ml	20ng/ml
Poly I:C	TLR3	25ng/ml	-
LPS (*Salmonella Abortus Equi)*	TLR4	-	200ng/ml
E. Coli LPS	TLR4	20ng/ml	80ng/ml
Flagellin (*S. Typhimurium*)	TLR5	10ng/ml	400ng/ml
MALP-2	TLR6/2	10ng/ml	40ng/ml
Imiquimod (R-837)	TLR7	0.1µg/ml	5µg/ml
Resiquimod (R-848)	TLR7/8	0.1µg/ml	5µg/ml
CpG ODN	TLR9	0.5µg/ml	-

Beside cells treated with SAE LPS and *E. Coli* LPS, all of the samples treated with other reagent showed no induction in hypoxia (Fold induction ≤1). Although SAE LPS and *E. Coli* LPS induced IL-10 expression by 1.46 and 1.42 fold respectively, the decrease in induction levels in hypoxia were proven to be significant. Moreover, statistical analysis confirmed that MALP-2 stimulated IL-10 mRNA induction showed a significant decrease in hypoxia.

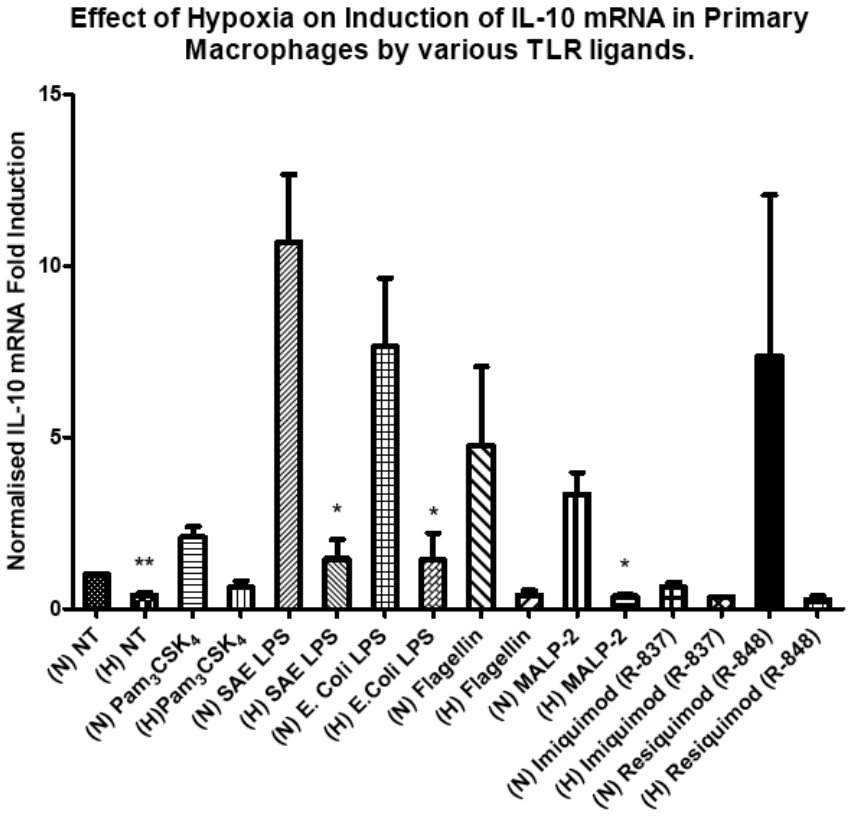


Figure 3.15: Effect of Hypoxia on Induction of IL-10 mRNA in Primary Macrophages by various TLR ligands

