Interleukin-6 promoter polymorphisms, chromatin remodelling and gene expression in a monocyte-derived macrophage model of inflammation

by

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Abstract

Interleukin-6 (IL-6) is a pro-inflammatory cytokine produced by macrophages, endothelial cells, fibroblasts, vascular smooth muscle cells, T lymphocytes and adipocytes. IL-6 is regulated at the level of transcription. There are multiple regulatory elements in the IL-6 promoter. Three single nucleotide polymorphisms at position -597, -572 and -174 and a variable region of A's and T's at -373 have been identified in the IL-6 proximal promoter.

The THP-1 macrophage-like cell line was induced to express IL-6 mRNA in response to IFN-γ and LPS. Interferon-gamma induced IL-6 mRNA expression was inhibited by treatment with either atorvastatin or pravastatin. Monocytes extracted from the blood of healthy volunteers of known IL-6 promoter haplotype (+GG9/11G and -GG9/11G) were differentiated to macrophages ex vivo. These cells were cultured in the presence of IL-1β, LPS or IFN-γ, to induce IL-6 mRNA. Quantitative PCR was used to quantify the level of IL-6 mRNA. IL-6 mRNA in macrophages from individuals with the IL-6 promoter haplotype +GG9/11G was significantly induced in response to IL-1β when compared to -GG9/11G individuals. A similar though non significant effect was detected in response to IFN-γ. No significant IL-6 promoter haplotype-specific difference was observed in response to LPS.

Chromatin remodeling of the IL-6 promoter occurred within 30 minutes of stimulation and was specific to the region of the IL-6 promoter and the stimulus applied to the cells. A DNase I hypersensitive site was identified in
the region of the polymorphic AnTn variable tract and a potential DNA cruciform structure in the IL-6 promoter was resolved by T7 Endonuclease I. Atomic force microscopy was used to visualise DNA cruciform structure in IL-6 promoter haplotype constructs.

This work contributes to the understanding of the molecular mechanisms underlying the normal inter-individual differences in the macrophage IL-6 inflammatory response.
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Abbreviations

AFM  Atomic force microscopy
ARE  AU rich elements
C/EBP  CCAAT/enhancer-binding protein
CHART-PCR  Chromatin Accessibility Real Time PCR
CRP  C-reactive protein
c t  Cycle threshold
dTHP-1  Macrophage-like THP-1 cells
f  Frequency
FBS  Foetal bovine serum
RFLP  Restriction fragment length polymorphism
GRE  Glucocorticoid response element
hsp  Heat shock protein
IFN-γ  Interferon-gamma
IL-1ra  Interleukin-1 receptor antagonist
IL-1β  Interleukin-1 beta
IL-6r  Interleukin-6 receptor
LPS  Lipopolysaccharide
ng  nanogram
OR  Odds ratio
PBS  Phosphate buffered saline
PMA  Phorbol myristate acetate
PSQ  PyroSequencing
QPCR  Quantitative PCR
RFLP  Restriction fragment length polymorphism
sIL-6r  Soluble Interleukin-6 receptor
SNP  Single nucleotide polymorphism
THP-1  Monocytic THP-1 cells
UTR  Untranslated region
Chapter 1 Introduction

1.1 Overview
Interleukin-6 (IL-6) is a 26kDa inflammatory cytokine with biological actions in inflammation and the regulation of endocrine and metabolic functions. IL-6 is produced by immune and non-immune cells, including endothelial cells, fibroblasts, monocytes, macrophages and T lymphocytes (Figure 1.1). IL-6 is a member of a larger family of cytokines including interleukin-11, oncostatin-M, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotropin-1 and leptin (Papanicolaou et al, 1998; Papanicolaou and Vgontzas, 2000).

1.2 Actions of Interleukin-6
IL-6 stimulates the hypothalamic-pituitary-adrenal axis and is suppressed by glucocorticoids (Ray et al, 1990; Papanicolaou et al, 1996). IL-6 stimulates the release of growth hormone, inhibits thyroid-stimulating hormone and decreases serum lipid concentrations. When the body is under stress IL-6 is secreted and positively controlled by catecholamines, as seen for example during exercise (Papanicolaou et al, 1996). Animal studies have shown that the administration of IL-6 leads to fever, anorexia and fatigue. Circulating levels of IL-6 are increased in rheumatoid arthritis and osteoporosis. IL-6 increases body temperature and metabolic rate and is stimulated by insulin. C-reactive protein (CRP), an acute phase protein, is induced by IL-6, and is produced in the liver (Papanicolaou et al, 1998). More recently, IL-6 has been shown to inhibit the development of regulatory T lymphocytes, which provide protection against autoimmunity (Bettelli et al, 2006) and could implicate IL-6 in the development of autoimmune conditions, such as
rheumatoid arthritis, type 1 diabetes mellitus and inflammatory bowel disease.

Figure 1.1. Interleukin-6 and its downstream actions and targets
The figure shows the cells which produce IL-6 and the contribution IL-6 makes to downstream targets (adapted from Naka et al, 2002).

1.3 Interleukin-6 receptor
There are two forms of the interleukin-6 receptor (IL-6r); a trans-membrane receptor and a soluble form (sIL-6r) (Peters et al, 1998; Kerr et al, 2001). The sIL-6r is formed from the extracellular domain of the membrane-bound receptor, either as a result of proteolytic cleavage or by alternative mRNA splicing. Activation of either receptor by IL-6 induces homodimerisation of gp130, a trans-membrane receptor involved in signal transduction. Cells
which do not express the IL-6r can therefore be stimulated by IL-6 through the sIL-6r (Peters et al., 1996a; Peters et al., 1996b). Multimeric complexes of IL-6 are found in blood associated with plasma proteins and regulate the transport and bioavailability of IL-6 (May et al., 1992). These complexes have been shown to include the soluble IL-6 receptor (Ndubuisi et al., 1998).

1.4 Interleukin-6 gene

The IL-6 gene is located at chromosome 7p21 and comprises 5 exons and 4 introns (Sehgal et al., 1986; Yasukawa et al., 1987). There is a high degree of sequence conservation between the human and mouse IL-6 gene, particularly in the 5' promoter region, which is involved in the regulation of IL-6 gene expression (Tanabe et al., 1988).

Non-synonymous single nucleotide polymorphisms (SNPs) resulting in an amino acid substitution have been identified in exon 1, exon 2 and exon 3. A synonymous SNP was identified in exon 3. Thirty-eight intron SNPs have also been identified on dbSNP (http://www.ncbi.nlm.nih.gov/SNP). There is a variable length polymorphic AT-rich tract in the 3' untranslated region (UTR) of the IL-6 gene (Bowcock et al., 1989).

1.5 Interleukin-6 promoter

Early work showed that the 5' flanking region of the IL-6 gene contains regulatory elements (Ray et al., 1988) and an interaction with inducible factors (Isshiki et al., 1990), which are activated by other cytokines (tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), platelet-derived growth factor and...
epidermal growth factor) (Faggioli et al, 2004), second messengers and viruses (Ray et al, 1988). It is now known that there are multiple regulatory elements in the IL-6 promoter (Faggioli et al, 2004). These include NF-IL6, a member of the C/EBP (CCAAT/enhancer-binding protein) family (Akira et al, 1990), AP-1 (a specific DNA sequence that binds c-jun and c-fos heterodimers) and NF-κB (Figure 1.2). A glucocorticoid receptor response element (GRE) in the IL-6 promoter has also been demonstrated (Ray et al, 1990) and that activated glucocorticoid receptor represses IL-6 promoter activity. A protein-protein interaction between the glucocorticoid receptor and NF-IL6 transcription factors is thought to lead to the induction of the acute phase protein genes by cytokines and glucocorticoids (Nishio et al, 1993). NF-κB is activated by the pro-inflammatory cytokines TNF-α and IL-1 which induce IL-6 (Zhang et al, 1990). Electrophoretic Mobility Shift Assays have shown complexes of the p65 subunit of NF-κB, C/EBP and a mutated form of c-jun lacking a transactivation domain (Ray and Prefontaine, 1994), indicating a functional synergism and the importance of physical location of the transcription factors for the induction of IL-6 mRNA expression (Xia et al, 1997). Stimuli signalled through different pathways are co-ordinated by direct protein-protein interactions of NF-IL6 and other transcription factors, including NF-κB (Nishio et al, 1993), in the IL-6 promoter (Terry et al, 2000). There is an IL-1 response element consisting of a 14bp palindromic sequence (ACATTGCAACAATCT) in the region of -180 to -123 (Isshiki et al, 1990). Appendix I contains the annotated IL-6 promoter and gene for reference.
1.6 Interleukin-6 promoter polymorphisms

Three SNPs at position -597, -572 and -174 and a variable AT region at -373 have been identified in the IL-6 proximal promoter (Figure 1.2) and extensively studied in relation to disease association.

![Structure of the IL-6 promoter](image)

**Figure 1.2. Structure of the IL-6 promoter**

Schematic diagram showing polymorphisms at -597, -572, -373 and -174 and trans-regulatory elements (GRE glucocorticoid response element, AP-1 c-jun and c-fos binding region, MRE multiple response element, CRE cAMP response element, NF-IL6 nuclear factor IL-6 and NF-κB complex binding) within the IL-6 proximal promoter. The TATA box and transcription start site are indicated (adapted from Terry et al, 2000).

Eight IL-6 proximal promoter haplotypes have been identified (Terry et al, 2000), the four most common of which are shown in Table 1.1. Linkage disequilibrium (the relationship between genotypes at a pair of polymorphic sites (Carlson et al, 2004)) at the IL-6 locus defined the haplotype GG9/11G,
where individuals carrying the 9/11 allele would usually also have G at -597, G at -572 and G at -174.

<table>
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<tr>
<th>Haplotype</th>
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<tr>
<td>-597A -572G 8A/12T -174C</td>
<td>0.36</td>
</tr>
<tr>
<td>-597G -572G 10A/11T -174G</td>
<td>0.24</td>
</tr>
<tr>
<td>-597G -572G 9A/11T -174G</td>
<td>0.18</td>
</tr>
<tr>
<td>-597G -572G 10A/10T -174G</td>
<td>0.12</td>
</tr>
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Table 1.1. Common haplotypes of IL-6 proximal promoter polymorphisms
Four common IL-6 promoter haplotypes and the frequency of each in a Caucasian population (Terry et al, 2000).

1.7 Polymorphisms and disease
Single nucleotide polymorphisms make up ninety percent of DNA variation in the human genome (Collins et al, 1998). Single nucleotide polymorphisms in coding or regulatory regions are more likely to cause functional changes in gene expression. Mapped SNPs are used as markers for finding functional SNPs in the genome, by analysis of linkage disequilibrium over large areas of the genome, and this can differ by population groups. An important review by Clark (Clark, 2004) makes the case that it is necessary to consider a haplotype, and not just a single genotype, when studying the potential of candidate genes in disease.
1.8 Interleukin-6 promoter polymorphisms and disease

Several groups have sought a link between the circulating plasma levels of IL-6, disease occurrence, severity or progression and genetic variation. This section reviews IL-6 expression, promoter polymorphisms and disease association.

1.8.1 Interleukin-6 and cardiovascular disease

Atherosclerosis is the result of a chronic inflammatory process (Ross, 1999). In an early study of human explants tissue culture supernatants of atherosclerotic abdominal aortic aneurysms, IL-6 production was shown to be increased when compared to normal aortic tissue (Szekanecz et al, 2004). Plasma levels of IL-6 and C-reactive protein were measured, as markers of risk for cardiovascular disease, among women participating in the Nurses’ Health Study and men participating in the Health Professionals Follow-Up Study, and who provided a blood sample at baseline (Pai et al, 2004). During the eight or six year follow up respectively, 239 women and 265 men had a non-fatal myocardial infarction or fatal coronary artery disease. The study demonstrated an increased risk of coronary artery disease associated with high levels of circulating IL-6 and CRP at baseline, after adjusting for the traditional cardiovascular risk factors of age, smoking, plasma lipid levels and the presence or absence of diabetes and hypertension.

A mouse model was developed to investigate the role of IL-6 in atherogenesis (Schieffer et al, 2004). Double knockout mice ApoE^{-/-}IL-6^{-/-} were fed a normal chow diet over the lifetime of the mice. Serum cholesterol
levels and subsequent atherosclerotic lesion formation were significantly increased in the ApoE^+IL6^+ mice compared with the ApoE^+IL6^- mice. Contrary to previous information, this study showed that a lifetime deficiency of IL-6 enhances the development of atherosclerosis, suggesting that baseline IL-6 levels are required for lipid homeostasis and vascular remodelling. IL-6 may effect its actions in the atherosclerotic lesion by increasing metalloproteinase-13 gene expression through the Janus kinase JAK2 cell signalling pathway, AP-1 binding activity and c-jun phosphorylation (De la Torre et al, 2005).

In a UK Caucasian population, 132 patients undergoing coronary artery bypass graft surgery were genotyped for the AnTn polymorphism by direct sequencing (Kelberman et al, 2004). Individuals homozygous for the IL-6 -373 9A/11T polymorphism had significantly higher post-operative IL-6 levels than 10A/11T homozygotes. This study also reports that the IL-6 promoter -373 AnTn polymorphic tract is conserved in length between primate species, suggesting that this region is functional, independent of the other single nucleotide polymorphic sites within the IL-6 promoter.

Stroke is a debilitating outcome of cardiovascular disease. Stroke incidence and severity has been studied in relation to IL-6 promoter polymorphisms (Chamorro et al, 2005). Stroke phenotypes was characterised with respect to the presence or absence of the IL-6 -174 CC genotype. Lacunar stroke affects the deep, penetrating cerebral arteries and often occurs due to hypertension. The prevalence of the -174 CC genotype, as determined by
PCR RFLP, was studied in 89 patients with lacunar stroke (19.1%), 82 with stroke due to large vessel disease (8.5%), 53 with cardioembolism (7.5%) and 49 with idiopathic stroke (14.3%), when compared to a matched control group with no history of stroke (n = 105, 8.6%). The -174 CC genotype was associated with lacunar stroke (OR 3.22, 95% CI, 9.09 to 1.12), whereas pooling of the patients with non-lacunar stroke did not show any association of the -174 CC genotype when compared with the control group (OR 1.01, 95% CI, 2.77 to 0.36). The authors propose that the deep cerebral arteries may be more susceptible to IL-6-mediated inflammation.

Forty-eight stroke patients recruited within 24hrs of onset were analysed for serum levels of IL-6r and sIL-6r and genotyped for the IL-6 promoter polymorphisms (Acalovschi et al, 2003). Exclusions were made due to infection, malignancy or admission to intensive care. C-reactive protein, fibrinogen and serum IL-6 were increased in stroke patients. Serum soluble gp130 levels were reduced in the first week after stroke onset but had returned to normal levels at follow-up. No difference was observed between patients and controls with respect to IL-6r. There was an observed difference in the frequency of the GG10/10G IL-6 promoter haplotype between patients and controls. The most frequent haplotype, AG8/12C, was associated with low serum IL-6 levels in stroke patients. Transfection of the astrocyte-like cell line U373 (a cell type important in the study of ischaemic stroke) with constructs of the known IL-6 promoter haplotypes showed a significantly lower IL-6 induction with the AG8/12C construct in response to an adenosine analog, a known stimulus of IL-6 transcription.
Increased levels of the 60kDa heat shock proteins (hsp60) were associated with severe risk of atherosclerosis (Pandey et al, 2004). Healthy, male blood donors (n=176) were recruited and genotyped for the IL-6-174 G/C SNP (Veres et al, 2002). Antibody levels to human hsp60 and *Mycobacterium bovis* hsp65 were determined by ELISA. Carriers of the IL-6 -174 C allele at this position had a significantly lower level of anti-hsp60 and anti-hsp65 antibodies.

Endothelial function was assessed by flow mediated dilatation, in response to endothelium-dependent or independent stimulation (Brull et al, 2002). The effect of the IL-6-174 G/C alleles on endothelial function was assessed in 248 healthy adult volunteers. The authors found a significant difference in the endothelial-dependent responses in smokers who were homozygous for the IL-6 -174 C allele. However, no serum IL-6 measurements were made and the authors speculate that the results observed in the smoking group are indicative of a more complex set of reactions and not a direct cause-effect.

Atherosclerosis not only affects the heart and brain resulting in heart attack and stroke, but can also affect other organ systems. The IL-6 -174 G/C SNP may be associated with a protective effect against renal artery occlusion (Weger et al, 2005). The frequency of the -174 C allele was significantly lower in 182 patients with renal artery occlusion than in 307 matched control subjects. Homozygosity for the C allele was associated with an odds ratio of 0.5 (95% CI, 0.28 to 0.89) for renal artery occlusion. A large study tested the association of known gene variants with acute renal injury following cardiac
surgery (Stafford-Smith et al, 2005). DNA was isolated from pre-operative blood and genotyped by mass spectrometry for 12 candidate polymorphisms. Caucasians and African Americans were analysed separately. The IL-6 -572 C and angiotensinogen -842 C alleles showed a strong association with renal injury in Caucasians (p<0.0001). A less stringent application of the criteria for significance identified four additional polymorphisms in Caucasians (ApoE -448 C, angiotensin receptor I -1166 C and eNOS -894T). The paper draws the conclusion that clinical assessment and identification of genetic variation could be used to predict post-cardiac surgery renal dysfunction.