After 4days of incubation in normoxia, cells were transferred to the condition indicated (H = hypoxia; N = Normoxia) for 24hrs. After the 24hr incubation in the designated condition, the cells were Not Treated (NT) or treated with different reagents indicated on the horizontal axis (Pam_3CSK_4 with final concentration of 20ng/ml, 200ng/ml SAE LPS, 80ng/ml E. Coli LPS, 400ng/ml Flagellin, 40ng/ml MALP-2, 5µg/ml Imiquimod, or 5µg/ml resiquimod) and returned to the indicated condition (N or H) for a further 4hr. The cells were harvested after the 4hr treatment with or without the TLR stimulant for RNA isolation, reverse transcription and quantification of IL-10 and β2M mRNA levels by real-time PCR. IL-10 level was normalized to β_2M level. Fold induction was obtained by dividing each normalized IL-10 expression level from the sample in the given condition by the normoxia non-treated (N NT) sample. Non-treated sample showed a 60% reduction (0.40FI) in hypoxia. This graph presents the mean ± SEM from data gathered from 4 independent experiments. Pam_3CSK_4 (TLR1/2 ligand) treated sample showed 2.1FI in IL-10 level in normoxia and 0.62FI in hypoxia. Stimulation with SAE LPS (TLR4 ligand) gave 10.68FI in normoxia and 1.46FI in hypoxia. Treatment with E. Coli LPS (TLR4 ligand) induced 7.65FI in normoxia and 1.42FI in hypoxia. Flagellin (TLR5 ligand) treated sample showed 4.67FI in normoxia and 0.39FI in hypoxia. Activation with MALP-2 (TLR6/2 ligand) induced IL-10 expression by 3.34 fold in normoxia and 0.36 fold in hypoxia. Imiquimod (TLR7 ligand) treatment induced 0.63FI in normoxia and 0.34FI in hypoxia, whereas resiquimod (TLR7/8 ligand) treatment induced 7.39FI in normoxia and 0.26FI in hypoxia. The data was further analyzed with Student's two-tailed, paired t-test. **p value<0.01 when compared to the normoxia sample treated with the same TLR ligand. *p value<0.05 when compared to the normoxia sample treated with the same TLR ligand.

4 Discussion

4.1 The blockage of IL-10 expression by 24hr hypoxia

Interleukin 10, previously described as cytokine synthesis inhibitory factor (CSIF), is an important immunosuppressive cytokine that down-regulates the synthesis of a variety of pro-inflammatory cytokines (Brightbill *et al.*, 2000). However, the regulation of IL-10 is not yet fully understood. Recent research by Staples et al. (personal communication with Bernard Burke) demonstrated that its regulation is altered in hypoxia ($O_2 \cong$ 0.2%). They showed that the LPS-induced IL-10 protein production was reduced by 78% (Staples, unpublished data) in hypoxia. In contrast, induction of the pro-inflammatory cytokine TNF was not reduced. The results presented in this thesis confirmed that the expression of human IL-10 was significantly reduced when PBMC were incubated in hypoxia (0.2% O_2) for 24 hours, compared to normoxia. In an experiment set with n = 8, the mean IL-10 level (basal IL-10 level) in non-treated cells dropped 68% in hypoxia, and 100ng/ml LPS failed to induce IL-10 expression (FI < 1) in hypoxia (Figure 3.2A). In a separate experiment set with n = 5, the basal IL-10 level dropped by 60% in hypoxia, and 200ng/ml LPS induced IL-10 expression only 1.30 fold in hypoxia (Figure 3.2B). In addition, 100ng/ml LPS induced 2.94fold IL-10 expression in hypoxia (when compared with non-treated, hypoxia sample (NT(H)) with 100ng/ml LPS, hypoxia sample (+LPS(H)); Figure 3.2A). 200ng/ml LPS induced 9.07fold IL-10 mRNA production in normoxia, but only 3.25fold in hypoxia (when compare LPS treated sample with non-treated sample; Figure 3.2B).

The finding that IL-10 basal and LPS-induced mRNA levels are decreased in hypoxia is novel but important. This indicated that the regulation of human immunity is subtle. Hypoxia that occurs in pathological conditions enables maximized production of mediators such as VEGF, versican and MMP-7 (Burke et al., 2003; B. Burke, personal communication) to enhance clearance of pathogens and tissue healing. While increased production of the pro-inflammatory cytokine TNF in response to LPS is not blocked by hypoxia (Figure 1.1; K. Staples and B. Burke et al., unpublished data), the production of IL-10, with its anti-inflammatory properties, is restricted in hypoxia. We hypothesise that this allows maximal levels of pro-inflammatory cytokines to be secreted to facilitate enhanced inflammation in hypoxic sites. As the pathological tissue is repaired and regains adequate oxygen supply and becomes normoxic, IL-10 production also is likely to resume, to down-regulate the inflammatory response which has now performed its function.