However, the role of IL-6 in cardiovascular disease is controversial. For example, a study of 57 patients post myocardial infarction showed no association with plasma levels of inflammatory markers including TNF-α, CRP or IL-6 (Annique et al, 2005). Other studies have similarly shown no clear evidence for the involvement of IL-6 promoter polymorphisms in cardiovascular risk and myocardial infarction (Bennet et al, 2003). The authors do however comment on the finding that serum IL-6 levels are increased in cases versus control for men but not for women. In this study matched controls for age, sex, smoking, body mass index, physical activity, biochemical markers and hormone replacement therapy (in women), suggest that the finding of increased serum IL-6 in male patients is associated with myocardial infarction.

Increasing evidence supports the hypothesis that acute infections, possibly mediated by IL-6, rather than the chronic inflammation identified with
atherosclerosis, may be associated with a transient increased risk of cardiovascular events. A study based on the United Kingdom General Practice Research Database, containing the records of more than 5 million patients looked at a total of 20,486 patients with a first myocardial infarction and 19,063 patients with a first stroke, who had received the influenza vaccine (Smeeth et al, 2004). They concluded that there was no increase in the risk of myocardial infarction or stroke in the period after vaccination against influenza, tetanus or pneumococcus. However, it was noted that the risk of both events were significantly increased during the first 3 days of being diagnosed with systemic respiratory tract infection and to a lesser extent in those patients diagnosed with a urinary tract infection. The risk decreased in the following weeks. In this study, circulating IL-6 levels were not measured.

In a small study, subjects were vaccinated with Salmonella typhii to test the role of IL-6 -174 G/C in induction of IL-6 in vivo (Bennermo et al, 2004b). Individuals were homozygous for the -174 C (n=20) or G (n=18) alleles. Subjects homozygous for the G allele showed significantly increased serum IL-6 levels at 6, 8 and 10 hours after vaccination. However, the results do show large error bars, possibly due to the small sample size, and might suggest the additional importance of haplotype in looking for disease association.

Table 1.2 is a summary of the IL-6 promoter genotype with atherosclerotic disease.
<table>
<thead>
<tr>
<th>Study description</th>
<th>Number of participants</th>
<th>Population group</th>
<th>IL-6 promoter genotype</th>
<th>IL-6 promoter genotype association</th>
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<tr>
<td>Coronary artery bypass graft surgery</td>
<td>132</td>
<td>UK Caucasian</td>
<td>-373 AnTn</td>
<td>-373 9A/11T genotype had higher post-operative IL-6 levels</td>
<td>Kelberman et al, 2004</td>
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<td>Deep vessel lacunar stroke</td>
<td>89</td>
<td>Spanish</td>
<td>-174 C/G</td>
<td>-174 CC</td>
<td>Chamorro et al, 2005</td>
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<tr>
<td>Stroke</td>
<td>48</td>
<td>German</td>
<td>-597 A/G</td>
<td>Haplotype AG8/12C associated with low serum IL-6 levels in stroke patients</td>
<td>Acalovschi et al, 2003</td>
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<tr>
<td>Heat shock protein hsp60 in healthy male blood donors</td>
<td>176</td>
<td>Finnish</td>
<td>-174 C/G</td>
<td>-174 C had significantly lower level of anti-hsp60 and anti-hsp65 antibodies</td>
<td>Pandey et al, 2004</td>
</tr>
<tr>
<td>Endothelial function in healthy adults</td>
<td>248</td>
<td>UK</td>
<td>-174 C/G</td>
<td>Endothelial function affected in smokers of -174 C genotype</td>
<td>Brull et al, 2002</td>
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<tr>
<td>Renal artery occlusion</td>
<td>182</td>
<td>Austrian</td>
<td>-174 C/G</td>
<td>Lower frequency of -174 C allele in patients</td>
<td>Weger et al, 2005</td>
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</tbody>
</table>

Table 1.2. Summary of studies of IL-6 in atherosclerosis
List of key findings of IL-6 promoter genotype association with atherosclerosis.
1.8.2 Interleukin-6 and rheumatoid arthritis

Systemic onset juvenile chronic arthritis is a chronic, autoimmune, inflammatory disease with a fever spike once or twice a day, usually at the same time. It has been shown that serum IL-6 levels increase in line with the fever spike and decrease as the body returns to normal temperature. The authors propose that the regulation of IL-6 differs between patients and unaffected individuals and that dysregulation of IL-6 production is a function of IL-6 promoter polymorphisms (Fishman et al., 1998). This study used a reporter gene assay to study the effect of LPS or IL-1 in HeLa cells transfected with either the IL-6 promoter -373 8A/12T, -174 C or the -373 8/12, -174 G construct. A significant reduction in the response to LPS and IL-1 was observed in cells transfected with the -174 C allele compared to the G allele. In a Caucasian group of patients with juvenile chronic arthritis, there were significantly less of the IL-6 -174 GG genotype than in the control group, indicating that the -174 CC genotype may offer a protective effect in development of the disease.

In addition, disease outcome was correlated with genotype of the pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ and the anti-inflammatory cytokines IL-10 and TGF-β in juvenile rheumatoid arthritis (Oen et al., 2005). Genotypes were determined for patients with juvenile rheumatoid arthritis who were participating in a long-term outcome study. In a multivariate analysis the IL-6 -174 GG genotype was positively correlated with pain, whilst an association of the TGF-β codon 25 GG genotype showed a protective
effect against joint space narrowing. The results were inconclusive as to the usefulness of these SNPs as early prognostic indicators of disease.

In a study of juvenile idiopathic arthritis the association with IL-6 promoter polymorphism -174 GC was determined (Ogilvie et al, 2003). Three cohorts from UK, American and French families were recruited. The IL-6 -174 SNP was genotyped by RFLP, heteroduplex analysis or allelic discrimination. The IL-6 -373 AnTn polymorphism was determined by DNA sequencing. When the data from all three cohorts were taken together, a significant association between occurrence of arthritis in patients and the IL-6 -174 G allele was found (p=0.04). When subsets of the systemic juvenile idiopathic arthritis families were compared, the excess transmission of the -174 G allele was to patients more than five years of age at disease onset (p=0.007). No association was observed between the AnTn alleles or the -174 / -373 AnTn haplotypes. The US cohort had a similar significant transmission.

In a follow-up to this study in 2005 (Fife et al, 2005), this work was expanded by looking at other SNPs and polymorphisms in the IL-6 promoter. In addition to the -174, -572, -597 SNPs, the authors also analyzed 103 patients and one or both parents, for a SNP at -1363 G/T and a newly reported insertion/deletion -1480 [CT]. An over represented haplotype was identified in affected individuals, a 4-marker haplotype -1480[+CT], -1363G, -572G, -174G.
1.8.3 Interleukin-6 and obesity

IL-6 is produced by adipocytes (Vicennati et al, 2002) and circulating IL-6 is increased in obese individuals. The IL-6 -174 G/C and IL-6 -572 G/C promoter polymorphisms were studied in a largely male, hypertensive Swedish population (mean age 57 years), to investigate the association between these single nucleotide polymorphisms and obesity (Wernstedt et al, 2004). Body mass index, serum leptin levels, serum IL-6 levels, C-reactive protein, fasting blood glucose and blood lipid levels were measured. A control group of younger non-obese females was also studied. In both groups the IL-6 -174 C allele (=0.46) was associated with higher body mass index and higher serum leptin levels. Obese patients undergoing gastric banding surgery (n=65) were genotyped for polymorphisms in leptin, IL-6 and adiponectin genes (Poitou et al, 2005). Serum concentrations of leptin, IL-6 and adiponectin and body mass index were measured before surgery and at follow up one year later. Weight loss following the surgery was associated with an increased circulating IL-6 concentration in carriers of the IL-6 -174 CC genotype. Carriers of the IL-6-174 CG or GG alleles showed a decrease in circulating IL-6 concentration. This suggests a role for IL-6 in body weight homeostasis.

In Swedish study of eighty-five men were genotyped for the IL-6 -174 G/C SNP and IL-6 gene expression was measured in subcutaneous adipose tissue. Higher fasting insulin levels and greater insulin resistance was observed in IL-6 -174 G allele carriers. Circulating adiponectin levels were lower in G allele carriers, whilst there was a significant increase in IL-6 gene
expression in adipose tissue. There was no difference between IL-6 -174 G or C allele carriers in plasma IL-6 (Yang et al, 2005). This suggests a tissue-specific regulation of IL-6 gene expression.

1.8.4 Interleukin-6 and type 2 diabetes mellitus
In a recent study, 188 cases versus 376 nested controls were drawn from a population study in Germany (Mohlig et al, 2004). Cases were free of diabetes at baseline but had developed the disease at follow up. Individuals with a body mass index greater than 28 and with the IL-6 -174 CC genotype had a five times greater risk of developing type 2 diabetes mellitus compared with other genotypes. Patients in this group were encouraged to lose weight to reduce the risk of developing diabetes.

In a Finnish study of 490 overweight patients with impaired glucose tolerance, genomic DNA was genotyped for SNPs in the TNF-α and IL-6 genes (Kubaszek et al, 2003b; Kubaszek et al, 2003a). Subjects with both the TNF-α -308A and IL-6 -174C alleles showed a greater risk of developing type 2 diabetes.

In a Danish study (Hamid et al, 2005), 6,164 subjects from the Inter99 cohort in the prevention of cardiovascular disease were recruited as controls provided that they had normal glucose tolerance and had no signs of metabolic syndrome, as defined by the World Health Organisation. Cases of diabetes were recruited from a diabetes clinic (n=1389). Cases and controls were genotyped for the IL-6 promoter SNPs at position -597, -572 and -174.
The IL-6 -597 and IL-6 -174 SNPs were shown to be in strong linkage disequilibrium (0.95). The IL-6 -174 G allele was associated with insulin resistance and the IL-6 -572 C allele was associated with increased serum insulin release during the oral glucose tolerance test. The authors report that the -597 G, -572 C, -174 G haplotype was more commonly associated with the type 2 diabetes patients. This is an unexpected finding as previous studies of haplotype frequencies show that this haplotype is uncommon (frequency = 0.05) (Terry et al, 2000). A study from northern Spain recruited Caucasian subjects for a prospective study of the IL-6 -174 G/C SNP and insulin sensitivity (Fernandez-Real et al, 2000). Following an oral glucose tolerance test the area under the curve of serum glucose concentrations showed an allele-specific effect (CC 6.3mmol/l, CG 9.3mmol/l, GG 9.7mmol/l).

A large meta-analysis covering more than 20,000 participants in 21 studies from eight European countries showed that individuals carrying either the IL-6 -174 GC or CC alleles were associated with a decreased risk of diabetes. There was no association with the IL-6 -572 G/C polymorphism (Huth et al, 2006). Furthermore, an association was demonstrated between metabolic syndrome and pre-diabetes in IL-6 -174 C allele carriers (Stephens et al, 2007).

1.8.5 Interleukin-6 and inflammatory bowel disease

The differential production of cytokines linked to SNPs in the promoter regions of cytokine genes has prompted studies of association in known
chronic inflammatory diseases. In a study of 193 patients with inflammatory bowel disease, including 138 with Crohn's disease and 55 with ulcerative colitis, no association was found between the cytokine gene polymorphisms studied and inflammatory bowel disease susceptibility, when compared to 92 controls (Cantor et al, 2005). However, the authors do note an association between disease phenotype and the site of Crohn's disease, and the IL-6 -174 G/C polymorphism. Patients with ileocolonic Crohn's disease were more likely to carry the IL-6 -174 GG genotype than non-ileocolonic Crohn's disease. Patients with ileal Crohn's disease were more likely to possess the IL-6 -174 GC genotype compared to those with non-ileal disease. An increased number of Crohn's disease patients with isolated colonic disease possessed the IL-6 -174 CC genotype compared to those with non-isolated colonic disease.

1.8.6 Interleukin-6 and aging
Chronic inflammation is associated with several neurodegenerative diseases of aging, including Alzheimer's disease, Parkinson's disease, non-Parkinson's dementia and age-related macular degeneration, possibly due to genetic variation and dysregulation of the balance between IL-6 and IL-10 (Caruso et al, 2004; McGeer and McGeer, 2004).

In a study of 362 eighty year old subjects in Denmark (Bruunsgaard et al, 2004), of whom 29 were excluded due to illness, the authors found an association of the IL-6 -174 C allele with mortality in octogenarians. This association was complex and interacted with smoking status. The IL-6 -174
GG genotype showed a protective effect against age-related pathology. In a similar study in Denmark, this finding is corroborated, as the authors observed a significant increase in the IL-6 -174 GG genotype in longevity (Christiansen et al, 2004).

Interestingly, a study conducted in Italy attempted to reproduce previous work on the effect of IL-6 -174 CC genotype on longevity (Capurso et al, 2004). However, the authors did not show any association with the IL-6 -174 C polymorphism and longevity, which they speculate could be related to environmental differences between the populations in Denmark and Italy.

### 1.8.7 Interleukin-6 and other studies

In other studies of the IL-6 -174 SNP, it has been shown that this single nucleotide polymorphism is associated with increased production of IL-6 from leukocytes stimulated ex vivo with lipopolysaccharide for 24 hours (Rivera-Chavez et al, 2003). Leukocytes from individuals homozygous for the G alleles at IL-6 -597 and IL-6 -174 (that is, -597 G, -174 G) showed the greatest production of IL-6, whilst the cells from individuals homozygous for the A allele at IL-6 -597 and the C allele at IL-6 -174 (-597 A, -174 C) showed the lowest response.

Chronic obstructive pulmonary disease is characterized by an abnormal inflammatory reaction to inhaled particles and fumes. Promoter polymorphisms in genes coding for the inflammatory markers TNF-α, TNF-β, IL-6 and IL-10 were studied in a case-control study, of 113 patients
diagnosed with chronic obstructive pulmonary disease and 113 age, sex and smoking-matched patients hospitalised with severe coronary heart disease without pulmonary disease and 243 healthy population controls (Seifart et al, 2005). The results did not show any significant differences in the genotype distribution of the tested SNPs between the chronic obstructive pulmonary disease patients and matched coronary heart disease patients. However, a comparison with the population controls revealed a significant correlation with the IL-10 -1082 G allele being more common in the chronic obstructive pulmonary disease patients (OR 1.66, 95% CI, 1.01 to 2.75).

Patients with pulmonary hypertension and chronic obstructive pulmonary disease had greater serum IL-6 levels than controls and were associated with the IL-6 -174 GG genotype (Eddahibi et al, 2006). Moulds are associated with incidence of asthma and allergy. There was an IL-6 / IL-10 imbalance as measured by ELISA in cell culture supernatants from the human Mono-Mac 6 cell line, stimulated with mycotoxins with or without LPS treatment (Johannessen et al, 2005). In a group of chronic obstructive pulmonary disease cases versus matched controls, there was an association with the IL-10 1082 G allele, but not IL-6 174, and decreased IL-10 levels (Seifart et al, 2005). These data point to the contribution of IL-6 / IL-10 homeostasis in regulating inflammation.

Glucose-6-phosphate dehydrogenase deficiency is associated with resistance to the malaria parasite in African American populations. Seventy-one percent of glucose-6-phosphate dehydrogenase deficient men studied
(n=111) carried the IL-6 -174 G allele. The presence of the IL-6 -174 G allele, together with the presence of the IL-10 -592 A and IFN-γ +874 A allele, is predominant in individuals with ancestry from malaria-endemic regions (Upperman et al, 2005), suggesting a protective effect of these polymorphisms.

Protein gp41 isolated from HIV and incubated with monocytic THP-1 cells resulted in rapid IL-6 gene expression which was downregulated by endogenous IL-10 (Takeshita et al, 1995). The cells were cultured over 7 days. IL-6 increased over days one to four and then IL-10 increased whilst L-6 decreased. This was consistent with data from CD4+ T cells (Weimer et al, 1998).

The cause of recurrent spontaneous abortions are largely unexplained. A case-control study in a southern Brazilian population of 57 women with recurrent spontaneous abortions showed an increased frequency of the IL-6-174 CC genotype (18%) when compared with 74 matched controls (4%) (Von Linsingen, Bompeixe and Bicalho, 2005).

A small study of 29 patients with renal cell carcinoma and 50 healthy controls suggests that the IL-6 -174 CC genotype may be protective for renal cell carcinoma (Basturk et al, 2005).

The rare IL-6 promoter haplotype -597G, -572C, -174G in a Jamaican Caribbean population was associated with susceptibility to the human T
lymphotrophic virus type I among children born to HTLV I-positive mothers (Brown et al, 2006).