Despite hypoxia is observed mostly in pathological tissues, low oxygen tension can still be found in normal tissues such as spleen. Caldwell *et. al.* (2001), reported that T cell activation in hypoxic conditions in spleen may lead to different patterns of lymphokine secretion and accumulation of cytokines. Similarly, hypoxia in normal tissue may also alter the IL-10 production pattern as in pathological tissues (i.e. IL-10 suppression). If infection takes place in tissues with low oxygen tension, the low-oxygen level may cause prolonged inflammation due to the unavailability of IL-10. Hence, in addition to the suppression of IL-10 in hypoxia, there must be a counteract mechanism to prevent autoimmunity in these normal tissues.

4.2 Dissecting the human IL-10 promoter

The expression of IL-10 has been reported to be regulated by AP-1, ATF-1, C/EBP, CREB-1, Sp1, Sp3, c-Maf, STAT3, transcription factors, and others (Gollnick *et. al.*, 2001, Brenner *et. al.*, 2003, Platzer *et. al.*, 1999, Brightbill *et. al.*, 2000; Tone *et. al.*, 2000, Cao *et. al.*, 2005, Ziegler-Heitbrock *et. al.*, 2003; Benkhart *et. al.*, 2000). Several transcription factors including NF-κB and ATF-1 are known to be activated in hypoxia (Shi e. al., 1999, Brenner *et. al.*, 2003). Each transcription factor has its consensus sequence within the IL-10 promoter. Knowing that hypoxia down-regulates the expression of IL-10 with or without LPS stimulation, we wanted to examine which transcription factors are involved in this negative regulation.

Our strategy was to determine the potential IL-10 regulatory element by studying promoter reporter constructs. By studying the promoter constructs, we may narrow down the involved region and sequences on the promoter. The hypoxia-controlled transcription regulator may then be identified with additional techniques such as shift assay and chromatin immunoprecipitation (ChIP) with the aid of bioinformatics.

To analyze the promoter, we generated two promoter constructs by cloning -4kb (from -1bp to -4kb; +1bp refers to the first transcribed base in transcription) and (-1bp to -7kb) IL-10 promoter regions into the pGL4 reporter vector. Together with the previously existing -4kb (reported to contain mutations) and -1kb IL-10 constructs kindly provided by Professor Zeigler-Heitbrock, efforts were made to optimize the transfection experiments. We aimed to verify each condition to optimize the activity of the promoter constructs and their sensitivity to stimulants.

Initially, each experiment was carried out with 2.0 · 10^6 cells/well (cell concentration = 10^6 cells/ml) in Iscove's Dulbecco's medium in normal 6-well cell culture plates, and the incubation time was the same with the PBMC experiments. Unfortunately, the luciferase activity level was low and close to the background level (Figure 3.5). By shifting the experiment to another system using shallow medium (1ml medium/well) to minimize "accidental" hypoxia and increasing the cell density to 5 · 10^6 cells in each well in 6-well low-attachment plates, we successfully obtained good luciferase activity levels. However, we found out that the constructs were not inducible by LPS (Figure 3.7, Figure 3.9). Modifications of the experiment including different incubation times, transfection agent (i.e. FuGENE 6®), LPS dosage, filtering the medium (Schaefer *et. al.*, 2008), making new reporter constructs with the addition of 150bp downstream of the original ending site (*i.e.* -622bp & -860bp IL-10 constructs; Figure 3.3; Ma *et. al.*, 2001). Therefore, the difference in promoter activity in hypoxia, if there was any, would not be able to be identified if the constructs failed to be inducible by LPS. We suggest that it may be the mutations on the constructs that caused the -4kb IL-10pGL3, -4kb IL-10pGL4 and -7kb IL-10 constructs not able to be induced. Yet, the -860bp IL-10 and -1kb IL-10 construct, which are free of mutation were not inducible by LPS either. The cause of the non-inducibility of the constructs remains unknown. Various conditions may cause the failure that occurred. These may include the mutations occurring in the constructs, the ability of the plasmid to work in the host cells (Ma *et. al.* 2001) used pGL3B basic reporting vector), the health of the cells, donors, and other unknown causes.

An alternative approach to study promoter activity is by using adenovirus transduction to deliver the construct DNA into the cells. The -195bp and -4kb IL-10 adenovirus (-195bp IL-10 Adv and -4kb IL-10 Adv respectively) kindly provided by Professor Ziegler-Heitbrock worked well with robust inducibility after treatment with LPS. In an experiment with moi = 10, however, the blockage of IL-10 promoter activity by hypoxia was not seen. SAE LPS induced the -195bp promoter activity by 9.53 fold in normoxia and 11.97 fold in hypoxia (Figure 3.11A).Hypoxia has no effect on the -195bp Adv constructs according to the result. Hence the result suggested that the negative regulator that caused the reduction of IL-10 expression in hypoxia may be further upstream of the -195bp promoter region.

Similar to the -195bp Adv construct, the -4kb promoter construct was induced by 92.4 fold in normoxia and 114.2 fold in hypoxia after 6hr LPS treatment (Figure 3.11B). That is, the blockage on the activity of the -4kb Adv IL-10 construct in hypoxia was not observed.

We used an moi (multiplicity of infection) of 10 in the first sets of transductions, and with the robust transduction and numerous copies of the promoter constructs, binding and promoter activities that overcame the potential inhibition brought out by hypoxia may be expected (Semmes *et. al.*, 1996). However, the effect of hypoxia on the -4kb Adv constructs was not yet proven to be statistically significant after six experiments (Figure 3.13). Interestingly, according to statistical analysis (Figure 3.11B), the LPS-stimulated -4kb Adv construct showed significant higher induction in hypoxia than in normoxia when moi = 10. Yet, this effect was not seen when moi was decreased to 1 (Figure 3.13).

In either experiment setups with moi = 1 or moi = 10, the down-regulation of IL-10 promoter activity we expected was not observed in hypoxia. This implied that the hypoxia-

regulated negative-regulating element may be further upstream of the -4kb IL-10 promoter region. Nevertheless, our results from another experiment (see Figure 3.13), showed hypoxia blockade in the -4kb construct. Therefore, the conclusiveness of the result from Figure 3.11 setup should be in doubt.

4.3 The role of HIF-1 in IL-10 regulation

HIF-1, an important transcription factor that is induced in hypoxia, is known to participate in several different signaling pathways. It is usually referred to as a transcription activator that initiates gene expression (Sutter *et. al.*, 2000; Brahimi-Horn & Pouyssegur, 2006). To date, whether it has any direct or indirect effect on the regulation of IL-10 in hypoxia has not yet been reported.

We hypothesized that the inhibitory effect of hypoxia on IL-10 production and induction may be related to HIF-1. By inducing HIF-1 protein in normoxia using $CoCl_2$ or DFO (Maxwell *et. al.*, 1999), we compared the IL-10 expression patterns and determined the role of HIF-1 in the regulation of the IL-10 gene with the aid of statistics.

In the endogenous IL-10 experiment (Figure 3.12), the results showed that the basal expression level of IL-10 mRNA treated with either $CoCl_2$ or DFO are significantly (p value<0.01) lower than un-treated samples. That is, treatment with $CoCl_2$ or DFO down-regulates IL-10 mRNA expression. On the other hand, in response to LPS stimulation, $CoCl_2$ treated cells behaved the same as hypoxia–treated cells; LPS was not able to induce IL-10 production in $CoCl_2$ treated sample even in normoxia (Figure 3.12). However, the suppression of LPS-induced IL-10 expression was not observed in DFO treated samples. Both $CoCl_2$ and DFO induce HIF-1 protein by stabilizing the HIF-1α subunit. The result

obtained from our experiments (Figure 3.12) inferred that HIF-1 may be involved in the diminishment of basal IL-10 expression. The $CoCl_2$ treated sample seemed to confirm that HIF-1 did play a role in both decreasing the IL-10 basal mRNA level and decreasing the up-regulation of IL-10 expression by LPS in hypoxia. The basal IL-10 mRNA level decreased 44% after treatment with $CoCl_2$ when compared to the normoxia negative control (N). LPS failed to induce IL-10 production in $CoCl_2$ treated samples (the IL-10 mRNA level in $CoCl_2$ +LPS (N) sample was even lower than the IL-10 mRNA level in the $CoCl_2$ (N) sample (Figure 3.12)).