Early studies have shown that oestrogens inhibit TNF-α-induced IL-6 expression (Galien et al, 1996) and that 17β-estradiol inhibits IL-6 expression (Pottratz et al, 1994), mediated by the C/EBP and NF-κB sites in the IL-6 promoter (Stein and Yang, 1995), possibly in a tissue-specific manner. This is important for the study of osteoporosis and bone mineral density, particularly in post-menopausal women (Ferrari et al, 2004; Moffett et al, 2004), where lifestyle and dietary factors influenced the effect of IL-6 promoter polymorphism -174 G/C. Bone mineral density is lowest amongst women with the IL-6 -174 GG genotype compared to the CC genotype.

A Danish study of young females showed an association of -174 C with type I diabetes mellitus, which decreases with age possibly due to the increase of 17β-estradiol with puberty (Kristiansen et al, 2003), and this was confirmed by a more recent study (Gillespie et al, 2005) of 521 female patients recruited from the UK-BOX study diagnosed with type I diabetes before the age of 21, although the Danish female IL-6-174 CC population have an earlier age of onset.

1.8.8 Interleukin-6 and population studies

It has previously been shown that population differences account for some of the contradictory information on the effects and correlation of the IL-6
promoter polymorphisms. In particular, studies on Japanese populations vary from Caucasian population studies.

Japanese individuals with chronic periodontitis, n=112, and non-CP controls (n=77) were genotyped for four SNPs in the IL-6 promoter (-597, -572, -190, -174) and the -373 AnTn polymorphic tract (Komatsu et al, 2005), in order to investigate an association of the IL-6-373 AnTn polymorphism with inflammatory disease and serum IL-6 levels. The -190 SNP is not found in Caucasian populations. In this study, all the subjects were homozygous for the -597 G allele, -190 C allele and -174 G allele. The -572 and -373 polymorphisms were in Hardy Weinberg Equilibrium and in linkage disequilibrium. The frequency of the IL-6 -373 9A 11T allele was found to be significantly higher in individuals without chronic periodontitis. Serum IL-6 levels assessed by ELISA were shown to be lower in association with the -572 G and -373 9A 11T haplotype. It is suggested that in this population the -572 G, -373 9/11 haplotype could be associated with a decreased risk of chronic periodontitis. A meta-analysis of prospective and retrospective studies showed that periodontal disease may increase the risk of cardiovascular disease and stroke (Meurman et al, 2004).

1.8.9 Studies showing no association with IL-6 promoter polymorphisms

The search for prognostic markers for disease outcome is ongoing. The number of studies showing no association between cytokine gene polymorphisms and disease outcome is almost as large as the number of
studies published which show one or other SNP may be significantly associated with disease risk and/or outcome. I include the following examples of this negative association.

Researchers genotyped paediatric patients on the heart transplant list for end-stage ventricular failure due to cardiomyopathy or congenital heart disease (Webber et al, 2002). Single nucleotide polymorphisms in TNF-α, IL-10, IL-6, TGF-β and IFN-γ were assessed by PCR RFLP. Patients with ventricular failure after transplantation for dilated cardiomyopathy (n=37) or congenital heart disease (n=34), fifteen patients transplanted for structural disease, such as hypoplastic left heart syndrome without ventricular failure, and an additional eight patients with congenital cardiomyopathy, were compared to data from healthy children. No differences in genotype distribution between the groups was found, although this should not be surprising as cardiomyopathy is not an atherosclerotic disease.

A study in Chinese children with immune thrombocytopenic purpura (fifty children with acute immune thrombocytopenic purpura and thirty children with chronic immune thrombocytopenic purpura) versus one hundred healthy individuals failed to show an association with the IL-6 -572 G/C polymorphism (Wu et al, 2005). In the same study a comparison of IL-4 and IL-10 SNPs with healthy controls showed significant differences. The presence of the IL-4 and IL-10 SNPs may increase the risk of developing childhood chronic immune thrombocytopenic purpura.
In a study published in 2004 (Bennermo et al, 2004a), the authors comment on the limitations of their and other studies and that the presence or absence of IL-6 -174 G/C alleles in particular is not predictive. In this study, a borderline association was reported between the IL-6 -572 G polymorphism and a prediction of cardiovascular risk.

A meta-analysis of 5,383 diabetes cases and 12,069 controls showed no association with the IL-6 -174 G/C SNP. The authors also looked at seven SNPs, three 5' promoter, one in intron 3, one in exon 5 and two in 3' UTR and found no association in either men or women as single polymorphisms or as haplotypes with type 2 diabetes mellitus. All the SNPs were in linkage disequilibrium (D' greater than 0.95) and there was no association with plasma IL-6 levels (Qi et al, 2006).

An important point is made by the authors of a study where no association was found between the IL-6-174 G/C, IL-10-1082 G/A and TGFβ-509 C/T polymorphisms and the premature onset of ischaemic heart disease in an Irish population (McGlinchey et al, 2004). Future work will have to pay attention to both large scale case versus control studies and transmission disequilibrium test, or family-based methods, to ensure consistency of results.

From the literature it is becoming clear that, in the study of the IL-6 promoter, the haplotype and not just a single base change, could affect an individual's IL-6 response. This suggests an important level of regulation influenced by
haplotype (Terry et al, 2000; Acalovschi et al, 2003). The contradictory results of disease association with IL-6 promoter polymorphisms arise not just from gene-environment interactions in different population groups, but also from the small sample size in some studies, the lack of disease severity and progression stratification when comparing disease-polymorphism association, and the emerging importance of studying a haplotype to assess functional genomics.

1.9 Hypothesis
IL-6 proximal promoter haplotype influences the normal ex vivo individual macrophage inflammatory response.

1.9.1 Objectives
1. To establish the THP-1 cell line as a monocyte-macrophage model of the IL-6 inflammatory response.
2. To quantitate IL-6 gene expression in primary monocyte-derived macrophages from healthy individuals of known IL-6 proximal promoter haplotype.
3. To investigate possible mechanisms of IL-6 proximal promoter activation.
Chapter 2 Materials and Methods

2.1 Suppliers

Suppliers used are listed below unless otherwise stated in the text.

<table>
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<tr>
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2.2 Ethics

This study was approved by the NHS South West Surrey Local Research Ethics Committee (04/Q1909/19) and the Advisory Committee on Ethics of the University of Surrey (EC/2004/58/SBMS).

2.3 Recruitment of volunteers

Twenty-seven healthy volunteers (n=11 male, n=16 female) working or studying at the University of Surrey or the Royal Surrey County Hospital and
who had previously participated in a study to investigate inflammatory factors in the metabolic syndrome were recruited. In the initial study, lipids, fasting glucose and C-reactive protein were measured in these volunteers and only those whose serum levels of these analytes were within the reference range for healthy subjects were invited to participate in this study, to avoid those with incipient disease. Any individuals with abnormal levels were referred to specialists as part of the previous study.

2.4 Experimental design

Volunteers were genotyped for the IL-6 promoter polymorphisms. Individuals of known haplotype were recalled for ex vivo culture of monocyte-derived macrophages. IL-6 gene expression, promoter chromatin remodelling and secondary structure were assessed in the THP-1 cell line and primary monocyte-derived macrophages.

2.5 Genomic DNA extraction

Genomic DNA was extracted from white blood cells of consented healthy volunteers using Genecatcher (Invitrogen), according to the manufacturer’s instructions. Briefly, DNA is captured from lysed cellular matter using positively charged magnetic beads at low pH and eluted into TE buffer at pH 8.5.
2.6 Genotyping for IL-6 promoter polymorphisms

Volunteers were genotyped for seven IL-6 promoter polymorphisms as well as two intron polymorphisms (Table 2.1). The THP-1 cell line was also genotyped for the IL-6 proximal promoter polymorphisms.

In addition to the four proximal polymorphisms, three distal promoter polymorphisms at position -1586, -1480 and -1363 were included. The single nucleotide polymorphism at -1586 was previously validated on dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). The -1480in/del and -1363 single nucleotide polymorphisms were included in an analysis of juvenile systemic arthritis trio families (Fife et al, 2005). Two non-promoter SNPs were also genotyped in intron 2 and intron 3 of the IL-6 gene as these were not in linkage disequilibrium with the promoter polymorphisms. All polymorphisms were in Hardy Weinberg Equilibrium, that is, there was no deviation from the expected frequency. Frequency of the polymorphisms were consistent with reported values.

Table 2.1. Methods for genotyping IL-6 polymorphisms

The table shows the IL-6 promoter polymorphisms and IL-6 intron polymorphisms and the method used to genotype each polymorphism (PSQ=PyroSequencing, RFLP=restriction fragment length polymorphism). IL-6 promoter polymorphisms are identified according to their position 5' to the start of transcription. The dbSNP identification number is also given (rs).
2.6.1 Restriction fragment length polymorphism (RFLP)

RFLP assays were used to genotype the IL-6 promoter -1480, -597, -572 and -174 SNPs. The assays for -597, -572 and -174 were described previously (Khwaja, PhD thesis, Oxford 2005), whilst the assay for the -1480 was developed for this project, according to Table 2.2. The primer sequences for all PCR reactions are listed in Table 2.3. All PCR reactions were carried out in 25µl reactions.

The assay for the IL-6 -1480 SNP is described. A PCR product of 197bp was digested to fragments of 153bp and 44bp with BsmA I (New England Biolabs) which recognizes the site 5'-GTCTC-3'. The restriction enzyme digestion condition was optimized in a 25µl reaction volume for 3 hours at 55°C, according to the manufacturer’s recommendations.

2.6.2 Fragment analysis

The IL-6 -373 AnTn polymorphism was genotyped using a method of fragment size analysis (Khwaja and Green, 2006) at the Wellcome Trust Centre for Human Genetics, Oxford. This method allows differences in length of one base pair to be resolved.

A structural motif ‘GTTTCTT’ was included on the 5’ end of the forward PCR primer to ensure complete non-templated adenylation of the 3’ end of the reverse PCR strand. A fluorescently labelled reverse primer was used to label the PCR product. The fluorescently labelled product was digested with
Tsp509 I, which recognizes the site ^AATT, and the fragment lengths analysed by capillary electrophoresis.

2.6.3 PyroSequencing

PyroSequencing (PSQ) is sequencing by synthesis. A complementary strand is built up with the addition of dNTPs in sequence. Each incorporated dNTP leads to the release pyrophosphate which is converted to ATP. ATP mediates the conversion of luciferin to oxyluciferin by luciferase. This visible light signal is detected by a camera and is seen as a peak on the accompanying pyrogram, the height of which is proportional to number of nucleotides incorporated.

PyroSequencing assays were used to genotype the IL-6 promoter -1586 and -1363 SNPs, and the IL-6 intron 2 and intron 3 SNPs. The assay for -1363 was described previously (Fife et al, 2005). Assays for -1586 and intron 2 and 3 were designed using the online SNP Primer Design from Pyrosequencing AB version 1.0.1 (Biotage). The sequences of the PCR primers and PSQ sequencing primers are listed in Table 2.4.

A short region of DNA around each SNP of interest was amplified by PCR using a biotinylated primer in a 50μl reaction volume from 10ng genomic DNA. Biotinylated PCR product (40μl) was captured on 3μl streptavidin-coated sepharose beads (Amersham) with 37μl binding buffer (Biotage) at room temperature by shaking for 5 minutes and immediately captured onto the probe tips of the vacuum tool.
The captured beads and PCR product were denatured by flushing for 5 seconds each with 70% EtOH (v/v) and denaturation buffer (0.2M NaOH) and washed twice in washing buffer (Biotage).

The template DNA was released into 40μl annealing buffer (Biotage) with 5μl sequencing primer (10pmol/μl) in a pyrosequencing 96 well plate. The sequencing primer was annealed to the PCR product for 2 minutes at 80°C and cooled to room temperature for 5 minutes and transferred to the PSQ96 for genotyping.

The sequence around the SNP was input into the PSQ96 software which calculated the dispensation order of the dNTPs (dATPαS, dCTP, dGTP, dTTP), enzyme (DNA polymerase, ATP-sulfurylase, luciferase, apyrase) and substrate (luciferin, adenosine 5' phosphosulfate) reagent volumes required for each assay depending on the sequence and the number of samples.
<table>
<thead>
<tr>
<th>-1480</th>
<th>-597</th>
<th>-572</th>
<th>-174</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR produces a 197bp product.</td>
<td>PCR produces a 163bp product.</td>
<td>PCR produces a 163bp product.</td>
<td>‘Touch-down’ PCR to produce 639bp product.</td>
</tr>
<tr>
<td>Restriction enzyme digestion using BsmA I (NEB).</td>
<td>Restriction enzyme digestion using Type II Fok I (NEB).</td>
<td>Restriction enzyme digestion using BsrB I (NEB).</td>
<td>Restriction enzyme digestion using Nla III (NEB).</td>
</tr>
<tr>
<td>-CT/CT (bp)</td>
<td>-CT/CT (bp)</td>
<td>+CT/CT (bp)</td>
<td>All (bp)</td>
</tr>
<tr>
<td>195</td>
<td>195</td>
<td>153</td>
<td>GG</td>
</tr>
<tr>
<td>153</td>
<td>163</td>
<td>116</td>
<td>GC</td>
</tr>
<tr>
<td>44</td>
<td>47</td>
<td>47</td>
<td>CC</td>
</tr>
<tr>
<td>102</td>
<td>163</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>233</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>121</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. PCR-RFLP assays for genotyping IL-6 promoter polymorphisms

The table lists the PCR product produced and the restriction enzyme used for each genotyping assay for the IL-6 -1480 [CT] in/del, -597 G/A, -572 G/C and -174 C/G SNPs. The expected digestion products (bp) for each genotype are also shown.
<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1480 5'-CAG TGG CTT CGT TTC ATG C-3'</td>
<td>5'-TTG GGG AAA GTG AGG TCA TC-3'</td>
</tr>
<tr>
<td>-597 5'-GGA GAC GCC TTG AAG TAA CTG C - 3'</td>
<td>5'-GAG TTT CCT CTG ACT CCA TCG CAG - 3'</td>
</tr>
<tr>
<td>-572 5'-GGA GAC GCC TTG AAG TAA CTG C - 3'</td>
<td>5'-GAG TTT CCT CTG ACT CCA TCG CAG - 3'</td>
</tr>
<tr>
<td>-174 5'-GGG CTG CGA TGG AGT CAG AG - 3'</td>
<td>5'-TCC CTC ACA CAG GGC TCG AC - 3'</td>
</tr>
</tbody>
</table>

Table 2.3. PCR primers used for genotyping IL-6 promoter polymorphisms
The forward and reverse primer pairs for each IL-6 promoter polymorphism genotyping PCR reaction are shown.
<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Sequencing primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1586</td>
<td>5'-BIO-CAG TGG CTT CGT TTC ATG C-3'</td>
<td>5'-TTG GGG AAA GTG AGG TCA TC-3'</td>
<td>5'-CCT GTC TTA AGC AAC GTA-3'</td>
</tr>
<tr>
<td>-1363</td>
<td>5'-GCA GAG GAC CAC CGT CTC-3'</td>
<td>5'-BIO-AAT CGG TTT CTT TGC TTT GC-3'</td>
<td>5'-CAA CAG AGG TCA CTG TTT-3'</td>
</tr>
<tr>
<td>Intron 2</td>
<td>5'-GAT CCT TCC TGC TGG AAC ATT-3'</td>
<td>5'-BIO-AAG CTA CAA CTC ATT GGC ATC CT-3'</td>
<td>5'-GTG TGC CAG GCA CTT TA-3'</td>
</tr>
<tr>
<td>Intron 3</td>
<td>5'-AGA TCC AGG CAG CAA CAA AAA GT-3'</td>
<td>5'-BIO-TGA GAG TAC CTT TCC CAG GAT GA-3'</td>
<td>5'-TCA TGA GGA GGC CAA-3'</td>
</tr>
</tbody>
</table>

Table 2.4. Primers used for PSQ genotyping of IL-6 promoter -1586 and -1363 and IL-6 intron 2 and intron 3

The table shows the primers used for the PCR reactions prior to PSQ genotyping. Each reaction has a biotinylated primer which produces a biotinylated PCR product required for the PSQ protocol.
2.7 THP-1 cell line culture

THP-1 monocytic cells were cultured in suspension at 37°C, 5% CO₂ in a humidified incubator with RPMI1640 with Glutamax I (stabilized essential amino acid L-glutamine) (Invitrogen), 10% (v/v) heat inactivated foetal bovine serum (FBS) (Invitrogen) and 1% (v/v) penicillin-streptomycin (5000U/ml penicillin G sodium, 5000µg/ml streptomycin sulphate) (Invitrogen). The stocks of THP-1 cells tested clear for Mycoplasma infection (M. arginini, M. hyorhinis, M. laidlawii, M. orale).