The DFO treated sample showed a minor and yet statistically significant reduction in basal IL-10 production. However, LPS successfully induced IL-10 production in DFO treated cells and gave 5.49 fold induction when compared to (N), or normoxia non-treated samples (Figure 3.12). In other words, HIF-1 activation by DFO treatment was not shown to be able to block the LPS-induced production of IL-10 as expected, and the LPS induced IL-10 mRNA level was not significantly different from the control (+LPS(N)). This result showed in Figure 3.12 may not completely rule out the role of HIF-1 in the blockage of IL-10 expression, but the role of HIF-1 in LPS-induced IL-10 production was not verified. The result (Figure 3.12) suggested that both $CoCl_2$ and DFO both were able to reduce the basal production of IL-10. Since both $CoCl_2$ and DFO showed a common effect – down-regulation of the basal transcriptional level of IL-10, which is similar to the IL-10 expression level observed in hypoxia, it is reasonable to assume that HIF-1 may take part in IL-10 regulation, at least at the basal transcription level. However, other than HIF-1, $CoCl_2$ and DFO induce different genes that participate in different signalling pathways or cellular functions (Bezdudnaia & Kaliman, 2008; Reyes *et al.*, 2008), and the different result we

obtained may be caused by pathways activated by the different HIF-1 inducing agents. $CoCl_2$ had similar effect of blocking the LPS-induced IL-10 production with hypoxia but DFO only caused the diminishment of basal IL-10 expression. Furthermore, Lombaert *et. al.* (2008) reported that besides apoptosis, addition of cobalt to PBMC may lead to various stress/defence responses and downregulation of immune responses. Therefore, $CoCl_2$, may activate other pathways that eventually blocks the IL-10 expression. For this, we are not able to make a solid conclusion and tell whether or not the decrease in IL-10 level in hypoxia is caused by the production of HIF-1 from our results since addition of $CoCl_2$ successfully blocked IL-10 up-regulation by LPS while DFO did not produce the same result.

Alcantara*et. al.* (2001) suggested that the expression of at least 11 genes was inhibited greater than 50% by iron deprivation after treatment with DFO. Although DFO successfully reduced the IL-10 basal level in non-activated cells, it failed to block the massive up-regulation of IL-10 in response to LPS stimulation. More work and study needs to be done to clarify the role of HIF-1 in LPS activated IL-10 expression.

On the other hand, it has been suggested based on experiments carried out after my project was completed (Bernard Burke, personal communication) that the DFO stock I used may have been inactivated by repeated freeze-thawing and repeated use of a single aliquot. To test this, one could assess the expression of the genes that are activated by HIF-1 such as GLUT-1 and VEGF (Murdoch & Lewis, 2005) to see the potency of the HIF-1 inducing agent (i.e. HIF-1).

The results from Figure 3.12 suggested that HIF-1 may be involved in the IL-10 basal transcription level, we wanted to see whether this effect could be observed on an IL-10

promoter luciferase reporter construct. Previously, we were not able to see demonstrate an effect of hypoxia on IL-10 promoter constructs (Figure 3.11).