2.7.1 Differentiation of THP-1 cells

THP-1 monocytic cells (passage 2 to 4) were differentiated to macrophage-like cells by phorbol myristate acetate (PMA) (Tsuchiya et al, 1982) as follows: the cells were pelleted by centrifugation at 1000rpm for 5 minutes at room temperature, washed with 50ml sterile PBS and counted using a haemocytometer. The cells were resuspended in RPMI1640 with Glutamax I, 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin (5000U/ml penicillin G sodium, 5000µg/ml streptomycin sulphate) to a density of 4 x 10⁵ cells/ml, seeded at 2.4 x 10⁶ cells per well and cultured with PMA at a final concentration of 10nM for 72 hours.

2.7.2 Stimulation of THP-1 cells

Monocytic (in suspension) and macrophage-like (adherent) THP-1 cells at a density of 2.4 x 10⁶ cells per well in a 24 well tissue culture plate were treated with either 2.5ng/ml IL-1β, 100U/ml IFN-γ or 100ng/ml LPS (Table 2.5).
Table 2.5. Inflammatory stimuli used for inducing IL-6 in THP-1 cells
The table summarises the inflammatory stimuli and concentrations used in the treatment of THP-1 cells to induce IL-6.

2.7.3 Statin treatment
Stock concentrations of 20mM of pravastatin sodium (Sigma-Aldrich) and atorvastatin calcium (gift from Dr Graham Smith, AstraZeneca) were prepared in 100% DMSO and stored at -20°C. The statins were diluted in cell culture medium to a final concentration of 1μM, 5μM and 20μM in 0.1% (v/v) DMSO. The untreated control was 0.1% DMSO (v/v) in cell culture medium.

2.8 Extraction of RNA
Cells were lysed using TRIzol (Invitrogen), a phenolic compound, at a concentration of 1ml/10cm² and the total RNA extracted according to the
manufacturer's instructions by chloroform phase separation and isopropanol precipitation.

Total RNA was resuspended in 20µl DEPC-treated water, by gentle tip mixing. Quantitation and A260/A280 ratio was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The RNA was treated with DNase I (Promega) according to the manufacturer's instructions, to remove any DNA contamination, divided into aliquots and stored at -80°C.

2.9 Quantitative PCR (QPCR)
Total RNA extracted from cells was used to measure gene expression levels in a one-tube, two-step Sybr Green system. The reverse transcriptase enzyme was active at 50°C and the Taq polymerase was activated at 95°C.

2.9.1 Primer design
Primers for the quantitative PCR step were designed previously (Khwaja, PhD thesis, 2005, Oxford). The IL-6 primer pair spanned an intron-exon boundary to ensure the quantitation of mRNA expression and not genomic DNA.

The MLN51 primer pair flanked a large intron to ensure that only specific mRNA expression products were quantitated. The primer sequences were as follows:
IL-6_LH 5'-CTA GAT TCT TTG CCT TTT TCT GC-3'
IL-6_RH 5'-GAT TCA ATG AGG AGA CTT GCC-3'
MLN51_LH 5'-CAA GAG TGC TGA GGA GTC GG-3'
MLN51_RH 5'-TCA TTA GCT TCT GAT TTC AG-3'

2.9.2 QPCR method
RNA was diluted in RNase-free water to 20ng/3μl. All RT-QPCR reactions were carried out in 12.5μl reaction volume using the QuantiTect Sybr Green RT-PCR kit (Qiagen), according to the manufacturer's instructions. The cycling parameters were as follows: 50°C for 30 minutes, 95°C for 15 minutes, 95°C for 15 seconds followed by 58°C for 1 minute for 40 cycles. A melt curve of the QPCR product was produced for each reaction to show the specificity of the reaction.

2.10 Measurement of IL-6 in tissue culture supernatants and plasma
Supernatants were collected from THP-1 cells and primary monocyte-derived macrophages in culture. The supernatants were divided into aliquots and stored at -80°C.

Plasma was separated from whole blood by centrifugation at 1500rpm (500g) for 20 minutes at room temperature. The white blood cell layer (buffy coat) was removed for genomic DNA extraction. The plasma was removed, divided into aliquots and stored at -20°C.
2.10.1 IL-6 high sensitivity ELISA

IL-6 protein was measured in duplicate in cell culture supernatants and plasma using the QuantiGlo Chemiluminescent sandwich ELISA kit (R&D Systems), according to the manufacturer’s instructions. A standard curve ranging from 0.48pg/ml to 30,000pg/ml was produced from recombinant human IL-6 according to the manufacturer’s instructions.

2.11 Primary monocyte-derived macrophage culture

2.11.1 Isolation of mononuclear cells from whole blood

Blood (40ml) was collected from the volunteers by venepuncture, into EDTA coated tubes to prevent coagulation. The blood was immediately diluted 1:1 with sterile PBS at room temperature. Diluted blood was layered onto an equal volume of Histopaque-1077 (Sigma-Aldrich) and the peripheral mononuclear cells isolated by density gradient centrifugation, according to the manufacturer’s instructions.

The mononuclear cell pellet was resuspended in warmed 5ml serum-free medium RPMI1640 with GlutaMax I (Invitrogen), 1% (v/v) penicillin-streptomycin (5000U/ml penicillin G sodium, 5000μg/ml streptomycin sulphate) (Invitrogen).

Viable cells were counted using 0.4% (v/v) Trypan blue exclusion (Invitrogen) in a haemocytometer. Cells were seeded at a density of 2 x 10^6 cells/well in a 24-well plate and cultured for 2 hours to allow adherence. After 2 hours the medium was removed and replaced with RPMI1640 with Glutamax I, 10%
(v/v) FBS, 1% (v/v) penicillin-streptomycin (5000U/ml penicillin G sodium, 5000µg/ml streptomycin sulphate).

2.11.2 Ex vivo primary cell culture

The primary cells were cultured for 8 days in RPMI1640 with Glutamax I, 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, at 37°C, 5% CO_2 in a humidified incubator. The medium was replaced daily for 4 days and then at day 6 and at day 8 prior to stimulation, when the monocytes had differentiated into macrophages (Kaplan and Gaudernack, 1982; Ohyama et al, 2000; Malaguarnera et al, 2005). The cells were then stimulated as described for the THP-1 cells in section 2.7.2.

2.12 Chromatin accessibility real time PCR (CHART-PCR)

Briefly, intact nuclei were extracted from cells in culture. The nuclei were digested with DNase I, which preferentially digests regions of chromatin that are more open as a result of active transcription (Rao et al, 2001; Brettingham-Moore et al, 2005; Weinmann et al, 1999). Quantitative PCR was used to measure the amount of DNA not digested by DNase I, that is the condensed, inactive chromatin, across the IL-6 promoter, as summarised in Figure 2.1.
Figure 2.1. Summary of CHART-PCR assay
DNase I preferentially digests regions of open chromatin resulting in less product as determined by QPCR. Conversely, in closed chromatin there is no accessibility to DNase I and consequently more QPCR product.

2.12.1 Nuclei extraction
Intact, functional nuclei were extracted following lysis of the cell membranes and recovery of the nuclear fraction by centrifugation, using the Nuclei EZ preparation kit (Sigma-Aldrich). The manufacturer's instructions were followed but scaled down to extract nuclei from $2 \times 10^5$ cells, as described below.
Adherent cells in a monolayer were washed with 1ml cold sterile PBS and lysed with 400μl Nuclei EZ lysis buffer. The lysed cells were transferred to a 1.5ml tube and incubated on ice for 5 minutes.

Cells cultured in suspension were pelleted by centrifugation at 500g at 4°C for 5 minutes. The supernatant was discarded and the pellet washed once with ice-cold PBS before lysing in 400μl Nuclei EZ lysis buffer on ice for minutes.

The lysis products of either monolayer or suspension cultures were centrifuged at 500g at 4°C for 5 minutes. The supernatant containing cytoplasmic components was discarded, leaving a pellet of nuclei. The pellet was resuspended in 20μl Nuclei EZ storage buffer. An aliquot of nuclei was set aside for counting with Trypan blue, where the nuclei stain blue due to the permeable membranes. The nuclei were stored in aliquots at -80°C.

2.12.2 DNase I digestion

The nuclei were treated with DNase I (Promega) for 10 minutes at 37°C, according to the manufacturer’s instructions. The reaction was stopped by adding 1μl 20mM EGTA pH8.0. The nuclei were kept on ice before and after DNase I digestion.

2.12.3 Preparation of genomic DNA from nuclei

Genomic DNA was extracted from DNase I digested nuclei using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer’s instructions.
Briefly, the nuclei were lysed by proteinase K digestion prior to immobilization of the DNA to a membrane filter. After washing, DNA was eluted from the membrane and quantitated using a NanoDrop spectrophotometer (NanoDrop Technologies).

2.12.4 CHART-PCR assay design

A 650bp annotated sequence of the proximal IL-6 promoter is shown in Figure 2.2. PCR primers for three regions of the IL-6 promoter (Figures 2.2 and 2.3) were designed using Primer3 (Rozen and Skaletsky, 2000), a web-based application (http://primer3.sourceforge.net/) (Table 2.6). Primers were designed to amplify each of the regions in red (1, 2 or 3) and to quantitate the chromatin accessibility in each region of the IL-6 promoter.

Quantitative CHART-PCR was carried out using the QuantiTect Sybr Green PCR kit (Qiagen) according to the manufacturer’s instructions. The cycling parameters for Region 1 were as follows: 95°C for 15 minutes, 95°C for 30 seconds followed by 64°C for 30 seconds for 40 cycles. The cycling parameters for Region 2 and 3 were: 95°C for 15 minutes, 95°C for 30 seconds followed by 58°C for 30 seconds for 40 cycles. A melt curve of the QPCR product was produced for each reaction to show the specificity of the reaction.

2.13. T7 Endonuclease I digestion

Nuclei were digested with T7 Endonuclease I (New England Biolabs), a cruciform structure resolving enzyme, for 30 minutes at 37°C, according to
the manufacturer's instructions. Genomic DNA was extracted from T7 Endonuclease I digested nuclei using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's instructions. Quantitative PCR was carried out as described in section 2.12.4.

5'-gaagaaagtgcgagaagccacgogtgqqaaaaaagagtcacacactccaa

CHART_597_572_FOR REGION 1+ -597 A/G
cctggagacg ccttgaagta actgcaagaa attggagggt ggccaggcaag
-572 G/C

tctacaacacgccttcaca gggagagccgaacacagaa gaactcagat

gactgtagt attacctctt cctataccccc agcttgggg ggcgctgcgtg
CHART_597_572_REV CHART_373_FOR REGION 2-
gagtcagagggaaactcagtt cagaaacactt tttgtttttta caaatcaaa
-373 AnTn
ttaactggaa cgctaaatctc tagcctgtta atctggcaact tgb3oaane

CHART_572_373_REGION 2-
ttggttttta caaatacaaa
ttaactggaa cgctaaatctc tagcctgtta atctggcaact tgb3oaane

CHART_373_REV

aaaactcgt gcatgacttc agctttactc tttgtcaaga catgccaaag

tgctgatcctaataaaaaagaaaaaagaa agtaaaggaa gagtgggttct

CHART_174_FOR REGION 3-
gttettgac getagcctca atgacgacct agtgcact tttcccctta
-174 C/G

gttgtgcttt gcagatgcta aaggagcgcac atgcaacaat cttataagg
CHART_174_REV

tttcaaatcagccgctctgtgccct accotoaacc tocaccacag

atatatcaaa tgtgggatttt tcccatgagt ctcaatatta gagtctcaac-3'

Figure 2.2. Annotated IL-6 promoter sequence

CHART-PCR primers are shown in red. The four common IL-6 promoter polymorphisms at position -597, -572, -373 and -174 are highlighted. Regions 1, 2 and 3 are indicated (→).
Figure 2.3. IL-6 proximal promoter showing CHART-PCR regions

Schematic representation of the IL-6 promoter showing the polymorphic regions in relation to the CHART-PCR regions (—) (adapted from Terry et al, 2000).
<table>
<thead>
<tr>
<th>Region 1</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-GGA GAC GCC TTG AAG TAA CTG C - 3'</td>
<td>5'-GAG TTT CCT CTG ACT CCA TCG CAG - 3'</td>
</tr>
<tr>
<td>Region 2</td>
<td>5'-AAA CTC AGT TCA GAA CAT CTT TGG - 3'</td>
<td>5'-AGC TGA AGT CAT GCA CGA AG - 3'</td>
</tr>
<tr>
<td>Region 3</td>
<td>5'-GCC TCA ATG ACG ACC TAA GC - 3'</td>
<td>5'-GTG GGG CTG ATT GGA AAC - 3'</td>
</tr>
</tbody>
</table>

Table 2.6. PCR primers used for IL-6 promoter CHART-PCR
The forward and reverse primer pairs for CHART-PCR for each IL-6 promoter region.
2.14 Atomic Force Microscopy

Atomic force microscopy (AFM) allows the resolution of molecules in the nanoscale. Briefly, a cantilever probe scans the surface of the molecule. Forces between the molecule and the probe are detected by a laser and interpreted as topographical features of the molecule.

2.14.1 Sample preparation

A mica slide (Agar Scientific) was freshly cleaved using Sellotape™. One hundred microlitres of dilute (0.001% v/v) 3-Triethoxysilylpropylamine (Sigma-Aldrich) was dropped onto the cleaved surface and incubated for 20 minutes at room temperature, to increase the adsorption of the DNA to the mica slide (Umemura et al, 2001). The slide was rinsed three times with 1ml MilliQ water and dried with a gentle flow of nitrogen gas.

Five microlitres (2 μg) of supercoiled plasmid DNA was dropped onto the prepared mica surface and allowed to adhere for 2 minutes at room temperature. The slide was rinsed three times with 1ml MilliQ water and dried with a gentle flow of nitrogen gas.

2.14.2 Scanning conditions

DNA molecules were scanned on the atomic force microscope (AFM) (NT-MDT) using a cantilever of length 100mm, width 35mm and spring constant (κ) 5.5 – 22.5 (NSG-10, NT-MDT), where the greater the spring constant, the stiffer the cantilever. This affects the scanning properties of the cantilever. The natural resonance (frequency) of the cantilever was determined in the
range of 190 -- 325 kHz and was typically between 265 and 268 kHz. The magnitude of the peak signal from the cantilever was set within a limit of 20 - 25 nAmp. The phase angle of the cantilever oscillation was adjusted to 90° to the frequency.

DNA molecules adhered to mica slides were visualised by scanning in air in tapping mode at 1.56Hz. The images were captured as height (topography) or phase (stiffness) files using the NOVA959 software and converted to JPEG format.

2.15 Statistical analysis
Statistical significance was set at P<0.05 and P values are quoted to one significant figure. One-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni post hoc test, were used to compare values in three or more categories. The two-tailed unpaired t test was used to compare measurements in two categories. All analysis was done using GraphPad Prism version 4.00 for Windows, GraphPad Software (www.graphpad.com).
Chapter 3 IL-6 gene expression in THP-1 cell line

3.1 THP-1 cells

THP-1 cells were established from the peripheral blood from a 1 year old Japanese boy with acute monocytic leukaemia (Tsuchiya et al, 1980). The cells are monocytic in morphology and can be differentiated into macrophage-like cells using phorbol myristate acetate, a protein kinase C activator. The cell stocks used in this work were obtained by Dr Green from the Wellcome Trust Centre, Oxford University. The cells were cultured and differentiated to macrophage-like cells as described in section 2.7.

3.1.1 Haplotype determination of THP-1 cells

Genomic DNA was extracted from $5 \times 10^6$ cultured THP-1 cells in suspension, using the DNEasy Tissue kit (Qiagen), according to the manufacturer’s instructions. Briefly, the cells were lysed to release the nucleic acid and treated with proteinase K to remove cellular protein. DNA was bound to the spin column membrane, washed and finally eluted into TE buffer. DNA concentration (ng/μl) was estimated using the Nanodrop spectrophotometer.

The THP-1 IL-6 promoter was genotyped as described in section 2.6.1 and the haplotype assigned as -597G / -572C / -37310A10T / -174G written as GC10/10G (homozygous), which is rare in a Caucasian population. The THP-1 cell line was derived from a Japanese boy and this accounts for the rare haplotype.
3.1.2 Measuring IL-6 mRNA expression in THP-1 cells

Monocytic (THP-1) (in suspension) and macrophage-like (dTHP-1) (adherent) (Figure 3.1) cells at a density of $2.4 \times 10^6$ cells per well in a 24 well tissue culture plate were treated with either 2.5ng/ml IL-1β, 100U/ml IFN-γ or 100ng/ml LPS for 24 hours.

Total RNA was extracted from the cells as described in section 2.10. Interleukin-6 mRNA expression was quantitated using the QuantiTect RT-PCR Sybr Green kit (Qiagen) according to the manufacturer’s instructions in the ABI Prism 7000 (Applied Biosystems) instrument. Interleukin-6 mRNA expression was normalized to a MLN51 mRNA expression, a housekeeping gene which has been shown not to respond to cytokine stimulation (Hamalainen et al, 2001; Khwaja, PhD thesis, Oxford, 2005).