We hypothesised that the "endogenous IL-10 suppressor" activated in hypoxia, although sufficient to block both the basal and LPS induced IL-10 level from the endogenous IL-10 gene, was not able to block the prominent promoter activities from the large amount of exogenous IL-10 luciferase reporter construct DNA which we adenovirally transduced into the cells. Therefore, we decreased the moi to 1 and repeated the transduction experiment. Additionally, in Figure 3.13, we added the $CoCl_2$ and DFO treatment to the samples in order to examine whether the luciferase activity would be altered by inducing HIF-1 in normoxia. Interestingly, we found out that in stark contrast to the results observed in the endogenous IL-10 experiment (Figure 3.12), the DFO treated cells, transduced with either the -195bp or 4kb IL-10-luc adenoviruses, also showed marked reductions in luciferase activity (and thus the promoter activity) after LPS treatment. All of $CoCl_2$, DFO and hypoxia induce HIF-1 activation. Addition of $CoCl_2$ and DFO both diminished the -4kb IL-10 Adv activity. Despite the decrease in the activity of -4kb IL-10 Adv in hypoxia not being statistically significant, a decrease in the induction level of the -4kb Adv, hypoxia sample shown in Figure 3.13can be observed. Thus, the result (Figure 3.13) implied that the promoter element that causes the blockage effect of IL-10 in hypoxia may be located within the -4kb region of the IL-10 promoter, and either directly or indirectly, HIF-1 may be involved in this effect. This result is contradictory to the result showed in Figure 3.11, where the -4kb Adv construct had increased activity in hypoxia, despite the fact that HIF-1 should be activated in all of the conditions (i.e. hypoxia, $CoCl_2$ treated, and DFO treated). This may be due to the different HIF-1 level induced in hypoxia and to $CoCl_2$ and DFO treatments. In this case, although the

HIF-1 protein induced by $CoCl_2$ and DFO was able to sufficiently block the -4kb IL-10 Adv construct, the amount of HIF-1 protein activated in hypoxia may be inadequate to reduce the activities from the robust -4kb IL-10 Adv construct even when the moi was reduced to 1, which was induced 30 fold after stimulation, as mentioned earlier in this paragraph. The observed differences in the effect of DFO between different experiment sets also point towards the likelihood of there being differences in the tubes of DFO being used, due to freeze thawing, as discussed previously.

Several repeated experiments were performed with small modifications, but we failed to optimize the experiment and generate consistent results. Hence, no solid conclusion could be made with the data.

In order to further confirm the involvement of HIF-1 in the negative regulation of IL-10 in hypoxia, an alternative approach could be to inhibit HIF-1 production by using drugs including digoxin or other cardiac glycosides (Zhang *et al.*, 2008). With this experiment, we could examine if the blocking effect of IL-10 expression can be restrained by inhibiting the production or activity of HIF-1.

HIF-1 is commonly known as a transcription factor that plays an important role in upregulating a large number of genes in hypoxia (Burke *et. al.*, 2003; Jewell *et. al.*, 2001 Manalo *et. al.*, 2004). In addition, its suppressive role in gene expressions such as expression of equilibrative nucleoside transporter (ENT) was also reported (Eltzschig *et. al.*, 2005).As mentioned earlier, HRE core sequence is essential for the binding of HIF-1 and activation of the target genes, but the sequences adjacent to the HRE core is another factor that determines the effectiveness of the transcription by HIF-1. From the result obtained in

Figure 3.14, we may suggest that HIF-1 can bind on at least three locations on the IL-10 promoter: -2,171bp to -2,187bp, -6,842bp to -6,858bp on the antisense strand, and -6,843bp to -6,859bp on the sense strand. Unfortunately, the -7kb IL-10 constructs failed to work. Therefore we are not able to make any suggestion on the relationship between HIF-1 and the HREs located on the -6,842bp to -6,858bp, and -6,843bp to -6,859bp regions.

The -4kb IL-10 Adv construct showed decreased activity in both $CoCl_2$ and DFO treated samples, and HIF-1 should be activated in both conditions. Together, the results in Figure 3.13 and Figure 3.14 implied that HIF-1 may play a role in the hypoxia-induced IL-10 inhibition either in a direct or indirect way, and the negative element that caused the suppression of IL-10 expression may be located within the -4kb region. If HIF-1 exerts an effect in inhibiting IL-10 expression directly on the IL-10 promoter, it is possible that the HRE located between the -2,171bp and -2,187bp on the IL-10 promoter allows the binding. Further experiments are required to confirm whether this negative element locates in the -195bp IL-10 promoter region (i.e. to prove whether the hypoxia inhibition effect can be seen in the -195bp IL-10 Adv constructs). If the negative element locates within the -195bp region, we will need to reconsider how HIF-1 functions in the hypoxia to cause the blockade of IL-10. On the other hand, to confirm the physical interaction between HIF-1 and IL-10 promoter, binding assays such as Chromatin immunoprecipitation (ChIP) will be required.

4.4 The effect of hypoxia on IL-10 expression is observed with a range of different toll-like receptor ligands, and is not limited to induction by LPS

This important result (Figure 3.14) showed that the blockage effect of hypoxia was also seen in cells stimulated by other TLR ligands, and is not just limited to treatment with LPS. This figure (Figure 3.14) showed that IL-10 induction via TLR1, 2, 4, 5, 6, and 8 ligands was suppressed in hypoxia.

Tests with TLR3 ligand, poly I:C and TLR9 ligand, ODN CpG were eliminated since optimization was not successful. TLR3 is a receptor located in the cytoplasm which recognises double-stranded RNA from viral infection. It is able to bind intracellular Poly I:C, which is a synthetic analogue of double-stranded RNA (Wang *et al.*, 2007; Akira *et al.*, 2006; Fortier *et al.*, 2004). TLR9 is activated by bacterial DNA or its analogue (e.g. oligodeoxynucleotides, or ODN) containing unmethylated CpG dinucleotides. It primarily localizes in the endoplasmic reticulum (ER), and it complexes with CpG intracellularly when activated (Merrell *et al.*, 2006). However when outside of the cell, the ligands are no longer accessible to these intracellular TLRs (Akira *et al.*, 2006; Leifer *et al.*). Attempts were made to activate the cells by delivering the ligands into the cell (PBMC) with JetPEI® transfection reagent. A maximum level of 2μg of poly I:C and ODN CpG were delivered to cells for induction. Unfortunately, the induction was not able to be optimized; the cells were not activated with this approach (confirmed by looking at the TNF mRNA level; Valentinos Kounnis, University of Leicester, MSc thesis 2008; B. Burke, personal communication). For this reason, we decided to eliminate these two ligands, and to focus on the other TLR stimulants.

On the other hand, the TLR7 ligand imiquimod failed to induce IL-10 expression. Therefore, the effect of hypoxia on the TLR7-induced IL-10 production, if there is any, cannot not be

identified. On the other hand, only the down-regulation of IL-10 by hypoxia was considered significant in the SAE LPS, *E. Coli* LPS (which activate macrophages via TLR4), and MALP-2 (which brings macrophage activations via TLR6/2 heterodimer), treated samples due to limited number of experiment. More experiments would be able to confirm the significance of the influence of hypoxia on IL-10 levels in macrophages activated by different TLR ligands.

As mentioned in the introduction section, despite the different ligands and receptors involved in each pathway, the signals stimulating all of the toll-like receptors later converge toward the MyD88-dependent pathway. However, there is evidence indicating the presence of MyD88-independent pathway (Takeda & Akira, 2003). TLR4 and TLR3 were reported to be able to activate IRF3, JNK, and NFκB in MyD88-deficient mice (Kawai *et al.*, 1999; Alexopoulou *et al.*, 2001). Since LPS activates the cell via TLR4 ligand, which can transmit the signal to the nucleus by either MyD88-dependent or MyD88-independent pathway, the inhibition effect from hypoxia on IL-10 may take part in either pathway. Furthermore, it was reported that MyD88-dependent and MyD88-independent pathway both triggers the activation of NFκB and JNK, and both pathways are markedly synergistic in mouse study (Bagchi et al., 2007). Hence, it is also possible that hypoxia-induced effector protein acts on both pathways to cause the inhibition of IL-10 production, or the inhibition may be caused on the effector protein downstream of NFκB or JNK. If the down-regulation of IL-10 was caused by the effector protein(s) upstream of the NFκB activation in the MyD88-independent pathway, the blockage would not be able to be observed in none-

TLR4 and TLR3 activated cell. The experiment we conducted tested which pathway and at which stage may be involved in the hypoxia-induced blockage of IL-10.