Serial dilutions of total RNA extracted from differentiated THP-1 cells were used to produce standard curves for IL-6 and MLN51 mRNA expression (Figure 3.2). The log nanogram (ng) amounts of RNA (100,10,1,0.1,0.01) were plotted against the cycle threshold (ct) value (mean±SD) and a line of best fit derived, giving an equation to calculate amounts of RNA from the unknowns.
Monocytic THP-1 cells in suspension

Macrophage-like THP-1 cells in a monolayer

Figure 3.1. Phase contrast light microscopy of THP-1 cells
Monocytic (undifferentiated) THP-1 cells were cultured in suspension. Macrophage-like (differentiated) THP-1 cells were cultured as an adherent monolayer. Magnification x 200 before reproduction.
Figure 3.2. Standard curves for IL-6 and MLN51 mRNA expression
Repeated measurements of amplification of serial dilutions of known amounts of total RNA are used to produce the standard curves for each gene.
Figure 3.3 is an example of a PCR amplification plot for IL-6 mRNA in duplicate samples. The ct value is indicated. This is the cycle number at which the amplification becomes exponential, for this sample at 31 cycles of the PCR.

Figure 3.3. Amplification plot in duplicate samples, showing cycle threshold
RNA was reverse transcribed and amplified in a one step reaction using the QuantiTect SYBR Green RT-PCR kit. The graph shows the real time amplification plot for IL-6 and the ct value (\(c_t\)).
A melt curve or dissociation protocol was performed for all QPCR samples to confirm that a specific product was produced during amplification. Temperature in the wells was increased from 60°C to 95°C. Specific products dissociated at higher temperatures. An example of a specific product that dissociates at 80°C is shown in Figure 3.4.

**Figure 3.4. Dissociation plot of IL-6 mRNA in duplicate samples**

The dissociation plot is the derivative of the melt curve raw fluorescence data as calculated by the ABI Prism 7000 software. Sample shown from duplicate wells.
Although the RNA samples were treated with DNase I prior to QPCR to remove DNA contamination, any remaining DNA in the samples could have affected the quantitation of mRNA expression. Serial dilutions (1:1, 1:2, 1:4, 1:8) of total RNA were reverse transcribed and amplified in a one step reaction using the QuantiTect SYBR Green RT-PCR kit. Amplification of the sample with the greatest amount of RNA appeared first at cycle 24 as expected. The sample with the least RNA appeared last after 28 cycles (Figure 3.5).

![Figure 3.5. Amplification plots of serial dilutions of RNA](image)

The graph shows the real time amplification plot for IL-6 in one sample of RNA.
3.2 IL-6 mRNA expression in THP-1 cells

3.2.1 Induction of IL-6 mRNA expression in macrophage-like THP-1 cells treated for 24 hours

The THP-1 monocyte-macrophage cell line was assessed as a model of inflammation by differentiating monocyctic cells into macrophage-like cells and challenging them with known inflammatory stimuli. The level of IL-6 mRNA expression was quantitated as a marker of inflammation.

THP-1 cells were differentiated into macrophage-like cells and induced to express IL-6 mRNA (Table 3.1) when treated with IFN-γ (P<0.001) and LPS (P<0.05). There was a small though non significant induction of IL-6 with IL-1β.

<table>
<thead>
<tr>
<th>IL-6/MLN51 mRNA expression</th>
<th>Control</th>
<th>IL-1β</th>
<th>IFN-γ</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD n=3</td>
<td>3.1±0.43</td>
<td>4.0±0.035</td>
<td>6.6±0.70***</td>
<td>4.7±0.36*</td>
</tr>
</tbody>
</table>

Table 3.1 Induction of IL-6 mRNA expression in macrophage-like THP-1 cells treated for 24 hours

Macrophage-like THP-1 cells were stimulated for 24 hours and IL-6 mRNA expression quantitated by QPCR. *** P<0.001, * P<0.05
3.2.2 Induction of IL-6 mRNA expression in monocytic and macrophage-like THP-1 cells treated for 24 hours

IL-6 mRNA expression was quantitated in undifferentiated monocytic THP-1 cells and macrophage-like THP-1 cells to assess the difference in IL-6 mRNA expression between the cell types (Figure 3.6). Stimulation of macrophage-like THP-1 cells with interferon-γ resulted in a significant increase in IL-6 mRNA expression (P<0.001), compared to monocytic THP-1 cells. There was no significant difference in IL-6 expression between monocytic and macrophage-like THP-1 cells at baseline or when stimulated with IL-1β or LPS.

![IL-6 mRNA expression graph](image)

**Figure 3.6. Induction of IL-6 mRNA expression in monocytic and macrophage-like THP-1 cells treated for 24 hours**

Monocytic and macrophage-like THP-1 cells were stimulated for 24 hours and IL-6 mRNA expression quantitated by RT-QPCR. Mean±SD, n=3. **P<0.001**
3.2.3 Dose response of THP-1 cells to Interferon-γ

The induction of IL-6 mRNA expression by IFN-γ in macrophage-like THP-1 cells was further tested by comparing 100U/ml with ten times this dose at 2 and 24 hours of treatment (Figure 3.7). A ten fold increase in the dose of IFN-γ resulted in a significant four fold difference in IL-6 mRNA expression after 24 hours of treatment (P<0.001). There was no significant difference in the IL-6 mRNA expression at 2 hours of treatment with both 100U/ml and 1000U/ml.

![Graph showing fold change in IL-6 mRNA expression](image)

**Figure 3.7.** Fold change in IL-6 mRNA expression in macrophage-like THP-1 cells treated for 2 and 24 hours with 100U/ml and 1000U/ml IFN-γ. Macrophage-like THP-1 cells were treated with IFN-γ and IL-6 mRNA expression quantitated by QPCR. Mean±SD, n=3. *** P<0.001
3.3 *Inhibition of IL-6 mRNA expression in THP-1 cells*

The effect of two commonly prescribed statins was investigated on the endogenous and inducible IL-6 mRNA expression in the macrophage-like differentiated THP-1 cell line. Although the statin experiments were also done using IL-1β as the inflammatory challenge, no IL-6 gene expression was induced as described earlier. As LPS is a broad stimulus of IL-6 in macrophages, it was decided to stimulate the macrophage-like THP-1 cells with IFN-γ.

### 3.3.1 Effect of statins on endogenous IL-6 mRNA expression

Macrophage-like THP-1 cells were treated with either atorvastatin or pravastatin for 24 hours to assess the effect of statins on IL-6 mRNA expression *in vitro* (Figure 3.8). There was no significant difference in the level of IL-6 mRNA expression when treated with an increasing dose of either atorvastatin or pravastatin for 24 hours.

### 3.3.2 Effect of statins on IL-6 mRNA expression in macrophage-like THP-1 cells stimulated with IFN-γ

When macrophage-like THP-1 cells were stimulated with IFN-γ and treated with an increasing dose of statin for 24 hours, there was a significant dose-dependent response in the inhibition of IL-6 mRNA expression with atorvastatin (P=0.01) and with pravastatin (P=0.02) (Figure 3.9).
Figure 3.8. Fold change in IL-6 mRNA expression in unstimulated macrophage-like THP-1 cells treated with an increasing dose of statin. There was no significant difference in IL-6 mRNA expression quantitated by QPCR in macrophage-like THP-1 cells were treated with an increasing dose of atorvastatin (P=0.8) or pravastatin (P=0.6) (ANOVA) for 24 hours. Mean±SD, n=8.

Figure 3.9. IL-6 mRNA expression in IFN-γ stimulated macrophage-like THP-1 cells treated with an increasing dose of statin. Cells were treated with 100U/ml interferon-γ and an increasing dose of statin for 24 hours and IL-6 mRNA expression quantitated by QPCR. Mean±SD, n=8. ***P=0.01, *P=0.02 (ANOVA).
3.4 IL-6 gene expression in THP-1 cells

Monocytic and macrophage-like THP-1 cells were stimulated as before with IL-1β, IFN-γ or LPS for 16 hours to assess the IL-6 mRNA and protein expression in these cells, to compare the IL-6 gene expression between monocytic and macrophage-like cells. The shorter time of stimulation was chosen to allow comparison with transient transfection data of HA Khwaja (PhD thesis, Oxford, 2005).

3.4.1 IL-6 mRNA expression in THP-1 cells stimulated for 16 hours

The level of IL-6 mRNA expression was assessed after 16 hours of stimulation with IL-1β, IFN-γ or LPS in monocytic and macrophage-like THP-1 cells (Figure 3.10). There was no significant difference between monocytic and macrophage-like THP-1 cells at baseline or stimulated with IL-1β or IFN-γ for 16 hours. There was a significant difference (P<0.05) between the two cell types when stimulated for 16 hours with LPS. Overall, there was less IL-6 mRNA induction at 16 hours of stimulation when compared with the induction at 24 hours of stimulation. This suggests that there is a differential IL-6 mRNA response that is dependent on time and stimulus.

3.4.2 Measuring IL-6 protein concentration in cell culture supernatants

A composite standard curve was prepared from five different assays run in duplicate. The standard curve is shown in Figure 3.11. The pg/ml values of IL-6 protein expression were calculated from the spline of the fit of the standard curve.
Figure 3.10. IL-6 mRNA expression in monocytic and macrophage-like cells stimulated for 16 hours
Monocytic and macrophage-like THP-1 cells were stimulated for 16 hours and the levels of IL-6 mRNA expression quantitated by QPCR. Mean±SD, n=3. * P<0.05

Figure 3.11. Composite standard curve of IL-6 protein concentration
Serial dilutions of the IL-6 standard were used to produce the standard curve. The curve is a composite of five experiments (mean±SD), with each standard assayed in duplicate.
3.4.3 IL-6 protein expression in THP-1 cells stimulated for 16 hours

IL-6 protein expression was measured in cell supernatants from monocytic and macrophage-like THP-1 cells stimulated as described in section 3.1.2 for 16 hours (Figure 3.12). IL-6 protein expression was measured using a validated high sensitivity chemiluminescence ELISA (R&D Systems). There was a significant difference between the monocytic and macrophage-like cells when stimulated with LPS (P<0.001) for 16 hours. This was consistent with the LPS-induced IL-6 mRNA expression at 16 hours and with previous reports of LPS-induced IL-6 production in THP-1 cells (Suda et al, 1999).

![Figure 3.12. IL-6 protein expression in THP-1 cells stimulated for 16 hours](image)

The left and right axes in this chart show the difference in measured IL-6 protein in cell culture supernatants between macrophage-like THP-1 (dTHP-1) and monocytic (THP-1) cells respectively. Mean±SD, n=3, *** P<0.001.
3.5. Conclusions

There was a significant induction of IL-6 mRNA in macrophage-like THP-1 cells differentiated with PMA and stimulated with IFN-γ for 24 hours or LPS for 16 hours, whereas monocytic THP-1 cells expressed very low levels of IL-6 mRNA upon stimulation. There was a significant increase in the LPS-induced IL-6 protein expression in macrophage-like THP-1 cells, which was consistent with the increased IL-6 mRNA expression observed at 16 hours with LPS stimulation, and this time- and stimulus-dependent response of IL-6 gene expression is consistent with previous reports that the IL-6 gene is differentially activated.

Macrophage-like THP-1 cells activated by IFN-γ responded to statin treatment by down-regulating IL-6 mRNA expression and this could indicate a role for statins at the level of the atherosclerotic plaque by reducing local inflammation and having a potential effect on plaque stability. There was no quantitative difference in the overall effect of the two drugs, which suggests a possible class effect. These data provide an insight into the mechanisms of action of statins and provide further evidence for their beneficial anti-inflammatory effects.

Quantitation of IL-6 gene expression was validated in the THP-1 monocyte-macrophage cell line model of inflammation. In the next chapter, a primary-monocyte-derived macrophage model is developed to assess the role of IL-6 promoter haplotype on IL-6 gene expression.
Chapter 4 IL-6 expression in primary monocyte-derived macrophages

4.1 Ex vivo culture of monocyte-derived macrophages

Peripheral blood mononuclear cells from nine individuals (n=3 individuals possessing the high-responder GG9/11G allele, n=3 individuals heterozygous for the low-responder AG8/12C allele, and n=3 individuals homozygous for the AG8/12C allele) of known IL-6 proximal promoter haplotype (Table 4.1) were prepared on a density gradient from whole blood using Histopaque-1077, according to the manufacturer’s instructions. The haplotype frequencies (AG8/12C (0.35), GG10/11G (0.30), GG10/10G (0.18) and GG9/11G (0.17)) were consistent with the reported values (Terry et al, 2000).

Table 4.1 Haplotype assignment for volunteers recruited to the study (over page)

Volunteers are indexed 1-27 and IL-6 proximal promoter polymorphisms are indicated across the top of the table. Possession of the allele is indicated by X. Highlighted individuals were recalled for culture of primary monocyte-derived macrophages. Complete genotyping results are shown in Appendix III.
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The yield of peripheral blood mononuclear cells (1.19x10⁶/ml whole blood ± 0.44, mean±SD) was determined following Histopaque-1077 centrifugation and found to be within the normal range (lymphocytes 1.5 – 4 x 10⁶/ml, monocytes 0.2 – 0.95 x 10⁶/ml). Mononuclear cells were cultured as described in 2.11.1 and 2.11.2. The percentage (mean±SD) adherence (14.14 ± 5.28) and viability (95.16 ± 3.02) of monocyte-derived macrophages was determined at 8 days of primary culture, prior to stimulation.

Monocyte-derived macrophages were stimulated using interleukin-1β interferon gamma or LPS, according to Table 2.5. After stimulation, the cell culture supernatants were removed and stored at -80°C for ELISA analysis of cytokine production. RNA was extracted from the adherent cells for QPCR and in some cases, intact, functional nuclei were extracted for CHART-PCR.

4.2 IL-6 mRNA expression in primary monocyte-derived macrophages

The method for RNA extraction and optimization of the QPCR assay was described in sections 2 and 3 respectively.

A published assay of chitotriosidase mRNA expression (a biochemical marker of macrophage activation, associated with lysosomal diseases and an early response to infections including malaria) in monocyte-derived macrophages from healthy volunteers was used as a positive control to demonstrate that the method for preparing monocyte-derived macrophages and assaying mRNA expression could reproduce reported results (Malaguarnera et al, 2005). In contrast to the methods presented here the
authors enriched the monocyte population by using CD14 magnetic beads prior to differentiation, used the housekeeping gene GAPDH and calculated the fold change using the ct values instead of a standard curve. Primary monocyte-derived macrophages from healthy volunteers were stimulated with 100U/ml IFN-γ for 2, 4 and 24 hours. Although there were differences between the methods, the fold change over unstimulated control was similar to the published data (Figure 4.1).

Figure 4.1. Chitotriosidase mRNA expression in monocyte-derived macrophages
Monocyte-derived macrophages were stimulated for 2, 4 and 24 hours and chitotriosidase and MLN51 mRNA expression determined by QPCR. The graph shows the fold change over unstimulated chitotriosidase expression (mean±SD) of triplicate cell culture wells, assayed in duplicate. *P<0.05.

The primary monocyte-derived macrophage model was validated further by measuring the IL-6 mRNA expression in differentiating monocyte-
macrophages, as well by comparing the inter- and intra-individual differences in IL-6 mRNA expression.

4.2.1 IL-6 expression in unstimulated differentiating monocyte-derived macrophages

Total RNA was extracted from differentiating monocyte-macrophages at 48 hours, 96 hours and 192 hours after seeding out. QPCR was carried out as described previously. Cells in the first 48 hours of ex vivo primary culture showed increased IL-6 expression relative to the housekeeping gene MLN51. There was a significant reduction in IL-6 mRNA expression (P=0.01) over time from 48 hours to 192 hours (8 days). The level of IL-6/MLN51 expression dropped to basal level (P=0.004). There was a small though non-significant decrease in the level of MLN51 mRNA expression (P=0.09) (Figure 4.2 and Table 4.2).
Figure 4.2. Baseline IL-6 and MLN51 mRNA expression in differentiating monocyte-derived macrophages

IL-6 and MLN51 mRNA expression was measured in differentiating monocyte-macrophages from three individuals. The graph shows the mean of the three experiments in triplicate cell culture wells, assayed in duplicate (mean±SEM). *P=0.01 for IL-6, **P=0.004 for IL-6/MLN51.

<table>
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Table 4.2. Baseline IL-6 and MLN51 mRNA expression in differentiating monocyte-derived macrophages

Measured QPCR values (ng) of IL-6 and MLN51 expression and IL-6 normalized to MLN51 in differentiating monocyte-macrophages. *P=0.01, **P=0.004.
4.2.2 Inter-individual IL-6 mRNA expression in unstimulated primary monocyte-derived macrophages

RNA was extracted from monocyte-derived macrophages at 8 days + 0, 8, 24, 48 hours of ex vivo culture. The levels of IL-6 and MLN51 mRNA expression were measured by QPCR (Figure 4.3). There was little variability observed at each time point between the individuals.

Baseline IL-6 mRNA expression increased at 24 and 48 hours following differentiation, whilst there was little increase from 0 hours in the mean IL-6 expression in the group at 8 hours. There was a significant difference between 8 and 24 hours (P=0.04). Only one individual was cultured to 48 hours due to the limited number of cells available.

In further experiments stimulation with IL-1β, IFN-γ or LPS was carried out for 8 hours where the maximum induction in IL-6 expression could be observed.
The graph shows the IL-6 mRNA expression normalized to MLN51 in each individual (●) over time in ex vivo culture following differentiation (not all individuals were measured at all time points). The cells were not stimulated and the points show baseline IL-6 mRNA expression. Mean±SD is given for each time point.

The data at 8 hours were analysed to ascertain whether sex or age affect IL-6 mRNA expression. There was no significant difference between the unstimulated IL-6 mRNA expression in men and women (P=0.3) (Figure 4.4) and there was no significant difference between the unstimulated IL-6 mRNA expression in the 25-40 year or 41-60 year age groups (P=0.9) (Figure 4.5).
Figure 4.4. Effect of sex on baseline IL-6 mRNA expression in monocyte-derived macrophages
The graph shows the IL-6 mRNA expression normalized to MLN51 in each individual. The cells were not stimulated and the points show baseline IL-6 mRNA expression.

Figure 4.5. Effect of age on baseline IL-6 mRNA expression in monocyte-derived macrophages
The graph shows the IL-6 mRNA expression normalized to MLN51 in each individual. The cells were not stimulated and the points show baseline IL-6 mRNA expression.
4.3 IL-6 mRNA expression in stimulated primary monocyte-derived macrophages

4.3.1 Intra-individual IL-6 mRNA expression in stimulated primary monocyte-derived macrophages

It has already been shown that there was little variation in IL-6 mRNA expression between individuals at baseline (section 4.2.2). IL-6 mRNA expression in stimulated monocyte-derived macrophages was studied in one individual in three separate experiments (blood was taken on three different occasions over one year) to determine the intra-individual variability of the model (Figure 4.6).

The results show that there was little intra-individual variability when stimulated with IL-1β or IFN-γ. There was some variability in the LPS-induced IL-6 mRNA response although this was not significant.

A time-course of IL-1β stimulation in monocyte-derived macrophages from one individual showed that IL-6 mRNA appeared to peak between 4 and 24 hours of stimulation, whilst the housekeeping gene (MLN51) mRNA expression remained constant with stimulation at 2 (P<0.001), 4 (P<0.001) and 24 hours (P<0.01) (Figure 4.7).
Figure 4.6. Intra-individual fold change in IL-6 mRNA expression in stimulated monocyte-derived macrophages
Monocyte-derived macrophages were stimulated for 8 hours. Induced intra-individual IL-6 mRNA expression was normalized to MLN51 from three separate experiments over one year.

Figure 4.7. IL-6 and MLN51 mRNA expression in IL-1β stimulated primary monocyte-derived macrophages
Monocyte-derived macrophages were stimulated for 2, 4 and 24 hours and IL-6 and MLN51 mRNA expression determined by QPCR. Mean±SD of triplicate cell culture wells, assayed in duplicate. ***P<0.001, **P<0.01.
4.3.2 IL-6 mRNA expression in stimulated primary monocyte-derived macrophages

Nine individuals were recalled for the functional assay as described in section 4.1. Monocyte-derived macrophages were stimulated for 8 hours. IL-6 mRNA expression relative to MLN51 was measured by QPCR (Figure 4.8). There was a significant difference between the LPS- and IL-1β- (P<0.05) and IFN-γ-(P<0.05) induced IL-6 response.

![Figure 4.8. Induction of IL-6 mRNA expression in stimulated monocyte-derived macrophages](image)

The graph shows the fold induction over unstimulated IL-6 mRNA expression.

n=9, mean±SEM, *P<0.05.

A small study of three individuals was undertaken to look at the effect of priming monocyte-derived macrophages with IFN-γ on IL-6 mRNA
expression prior to adding LPS to the cell culture medium. There was no difference in the mean IL-6 mRNA expression between LPS alone (4.8 - 454) and when primed with IFN-γ for 12 (16 - 308) and 24 hours (2.5 - 295), but there was an increase compared to IFN-γ alone (0.54 - 4.8) (Figure 4.9).

![Graph showing IL-6 mRNA expression](image)

**Figure 4.9. IL-6 mRNA expression in IFN-γ primed monocyte-derived macrophages**

The graph shows the fold induction of IL-6 mRNA expression in individuals stimulated with IFN-γ alone, LPS alone and IFN-γ plus LPS.

**4.3.3 IL-6 mRNA expression in stimulated monocyte-derived macrophages by haplotype**

IL-6 mRNA expression was analysed by IL-6 SNP haplotype to investigate whether previous transient transfection analysis of haplotype-specific IL-6
promoter:reporter-gene constructs is consistent with the situation in ex vivo stimulated monocyte-derived macrophages. The previous findings were:

1. Reporter gene expression assays in the ECV304 cell line demonstrated that the IL-6 promoter GG9/11G haplotype showed higher IL-6 transcriptional induction by IL-1β (Terry et al, 2000).

2. IL-6 promoter-reporter constructs transfected into the human microvascular endothelial cell line (HMEC-1) respond to inflammatory stimuli in a haplotype-dependent manner; the highest transcriptional induction by IL-1β was associated with the GG9/11G and GC10/10G haplotypes and the lowest induction with AG8/12C and AG8/12G haplotypes (P<0.001) (Khwaja, PhD thesis, Oxford 2005; Khwaja, Terry, Green, manuscript submitted).

3. Quantitative RT-PCR analysis of the IL-6 mRNA from 60 carotid plaques taken at the time of carotid endarterectomy showed that the GG9/11G haplotype was the only haplotype associated with increased IL-6 mRNA in the plaque (P=0.005) (Khwaja, PhD thesis, Oxford 2005; Khwaja, Terry, Green, manuscript submitted).

IL-1β-, IFN-γ- and LPS-induced IL-6 mRNA expression were analysed in terms of haplotype (Figures 4.9, 4.10, 4.11). The group of individuals was assigned as possessing one or two copies of the GG9/11G allele (+GG9/11G) or none (−GG9/11G). There was a highly significant difference (P=0.0004) in the IL-1β-induced IL-6 mRNA response between the haplotype
groups. This was consistent with previous findings that the inducible IL-6 response in individuals with the +GG9/11G haplotype is greater than those with −GG9/11G haplotype (Figure 4.10). Although the mean of the +GG9/11G group (3.4) was greater than that of the −GG9/11G (1.3), this failed to reach statistical significance difference (P=0.06) in the IFN-γ-induced IL-6 mRNA response between the haplotype groups (Figure 4.11). The LPS-induced IL-6 response showed no significant difference between the haplotype groups (P=0.5) (Figure 4.12). LPS induces a strong inflammatory response and these results suggest that the mechanism of LPS action on IL-6 mRNA expression is not solely mediated through the IL-6 promoter.

![Figure 4.10](image)

**Figure 4.10. IL-6 mRNA expression in IL-1β stimulated monocyte-derived macrophages by haplotype**

The graph shows the fold induction of IL-6 mRNA expression following stimulation with IL-1β for 8 hours. ***P=0.0004.
Figure 4.11. IL-6 mRNA expression in IFN-γ stimulated monocyte-derived macrophages by haplotype
The graph shows the fold induction of IL-6 mRNA expression following stimulation with IFN-γ for 8 hours (P=0.06).

Figure 4.12. IL-6 mRNA expression in LPS stimulated monocyte-derived macrophages by haplotype
The graph shows the fold induction of IL-6 mRNA expression following stimulation with LPS for 8 hours (P=0.5).
4.4 IL-6 protein expression in human plasma and primary cell culture supernatants

4.4.1 IL-6 protein in human plasma

Plasma samples were collected from each individual recruited to the project. IL-6 protein levels were measured in twenty-five out of twenty-seven individuals to assess the plasma IL-6 expression in normal, healthy individuals. The IL-6 protein concentration in stored plasma samples was 1.3±0.48 pg/ml (mean±SD), which was within reported values for IL-6 in circulating plasma (Endler et al, 2004; Cardellini et al, 2007; Zalewska et al, 2006). One individual was observed at 3.0pg/ml which was still within the reported range. (Figure 4.13a).

These results were analysed to determine the effect of sex, age and haplotype on IL-6 plasma concentration (Figures 4.13b-d). There was no significant sex difference in plasma IL-6 (male 1.22±0.22, female 1.3±0.6) (Figure 4.13b). The volunteers recruited to this study were aged 25 to 60 years. The age range was split into two groups of roughly equal size for the analysis, 25-40 years (n=11, 1.2±0.39) and 41-60 years (n=14, 1.3±0.58). There was no significant difference in plasma IL-6 between the two groups (Figure 4.13c). The IL-6 promoter haplotype was assigned for each individual as possessing one or two copies of the GG9/11G allele (+GG9/11G) or none (−GG9/11G) (Figure 4.13d). There was no significant difference in plasma IL-6 concentration between the two defined haplotype groups (+GG9/11G 1.1±0.29, -GG9/11G 1.3±0.55).
Figure 4.13. Plasma IL-6 protein expression
IL-6 protein was measured in stored plasma samples. The graphs show the individual plasma IL-6 (pg/ml) concentrations.
4.4.2 IL-6 protein concentration in ex vivo cell culture supernatants

IL-6 protein was measured in supernatants from unstimulated monocyte-derived macrophages at 8 days of ex vivo culture + 2, 8 and 24 hours (Figure 4.14). There was no significant difference in the baseline IL-6 protein expression at 2 (2.6±1.2pg/ml, mean±SEM), 8 (10±3.8pg/ml) and 24 (8.3±2.2pg/ml) hours.

![Diagram showing IL-6 protein concentration over time](image)

**Figure 4.14. IL-6 protein concentration in cell culture supernatants from unstimulated monocyte-derived macrophages**

Basal IL-6 protein was measured in cell culture supernatants. Each individual value is the mean of the assay duplicates for the individual from triplicate cell culture wells.

IL-6 protein concentration was measured in primary cell culture supernatants collected after stimulation at 2, 8 and 24 hours (Figure 4.15). The peak IL-6 protein values were measured at 8 hours. IL-1β stimulated cells produced a significant increase in IL-6 (P=0.002) and there was an especially strong
Figure 4.15. IL-6 protein expression in ex vivo primary cell culture supernatants from stimulated monocyte-derived macrophages

The graphs show the mean measured IL-6 protein in individuals stimulated with IL-1β, IFN-γ or LPS for 2 hours, 8 hours and 24 hours. Each individual value is the mean of the assay duplicates for the individual from triplicate cell culture wells. *P=0.02, **P=0.002.
response to LPS (P=0.02). The LPS response was damped down by 24 hours, indicating that the IL-6 protein response is short-lived and this is consistent with its role as an early response gene. No significant difference in IL-6 supernatant concentration was observed at 2 or 24 hours of stimulation.

The data from the 8 hour supernatant collection were analysed by haplotype (Figures 4.16). Although there was no significant difference in IL-6 concentration between +GG9/11G and -GG9/11G individuals in the supernatants from stimulated cells, the +GG9/11G group showed a consistently greater concentration than the -GG9/11G group. There was a significant difference between the haplotype groups at baseline (P=0.04), suggesting a possible haplotype-specific effect on IL-6 production in non-activated monocyte-derived macrophages.

Figure 4.16. IL-6 protein concentration in supernatants from primary monocyte-derived macrophages
(over page)
The graphs show the individual IL-6 protein expression in monocyte-derived macrophages at baseline and when stimulated for 8 hours with IL-1β, IFN-γ and LPS. Each individual value is the mean of the assay duplicates for the individual from triplicate cell culture wells. *P=0.04.
There was no significant difference between the haplotype groups at 2 or 24 hours when stimulated with IL-1β, IFN-γ or LPS, although the number of individuals in these groups was small (n=4 and n=3 respectively).

A short time-course of IL-6 protein expression in IL-1β stimulated monocyte-derived macrophages was performed in two individuals (Figure 4.17), one from each haplotype group, to determine if the IL-6 concentration at 8 hours of stimulation was due to an accumulation of IL-6 over time in the cell culture supernatants. The values at 0.25 and 0.5 hours in +GG9/11G and 0.25 hours in −GG9/11G were below the detection limit of the assay. No significant difference was observed in the time points at 1 and 2 hours, however, there was a significant difference between the two individuals at 8 hours (P<0.001). This finding was not consistent with the lack of haplotype-specific IL-6 protein expression in the haplotype analysis of all individuals (Figure 4.16), and therefore cannot be confirmed as a haplotype-specific difference. Furthermore, a time-dependent IL-6 response was observed with increasing IL-6 concentration the longer the stimulation time with IL-1β.

Given the highly significant haplotype-specific IL-6 mRNA expression with IL-1β stimulation it would suggest that a similar response might be observed at the protein level. This was not the case, however, and could be due to a longer half-life in the cell culture environment than in vivo. These data do suggest that the IL-6 protein could be accumulating in the supernatant and this may account for the lack of haplotype-specific response.
Figure 4.17. IL-6 protein concentration in supernatants from monocyte-derived macrophages stimulated with IL-1β over a short time-course. The graph shows the IL-6 concentration (mean±SD) for an individual of each haplotype from triplicate cell culture wells, assayed in duplicate. ***P<0.001.

4.5 Conclusions

IL-6 mRNA was inducible in a primary monocyte-derived macrophage model, with low inter- and intra-individual variability. It was demonstrated that cells derived from individuals of either +GG9/11G or -GG9/11G haplotype varied little in their IL-6 mRNA expression at baseline. However, when these cells were exposed to IL-1β, IL-6 mRNA was induced in an IL-6 promoter haplotype-specific manner, with IL-6 mRNA expression in cells from +GG9/11G individuals more induced than -GG9/11G individuals and this is consistent with previous reports (Terry et al, 2000; Khwaja, PhD thesis, Oxford 2005; Khwaja, Terry, Green, manuscript submitted).
IL-6 protein concentration peaked after 8 hours of stimulation. All individuals were responsive to inflammatory challenge regardless of IL-6 promoter haplotype, which is an important positive control for this model. That is, all individuals were able to mount an inflammatory challenge at the protein level in spite of low mRNA expression levels. Stimulated and unstimulated monocyte-derived macrophages from +GG9/11G individuals produced more IL-6 protein ex vivo than -GG9/11G individuals, although this was not significant in stimulated cells.

A key inflammatory marker such as IL-6 requires fine control in vivo and it could therefore be a dysregulation of this fine control that accounts for the effects of IL-6 in inflammatory conditions, including atherosclerosis, inflammatory bowel disease and arthritis. One possible mechanism of haplotype-specific IL-6 mRNA expression is investigated in the next chapter.
Chapter 5 Chromatin remodelling in the IL-6 promoter

5.1 IL-6 promoter chromatin accessibility in THP-1 cells

In order to investigate a possible mechanism of action of IL-6 gene activation, the Chromatin Accessibility Real Time Quantitative PCR (CHART-PCR) assay was adapted (Brettingham-Moore et al, 2005) and validated in THP-1 cells. In this assay, whole, functional nuclei were extracted from the macrophage-like THP-1 cells and subjected to DNase I digestion. Chromatin is described as open (euchromatin) or closed (heterochromatin) depending on the state of gene activation, that is, open chromatin is an indication of transcriptional activity (Kuhnert et al, 1992). Open, active chromatin is more readily digested by DNase I than closed, inactive chromatin, thus the greater the digestion, the more open and active is the area of chromatin and the less PCR product is formed in the CHART-PCR assay.

5.1.1 Design of CHART-PCR assay

Three sets of primers were designed to span the proximal IL-6 promoter. The regions were named 1, 2 or 3, where region 1 contains the -597 A/G and -572 G/C SNPs, region 2 covers the -373 AnTn polymorphism and region 3 contains the -174 C/G SNP. Quantitative PCR was performed using the QuantiTect PCR Sybr Green kit on the ABI Prism 7000 (Applied Biosystems).

A standard curve was prepared for each region using serial dilutions of genomic DNA.
5.1.2 Optimisation of CHART-PCR assay

In monocytic and macrophage-like THP-1 cells at baseline, nuclei were extracted and treated with DNase I for 5, 10 and 15 minutes at 37°C to determine the optimum time of digestion with DNase I to leave quantifiable PCR fragments. The products of this DNase I digestion were subjected to electrophoresis on a 2% (w/v) agarose gel in 1X TBE buffer (Figure 5.1). An equal amount of template DNA (20ng) was added to each PCR reaction and equal amounts were loaded onto the gel. The product at 10 minutes was clearly greater (that is, less digested) than those at 5 and 15 minutes.

Figure 5.1. Agarose gel electrophoresis of PCR products of DNase I-digested nuclei
The figure shows the PCR products of IL-6 promoter region 1 digested for 5, 10 and 15 minutes from nuclei of macrophage-like (2-4) and monocytic (5-7) THP-1 cells. (1) DNA 50kb ladder.