We therefore suggest that the blockage effect on IL-10 is common in at least some of the TLR ligand-stimulated pathways, and hypoxia may trigger the effector that restrains IL-10 transcription via the downstream effector protein in the MyD88-dependent pathway. On the other hand, since both MyD88-dependent and MyD88 independent pathways eventually activate NFκB, JNK, and IR3 protein, the hypoxia-activated IL-10 inhibitor could also act on the effectors downstream of the NFκB, and JNK (Bagchi *et. al.*, 2007).

The finding that the hypoxia-dependent decrease in IL-10 production is not limited to LPS or TLR4 stimulated macrophages is another interesting finding. This indicates that the inhibition of IL-10 production by hypoxia is not limited to infections from specific pathogens, which is crucial to the host organism. Although we hypothesise that IL-10 production is decreased in hypoxia to allow enhanced inflammation and wound healing and pathogen removal, lack of IL-10 which suppresses inflammation may cause exaggerated tissue damage or even septic shock led by excessive TNF production (Gérard *et. al.*, 1993). Thus, our findings may also be beneficial to studies in immunity disorder treatment.

5. Conclusion

In conclusion, we confirmed that hypoxia was able to reduce IL-10 mRNA basal and TLR-ligand stimulated levels, in response not only to LPS (Figure 3.2) but also in response to a range of other TLR ligands (Figure 3.14).

Similar to the effects of hypoxia, HIF-1 inducing agents $CoCl_2$ and DFO were able to reduce the basal level of IL-10 (Figure 3.12). $CoCl_2$ treatment was also able to mimic the effect of hypoxia on LPS-stimulated IL-10 expression. In contrast, addition of DFO in the cell culture did not have notable effect on LPS-induced IL-10 production. Since the completion of my laboratory work, the theory was put forward that the DFO stock I used for some experiments was inactivated due to repeated freeze-thawing of a single aliquot. There is now evidence to support this idea (Bernard Burke, personal communication).

The down-regulation of the IL-10 promoter by $CoCl_2$ was also observed in exogenous IL-10 promoter adenovirus constructs (-4kb IL-10 Adv). Our result (Figure 3.13) showed that treatment with $CoCl_2$ significantly reduced the -4kb IL-10 Adv reporter gene (luciferase) activity after stimulation with LPS. Interestingly, DFO treatment also significantly diminished the LPS-induced -4kb IL-10 Adv activity. This result suggested that it is likely that HIF-1 may participate in the negative regulation of IL-10, despite $CoCl_2$ and DFO treatments having different effects in the endogenous IL-10 response in LPS-treated samples. Further approaches including study of IL-10 response in HIF-1 knocked out or silenced cells may help to finally confirm whether or not HIF-1 may be responsible for the reduction of IL-10 induction by TLR ligands in hypoxia.

The result we obtained (Figure 3.3~ 3.10) showed that the IL-10 plasmid promoter constructs we generated failed to show hypoxic blockade. Therefore we were not able to identify the regulatory element(s) on the IL-10 promoter that is (are) responsible for the hypoxia blockage effect. This may be due to causes including the mutations in the constructs, cell line used, plasmid activities, and the effects of the transfection reagents, etc. Our research focused on primary macrophages, which are relatively complex to use for transfection studies (personal communication with Bernard Burke). More efforts are required to choose the most appropriate transfection agents and to optimize the desired condition that enables the effect of hypoxia on IL-10 constructs to be observed.

This piece of research is important for the reason that IL-10 is a crucial immuno-suppressor that down-regulates the production of various pro-inflammatory cytokines such as TNF, IL-1 and IL-6, etc., which are involved in inflammations and several autoimmune diseases (Revel & Schattner, 1987; Stanton *et. al.*, 1985; Matsuda *et. al.*, 1989). IL-10 itself was also reported to be related to autoimmunity in NZB/W F1 mice due to its effects on other cytokines (Ishida *et. al.*, 1994). Hypoxia is a common outcome from pathological conditions. After thorough study of the relationship between hypoxia and IL-10, one may be able to identify the suppressor(s) that participate(s) in the downregulation of IL-10 expression and production. The results presented here may help to better understand IL-10 regulation. Continuation of this research may, in the longer term, make possible gene therapy that targets the hypoxic IL-10 suppressor mechanism in infected or other pathological tissue and helps to enhance the immune response.

6. References

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