The QPCR results of this one experiment are represented in Figure 5.2. In each case the chromatin from the macrophage-like cells was more open than
Figure 5.2. Time-course of DNase I treatment of nuclei from THP-1 cells
Each graph shows the qPCR product formed in region 1, region 2 and region 3 of the IL-6 promoter after DNase I digestion of nuclei from monocytic (▲) and macrophage-like (■) THP-1 cells. Each point is the mean of the assay duplicate (n=1).
the monocytic cells after 10 minutes of DNase I digestion and less PCR product was formed. The quantitative results confirmed the gel electrophoresis data.

All subsequent DNase I digestions were performed at 37°C for 10 minutes.

Figure 5.3 compares the endogenous DNase and exogenous DNase I digestion of nuclei from monocytic and macrophage-like THP-1 cells in region 1 of the IL-6 promoter. These data showed that chromatin in the IL-6 promoter region 1 was more open in the macrophage-like cells than in the monocytic cells at baseline and this was consistent with the results observed in Figure 5.2. More PCR product was measured in the nuclei from both the monocytic and macrophage-like THP-1 cells not treated with DNase I than the DNase I-treated nuclei (P<0.001). The amount of PCR product measured in nuclei from monocytic THP-1 cells not treated with DNase I was significantly greater than that measured in the macrophage-like cells (P<0.001).

The QPCR DNase I digestion values were normalized to the undigested values for all further analyses, to account for potential differences in PCR amplification.
Figure 5.3. IL-6 promoter region 1 chromatin accessibility at baseline in THP-1 cells
Quantitative PCR result (n=3, mean±SD) from IL-6 promoter region1 in nuclei from monocytic and macrophage-like cells treated at 37°C for 10 minutes with or without DNase I. ***P<0.001.

5.1.3 Chromatin accessibility in IL-6 promoter in THP-1 cells
THP-1 cells were cultured as monocytes or differentiated into macrophage-like cells as previously described. The cells were stimulated with IL-1β, IFN-γ and LPS for 16 hours. Nuclei were extracted as described in section 2.12.1 and DNase I digested at 37°C for 10 minutes.

CHART-PCR was carried out as described in section 2.12.4 in three regions of the proximal IL-6 promoter and the results are shown in Figure 5.4.
Figure 5.4. Chromatin accessibility in IL-6 promoter in THP-1 cells

The graphs show chromatin remodelling in region 1, region 2 and region 3 of the IL-6 promoter in monocytic and macrophage-like THP-1 cells. The PCR products were normalized to the undigested PCR value so that the chromatin accessibility could be compared across all three IL-6 promoter regions (n=3, mean±SD). **P<0.01, ***P<0.001.
In each region, the chromatin from macrophage-like cells was more open than the chromatin in monocytic cells (that is, less PCR product). In region 1 (P<0.01) and region 3 (P<0.001), there was a significant difference in the chromatin response to IL-1β. In addition, there was a significant difference in the chromatin between monocytic and macrophage-like cells in the control (unstimulated) samples (P<0.01), which was consistent with the baseline CHART-QPCR results in monocytic and macrophage-like cells in Figure 5.2.

To summarise the above data and to draw a comparison between monocytic and macrophage-like THP-1 cells, Figure 5.5 shows the chromatin accessibility profile from baseline and with stimulation for each cell type in all IL-6 promoter regions. No significant difference in the chromatin profile was observed between the regions of the IL-6 promoter. Overall, chromatin accessibility in the IL-6 promoter in nuclei from the monocytic cells was shown to be less open than that from macrophage-like cells. This was consistent with mRNA levels, where IL-6 induction was greater in macrophage-like cells than in monocytic cells. Chromatin was less accessible and therefore less active when the cells were stimulated with IL-1β compared to stimulation with IFN-γ and LPS in both monocytic and macrophages-like THP-1 cells and this finding was consistent with the IL-6 mRNA expression levels shown in Figure 3.10.
5.2 Chromatin accessibility in the IL-6 promoter in primary monocyte-derived macrophages

One possible mechanism of action for the differential IL-6 gene expression observed could be the state of the chromatin in the IL-6 promoter. As shown in the THP-1 model, chromatin accessibility was related to IL-6 gene expression, and the latter was therefore measured in the nuclei from primary cells.

5.2.1 Chromatin remodelling in the IL-6 promoter at 2 and 8 hours of stimulation

Four individuals (n=2 from each haplotype group) were re-called for this part of the study. Monocyte-derived macrophages were stimulated for 2 and 8
hours in the usual way and nuclei were extracted as described in section 2.12.1. The percentage nuclei yield from four individuals was 21.25±10.11 % (mean±SD). All CHART-PCR assays were performed in duplicate on nuclei from triplicate cell culture wells for each individual.

Figure 5.6 shows the dynamic response of the IL-6 proximal promoter chromatin to stimulation over time. There were differences in the profiles of stimulation across the regions and between 2 and 8 hours of stimulation. The chromatin in the control (unstimulated) samples in Region 2 at 2 and 8 hours was more accessible than in the other regions. This was consistent with the finding in macrophage-like THP-1 cells where the unstimulated chromatin in Region 2 was similarly more accessible than Regions 1 and 3 (Figure 5.4).

Chromatin in nuclei from monocyte-derived macrophages stimulated with IL-1β showed decreasing accessibility from Region 1 to 2 to 3 at 2 hours of stimulation. At 8 hours of stimulation with IL-1β there was a small increase in chromatin accessibility in Region 1 when compared to control. Chromatin accessibility decreased in Regions 2 and 3.

Stimulation of monocyte-derived macrophages with IFN-γ resulted in a small increase in chromatin accessibility in Region 1 at 2 hours which was further opened at 8 hours, when compared to the control. Chromatin was less accessible in Region 2 at 2 hours compared with 8 hours of stimulation with
IFN-γ. Chromatin accessibility increased in Region 3 compared to Region 2 at 2 hours, but was less accessible in Region 3 than in Region 2 at 8 hours. Stimulation of monocyte-macrophages with LPS for 2 hours showed that chromatin was less open in Region 1 and Region 3 than Region 2. By 8 hours of stimulation chromatin was similarly more open in Region 2 than Region 1, whilst the chromatin in Region 3 was dramatically more open, compared with Region 3 at 2 hours. This finding was consistent with the IL-6 gene expression data where LPS-stimulated monocyte-derived macrophages showed a significant increase in both mRNA and protein, compared to IL-1β and IFN-γ.

The changes in chromatin accessibility were however very small compared to the gene expression, suggesting a contribution to the fine regulation of IL-6 expression.

**Figure 5.6. Chromatin accessibility in IL-6 promoter in nuclei from monocyte-derived macrophages stimulated for 2 and 8 hours**

(over page)

The graphs show the chromatin accessibility across the three regions of the IL-6 promoter at baseline, stimulated for 2 hours and 8 hours with IL-1β, IFN-γ or LPS. The values are the mean±SEM of 4 individual experiments, where the DNase I digested QPCR values were normalized to the undigested QPCR values.
The fold change in chromatin accessibility at 8 hours was calculated (the change in chromatin accessibility with stimulation over control) and this was found to be significantly different between region 1 and region 2 at 8 hours of IL-1β stimulation (P<0.05). Region 2 was less accessible to DNase I digestion than region 1 and 3 when stimulated with IL-1β and INF-γ, but not with LPS stimulation (Figure 5.7). All three regions were accessible to DNase I digestion in the LPS stimulated cells, suggesting a possible mechanism for the greater inducibility of IL-6 with LPS stimulation.

Figure 5.7. Fold change in chromatin accessibility in the IL-6 promoter at 8 hours of stimulation
The graph shows the fold change in chromatin accessibility across the three regions of the IL-6 promoter stimulated for 8 hours with IL-1β, IFN-γ or LPS. The values are the mean±SEM of 4 individual experiments. *P<0.05.
5.2.2 Haplotype-specific IL-6 promoter chromatin accessibility

There were haplotype-specific differences in chromatin accessibility at both 2 and 8 hours of stimulation with IL-1β, IFN-γ or LPS (Figure 5.8). These were not statistically significant, most likely due to the small number of individuals studied (n=2 for each IL-6 promoter haplotype +GG9/11G and -GG9/11G).

Chromatin from the +GG9/11G group was clearly more accessible than the -GG9/11G group in region 3 at 2 hours when stimulated with LPS and at 8 hours at baseline and when stimulated.

There was little stimulus or haplotype variability in region 1, although at 8 hours of stimulation chromatin accessibility was greater in both groups of IL-6 promoter haplotype.

In region 2 the chromatin was more accessible in the -GG9/11G haplotype group, at baseline and with stimulation with IL-1β and IFN-γ but not with LPS, where the +GG9/11G group was more accessible at 8 hours.

Figure 5.8. Haplotype-specific chromatin accessibility in IL-6 promoter (over page)

The graphs show the haplotype-specific chromatin accessibility across the three regions of the IL-6 promoter at baseline, stimulated for 2 hours and 8 hours with IL-1β, IFN-γ or LPS. The values are the mean±SEM of 4 individual experiments (n=2 for each IL-6 promoter haplotype +GG9/11G and -GG9/11G), where the DNase I digested QPCR values were normalized to the undigested QPCR values.
5.2.2 Chromatin remodelling in the IL-6 promoter over a short time-course of IL-1β stimulation

A shorter time-course of IL-1β stimulation showed that there were time- and region- specific significant differences in chromatin accessibility in one +GG9/11G and one -GG9/11G individual (Figure 5.9).

In region 1 chromatin from the +GG9/11G individual was more open than that from the -GG9/11G individual from 0.25 hours to 8 hours (P<0.001).

In region 2, chromatin from the +GG9/11G individual was more open from 0.5 hours to 2 hours and showed a significant decrease in chromatin accessibility at 8 hours of stimulation with IL-1β, when compared to the -GG9/11G individual (P<0.001).

In region 3, chromatin from the +GG9/11G individual was more accessible to DNase I digestion in the early time-course but became less open by 2 hours of stimulation and equal to that in the -GG9/11G individual.

In all three regions, the chromatin from the +GG9/11G individual was more accessible to DNase I digestion than chromatin from the -GG9/11G individual, that is the chromatin was more open and therefore active, apart from at 8 hours in region 2 where the chromatin from the +GG9/11G individual became less accessible to DNase I digestion. In region 3 at 2 and 8 hours the chromatin accessibility was similar between the two individuals of different IL-6 promoter haplotype.
Figure 5.9. Chromatin accessibility in IL-6 promoter regions over a short time-course

The graphs show the fold change in chromatin accessibility (mean±SD) across region 1, region 2 and region 3 of the IL-6 promoter, in nuclei from cells stimulated with IL-1β over a short time-course. n=1 individual for each haplotype, from triplicate cell culture wells, assayed in duplicate. ***P<0.001.
These short time-course data were amalgamated with the 2 and 8 hour data from all individuals (n=4) to produce a composite graph of chromatin remodelling in the IL-6 promoter in nuclei from IL-1β stimulated monocyte-derived macrophages (Figure 5.10). These data demonstrated chromatin remodelling within 0.5 hours of stimulation in the three regions of the IL-6 proximal promoter. There was a significant difference in the chromatin accessibility at 8 hours between region 1 and region 2 (P<0.05); that is, region 2 was less accessible to DNase I digestion. A similar though not significant effect was observed with IFN-γ but not LPS stimulation at 8 hours (as shown in Figure 5.7).

Figure 5.10. Chromatin remodelling in the IL-6 promoter
The graph shows the fold change in chromatin accessibility (mean±SEM) in region 1, region 2 and region 3 of the IL-6 promoter, in nuclei from monocyte-derived macrophages stimulated with IL-1β over a short time-course (n=2 0.25 to 1 hour, n=4 2 to 8 hours from triplicate cell culture wells, assayed in duplicate). *P<0.05.
5.3 DNase I hypersensitivity in the IL-6 promoter

Using CHART-PCR allows a more sensitive quantitation of DNase I hypersensitivity than the traditional DNase I foot printing method (McArthur et al, 2001). It was observed that the CHART-PCR absolute values (nanograms of PCR product) from region 2 were lower than those from region 1 or region 3 in nuclei extracted from unstimulated primary monocyte-derived macrophages (Figure 5.11). The ratios of region 1:2 and region 3:2 were 22±10 and 17±7.2 (mean±SEM) respectively.

![Figure 5.11. Quantitative PCR product from IL-6 promoter regions](image)

Each region of the IL-6 promoter was quantitated by CHART-PCR in four individuals (mean±SEM) following DNase I digestion in nuclei from unstimulated monocyte-derived macrophages. *P=0.02.

This result was also seen in QPCR of genomic DNA extracted from white blood cells and not DNase I digested. The input template amount was equal, the reaction efficiency of each QPCR reaction was similar (Region 1 68.9%, Region 2 68.4%, Region 3 78.4%), the PCR product was specific as
determined by melt curve analysis and gel electrophoresis (no additional product was formed) and no difference was observed on gel electrophoresis compared to Region 1 and Region 3 (although this is not strictly quantitative). The PCR primers were checked by BLAST2 (Altschul et al, 1997) for sequence alignment within region 2 of the IL-6 promoter (http://zeon.well.ox.ac.uk/blast2/).

This low amplification of region 2 seems to be the result of an effective lack of template. This may be due to DNA secondary structure in region 2 that makes it less available as template in the QuantiTect Sybr Green QPCR system, as agents used in PCR such as DMSO to aid the Taq polymerase through secondary structure cannot be added to the quantitative Sybr Green QPCR reaction.

In chromatin from unstimulated primary monocyte-derived macrophages, DNase I-digested CHART-PCR values from each IL-6 proximal promoter region were normalized to the corresponding undigested values (to account for differences in PCR amplification) and the ratio of region 1:2 and 3:2 showed a two- to three-fold difference respectively. The ratio (mean±SEM) of region 1:2 (3.3±1.2) and region 3:2 (2.0±0.73) showed that there was increased digestion with DNase I in the IL-6 promoter region 2, compared to region 1 and region 3, that is that the chromatin in region 2 was more accessible to DNase I, suggesting that this region is a DNase I hypersensitive site and could be constitutively open in these cells.
5.4 Conclusions

The THP-1 cell line was found to be a useful model for studying the differences between monocytic and macrophage-like cells in a model IL-6 response to acute inflammation. The data from the THP-1 cell line work provide proof of principle for the primary monocyte-derived macrophage work to follow. Chromatin accessibility in macrophage-like THP-1 cells was greater than in monocytic cells and this was consistent with IL-6 gene expression levels in macrophage-like cells being greater than in monocytic cells. The chromatin from IL-1β stimulated THP-1 cells was less accessible to DNase I digestion, that is the chromatin was less active, which was consistent with the low IL-6 gene expression level in response to IL-1β.

In primary cells, there was a variable response across the IL-6 promoter such that each region of the IL-6 promoter was more or less accessible to DNase I digestion depending on stimulation with either IL-1β, IFN-γ or LPS for 2 or 8 hours. This could contribute to the difference in the IL-6 mRNA response to each stimulus. IL-6 promoter chromatin remodelling occurred within 30 minutes of stimulation with IL-1β, with the chromatin in the IL-6 promoter from the +GG9/11G individual more accessible than that from the −GG9/11G individual, suggesting a haplotype-specific response.

Although the measured effect of chromatin remodelling in the IL-6 proximal promoter was small when cells were treated with an inflammatory stimulus, the data does suggest that dynamic chromatin remodelling occurs across the IL-6 promoter and is consistent with the differential activation of IL-6.
Furthermore, the data showing that the IL-6 promoter region 2 in chromatin from unstimulated THP-1 macrophage-like cells and primary monocyte-derived macrophages was more accessible to DNase I digestion suggested that this region could include a DNase I hypersensitive site. As DNase I hypersensitivity has been shown to occur in regions of secondary structure including cruciforms (Ward et al., 1991), stem-loops or branches, the following chapter looks at secondary structure in the IL-6 promoter.
Chapter 6 Secondary structure in the IL-6 promoter

6.1 Cruciform structure in the IL-6 promoter

T7 Endonuclease I resolves DNA cruciform structures and Holliday junctions in a structure-dependent manner (Birkenbihl and Kemper, 1998) and was used to identify this secondary structure in the IL-6 promoter in intact nuclei isolated as described in section 2.12.1.

6.1.1 Validating the T7 Endonuclease I assay

The T7 Endonuclease I enzyme was supplied with a positive control (pUC(AT₉)) to assess reaction conditions. pUC(AT₉) is derived from the pUC19 plasmid with nine AT doublets inserted between the EcoRI site and PstI site. This sequence will form a cruciform structure.

One unit of enzyme converted > 90% of 1 μg of supercoiled cruciform pUC(AT₉) to > 90% linear form in 10 minutes at 37°C (Figure 6.1). Quantitative PCR of digested pUC(AT₉) using M13 universal primers confirmed the gel electrophoresis results (Figure 6.2). There was a significant difference between the percentage undigested and the percentage digested at 10, 30 and 60 minutes. The products of the QPCR reactions are also shown in Figure 6.1.
Figure 6.1. Agarose gel electrophoresis of pUC(AT₉) digested with T7 Endonuclease I

pUC(AT₉) plasmid DNA was digested with T7 Endonuclease I at 37°C for 10, 30 and 60 minutes. 1. Supercoiled DNA ladder, 2. Undigested supercoiled plasmid pUC(AT₉), approximately 2.7kb, 3. pUC(AT₉) digested for 10 minutes, 4. pUC(AT₉) digested for 30 minutes, 5. pUC(AT₉) digested for 60 minutes, 6. DNA 1kb ladder, 7-10. QPCR product of 2-5, 11. DNA 50bp ladder.
Figure 6.2. Percentage digestion of pUC(AT₉) with T7 Endonuclease I

pUC(AT₉) plasmid DNA was digested with T7 Endonuclease I at 37°C for 10, 30 and 60 minutes and quantitated using QPCR. n=3, mean±SD. ***P<0.001.

6.1.2 Cruciform structure in IL-6 promoter haplotype constructs

Constructs of the IL-6 promoter haplotypes were previously prepared in the pGL3 basic plasmid (Terry et al, 2000; Khwaja, PhD thesis, Oxford 2005; Khwaja, Terry, Green, manuscript submitted). The plasmids with AG8/12C and GG9/11G constructs were subjected to electrophoresis in a 1% (w/v) agarose gel to assess the level of supercoiled plasmid DNA (Figure 6.3) prior to digestion with T7 Endonuclease I and atomic force microscopy. There was a high degree of supercoiled DNA in both plasmid preparations. Supercoiled DNA is required for cruciform formation (Mikheikin et al, 2006).
Figure 6.3. Supercoiled plasmid DNA containing construct of IL-6 promoter haplotype AG8/12C and GG9/11G
Agarose gel electrophoresis of supercoiled pGL3 basic plasmid with (2) AG8/12C construct and (3) GG9/11G construct. There was a high degree of supercoiled plasmid DNA (→) at approximately 5.4kb (the theoretical size of the plasmid plus insert). (1) Supercoiled DNA ladder 10kb.

Figure 6.4. T7 Endonuclease I digestion of pGL3 plasmid DNA with IL-6 promoter construct AG8/12C
Plasmid pGL3 with IL-6 promoter construct was digested with T7 Endonuclease I at 37°C for 10, 30 and 60 minutes. 1. Supercoiled DNA ladder, 2. Undigested supercoiled construct, 3. Construct digested for 10 minutes, 4. Construct digested for 30 minutes, 5. Construct digested for 60 minutes. Linearised plasmid (←).
One microgram of supercoiled pGL3 basic plasmid containing the AG8/12C IL-6 promoter construct was digested with 1 unit of T7 Endonuclease I for 10, 30 and 60 minutes. The gel electrophoresis of this digestion is shown in Figure 6.4. There was a time-dependent increase in a single linear fragment produced from the plasmid digestion.

The percentage template remaining after digestion was quantitated by QPCR in region 1 and region 2 of the IL-6 promoter (Figure 6.5 and Table 6.1). There was a significant reduction in the amount of template available for QPCR in both region 1 and region 2 of the IL-6 promoter (P<0.001), indicating that both regions of the IL-6 promoter were affected by digestion of cruciform structure.

![Graph](image1)

**Figure 6.5.** Quantitative PCR of IL-6 promoter construct AG8/12C following T7 Endonuclease I

QPCR of IL-6 promoter region 1 and region 2 in supercoiled pGL3 plasmid with IL-6 promoter construct AG8/12C following digestion with T7 Endonuclease I for 10, 30 and 60 minutes at 37°C. Each experiment was in triplicate, assayed in duplicate. ***P<0.001.
Both region 1 and region 2 showed significant digestion with T7 Endonuclease I with no measurable template remaining in region 2 after 60 minutes digestion (Table 6.1), although region 2 was significantly more digested than region 1 at 10 minutes of digestion (P<0.001).

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<td>Region 1 (% mean±SD)</td>
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<td>Region 2 (% mean±SD)</td>
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Table 6.1. Percentage template remaining following T7 Endonuclease I digestion in region 1 and region 2 of IL-6 promoter

QPCR of IL-6 promoter region 1 and region 2 in supercoiled pGL3 plasmid with IL-6 promoter construct AG8/12C following digestion with T7 Endonuclease I for 10, 30 and 60 minutes at 37°C. Each experiment was in triplicate, assayed in duplicate. ***P<0.001, ND not detectable.

6.2 Cruciform structure in the IL-6 promoter in functional nuclei from primary monocyte-derived macrophages

Secondary structure and protein-DNA complexes are preserved in functional nuclei. Nuclei from unstimulated and IL-1β stimulated monocyte-derived macrophages from four individuals were digested with T7 Endonuclease I for 30 minutes at 37°C.
Quantitative PCR of the remaining template showed that all regions of the IL-6 promoter were digested and that there was no significant difference in digestion between the regions, although region 2 was more digested than region 1 and region 3, suggesting more secondary structure in region 2 (Figure 6.6). Although there was no significant difference between T7 Endonuclease I digestion in nuclei from unstimulated and IL-1β stimulated monocyte-derived macrophages, there was less digestion when stimulated. This suggests less secondary structure in stimulated conditions, perhaps allowing access for transcription factor binding.

Figure 6.6. T7 Endonuclease I digestion of IL-6 promoter in nuclei from primary monocyte-derived macrophages

Nuclei from unstimulated and IL-1β stimulated monocyte-derived macrophages were digested with T7 Endonuclease I and quantitated using QPCR. The results are the mean±SEM of four individuals, from triplicate cell culture wells and assayed in duplicate. The values given are the percentage template remaining after digestion in each region (mean±SEM).

The observation that all regions were strongly digested with T7 Endonuclease I was unexpected and suggests that either the T7
Endonuclease I reaction is not specific or that cruciform structures are present, but may not be confined to region 2 of the IL-6 promoter.

To test the possibility that the T7 Endonuclease I reaction was not specific for promoter secondary structure, a non-promoter region in intron 3 of the IL-6 gene was compared to the IL-6 promoter (Figure 6.7). The graphs show very clearly that in unstimulated and IL-1β stimulated monocyte-derived macrophages the intron 3 region is dramatically less digested than the IL-6 promoter regions 1, 2 and 3.

![Figure 6.7](image.png)

**Figure 6.7. T7 Endonuclease I digestion of IL-6 promoter and IL-6 intron 3 in nuclei from monocyte-derived macrophages**

Nuclei from unstimulated and IL-1β stimulated monocyte-derived macrophages were digested with T7 Endonuclease I and quantitated using QPCR. The results are the mean±SEM of four individuals, from triplicate cell culture wells and assayed in duplicate. ***P<0.001.

There was no significant difference in T7 Endonuclease I digestion in the IL-6 promoter regions between the IL-6 promoter haplotype +GG9/11G and −GG9/11G (data not shown).
6.3 DNA structure and folding predictions

The proximal IL-6 promoter sequence (650bp) (given in section 2.12.4 Figure 2.1) was entered into a web-based DNA folding prediction program to assess the predicted secondary structure. The sequence for two IL-6 promoter haplotypes (AG8/12C and GG9/11G) as well as the THP-1 haplotype sequence was entered into Mfold (Zuker, 2003; Mathews et al., 1999; SantaLucia, Jr., 1998) (http://www.bioinfo.roi.edu/applications/mfold/). It was assumed that the DNA is linear for the purposes of this prediction. The default reaction conditions were set at 37°C and 1M Na⁺.

The results of the folding predictions are given as ΔG (kcal/mol) (Gibbs free energy change). This is the energy required to initiate a spontaneous chemical reaction. A negative ΔG value denotes a spontaneous reaction. The greater the -ΔG, the greater the driving force behind the reaction and the most energetically favourable (Table 6.2).

There was a small difference in the ΔG between AG8/12C and GG9/11G. The THP-1 ΔG was marginally more energetically favourable than the GG9/11G and AG8/12C. To analyse the difference between AG8/12C and GG9/11G IL-6 promoter haplotype, the AG8/12C haplotype sequence was changed by one base at a time (Table 6.2). By changing the -597 A to G, the ΔG approached that predicted for GG9/11G, and by changing the -174 C to G the ΔG matched that predicted for the GG9/11G (ΔG=-68.67).
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<tr>
<td>-65.04</td>
<td>-65.98</td>
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<td>-64.60</td>
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<td>-64.26</td>
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Table 6.2. Gibbs free energy ($\Delta G$) for the most energetically favourable folding predictions of IL-6 promoter haplotype from MFOLD

The table gives the $\Delta G$ of the haplotype sequences predicted by MFOLD.

By changing one base at a time in the input sequence from AG8/12C to GG8/12C, the same $\Delta G$ was achieved as for the GG9/11G (bold).
Figures 6.8 represents the most energetically favourable folding prediction for each sequence and shows the overall difference between the AG8/12C and GG9/11G haplotypes and the THP-1 haplotype. No difference was seen in the AnTn region. The major difference occurred around the -597 SNP with a smaller difference at -174. Detailed annotated views of the AG8/12C and GG9/11G folded sequences are included in Appendix II. By changing the -597 A to G the folding profile changed to resemble that of the GG9/11G profile, and by changing the -174 C to G the profile matched that predicted for the GG9/11G.

This suggests that the -373 AnTn polymorphism alone does not contribute to the difference in the folding predictions observed.

Figure 6.8. Folding profiles of IL-6 promoter haplotype sequence
(over page)
The figures represent the lowest energy folding prediction for each IL-6 promoter sequence (AG8/12C -66.57 kcal/mol, GG9/11G -68.67 kcal/mol, THP-1 GC10/10G -69.89 kcal/mol, GG8/12C -68.35 kcal/mol, GG8/12G -68.67 kcal/mol). These predictions were derived from the MFOLD web-software by entering the sequence variants.
6.4 Atomic force microscopy of DNA cruciform structure

Secondary structure in supercoiled plasmid DNA was visualised using Atomic Force Microscopy (AFM). Further to the biochemical experiments with T7 Endonuclease I which demonstrated the presence of cruciform structure or structures in the IL-6 promoter, AFM was used to visually assess the secondary structure. In all images, the scale is in micrometres (μm) as indicated in the figures. A schematic diagram of cruciform extrusion in supercoiled DNA is shown in Figure 6.9.

![Figure 6.9. Cruciform structure in supercoiled DNA](image)

AT rich regions are extruded as cruciform structure (→) under conditions of negatively supercoiled DNA (adapted from Gellert et al, 1983).

6.4.1 Cruciform structure in pUC(AT₃)

pUC(AT₃) was shown to be digested by T7 Endonuclease I which resolved cruciform structures in DNA. A sample of undigested pUC(AT₃) (0.4μg/μl) was prepared on freshly cleaved mica treated with 0.001% (v/v) APS. Images were scanned in tapping mode at a rate of 1.56Hz. A representative series of images is shown with increasing resolution from 10μm x 10μm to 1μm x 1μm (Figure 6.10). These images showed a dense pack of supercoiled DNA plasmid molecules. By increasing the resolution, more structures became visible. At a resolution of 0.5μm x 0.5μm a molecule with
a cruciform shape was observed and this was further resolved at 0.3μm x 0.3μm and 0.2μm x 0.2μm (Figure 6.11).

The expected length of the relaxed plasmid would be approximately 1μm (0.33nm x 2.7kb). The supercoiled molecule visible in the 0.5μm image was measured at approximately 250nm in length, one quarter of the length of the relaxed molecule. Super coiling was well demonstrated in the 0.3μm image (*). Two twists were observed over a length of 50nm. One helical turn of DNA is approximately 3.4nm (10.4bp) in length (Wang, 1979), therefore the twists seen were not two helical turns but supercoiled twists comprising fourteen to fifteen DNA helices.

The small branched region indicated by the arrow could be a cruciform structure. It measured 30nm in length across the structure, approximately 340bp of supercoiled structure, although this could not be verified.

Figure 6.10. Atomic force microscopy images of undigested pUC(ATg) (over page)
Undigested pUC(ATg) scanned in tapping mode with increasing resolution from 10μm x 10μm to 1μm x 1μm.
Figure 6.11. High resolution atomic force microscopy images of undigested pUC(AT₉)
Undigested pUC(AT₉) plasmid visualised with AFM in tapping mode to a resolution of 0.2µm x 0.2µm. The high resolution images show supercoiled plasmid DNA (*) and possible cruciform structure (→).
The pUC(AT₉) positive control sample (0.4µg/µl) was digested with T7 Endonuclease I to disrupt the cruciform structure. Figure 6.12 is a representative series of AFM images of increasing resolution showing linear molecules, not densely packed as in the undigested sample. The molecules were measured at approximately 1µm in length, which was consistent with the expected length for the pUC(AT₉) (2.7kb).

At high resolution, it was observed that the secondary structure had been resolved by T7 Endonuclease I digestion and the molecules became more linear with no apparent supercoiled or secondary structure. These images were consistent with the biochemical data (Figure 6.13).

**Figure 6.12.** Atomic force microscopy images of T7 Endonuclease I digested pUC(AT₉)

(over page)

Digested pUC(AT₉) scanned in tapping mode with increasing resolution from 10µm x 10µm to 1µm x 1µm.
Figure 6.13. High resolution atomic force microscopy images of T7 Endonuclease I digested pUC(AT₉)
Digested pUC(AT₉) plasmid visualised with AFM in tapping mode to a resolution of 0.2μm x 0.2μm. The high resolution images show that secondary structure was resolved by digestion with T7 Endonuclease I.
6.4.2 Cruciform structure in IL-6 promoter haplotype constructs

A sample of supercoiled plasmid DNA with IL-6 promoter construct GG9/11G (0.5µg/µl) was prepared on freshly cleaved mica treated with 0.001% (v/v) APS. Images were scanned in tapping mode at a rate of 1.56Hz (Figure 6.14). The images demonstrate the increasing resolution of the supercoiled plasmid with the IL-6 promoter construct GG9/11G. The supercoiled structure of the plasmid was visible as a dense white area (→) and a branched or cruciform region (*) was observed in the AFM images.

In the 0.5µm image the molecule was measured at approximately 500nm. The relaxed plasmid would be expected to be approximately 2µm in length (5.4kb). The supercoiled molecule was therefore a quarter of the relaxed molecule.

A sample of supercoiled plasmid with the IL-6 promoter AG8/12C promoter construct (0.5µg/µl) was prepared for AFM as described. A representative series of images is shown in Figure 6.15. The branched regions were measured at approximately 30nm to 40nm which was consistent with the measurements in the pUC(AT₉) samples.
Figure 6.14: Atomic force microscopy images of IL-6 promoter haplotype construct GG911G. Supercoiled plasmid DNA was scanned by atomic force microscopy in tapping mode. The supercoiled structure of the plasmid was visible at 0.5μm x 0.5μm (→). A potential cruciform structure is seen (*).
Figure 6.15. Atomic force microscopy images of IL-6 promoter haplotype construct AG8/12C

The images show the increasing resolution of the supercoiled plasmid with the IL-6 promoter construct AG8/12C. The images show the supercoiled structure of the plasmid and branched or cruciform regions (↓).
6.5 Conclusions

The specificity of T7 Endonuclease I for cruciform DNA structure was not shown in this thesis, however it is an accepted method for determining cruciform topology (Mizuuchi, 1982; Panayotatos and Fontaine, 1987). An appropriate negative control to demonstrate the specificity of the reaction to the AT₉ insert, and not to another sequence within the plasmid, would be to digest the native pUC19 vector with T7 Endonuclease I under the same conditions. T7 Endonuclease I was shown to cleave the supercoiled pUC(AT₉) and putative cruciform structure was demonstrated in the IL-6 promoter in constructs and in intact nuclei from primary monocyte-derived macrophages.

T7 Endonuclease I digestion of the IL-6 promoter was shown to differ from the digestion of a non-promoter region, which suggests a promoter-specific cleavage of secondary structure. No haplotype difference was observed in the T7 Endonuclease I digestion in nuclei prepared from monocyte-macrophages. The non-significant increase in template remaining after T7 Endonuclease I digestion in nuclei from IL-1β stimulated monocyte-derived macrophages suggests a biological significance which could be due to individual variability or could be the result of a dynamic process which the promoter undergoes in conditions of stimulation, as the promoter becomes more accessible to transcription factor binding and active transcription.

DNA folding predictions suggest a haplotype-specific profile. Although the predicted folding patterns did not show specific cruciform structure, it did
predict a high degree of secondary structure, branches and stem loops. The limitations of the default reaction conditions suggest that a different profile could be expected under supercoiled conditions or where chromatin is under torsional stress by nucleosomes, for example. This analysis requires more sophisticated software and processing than was available here.

Although the AFM results were qualitative, it was clear that DNA secondary structure could be visualised and quantitated. Measurements were made and this provided an understanding of the topography of supercoiled plasmid DNA. Achieving high resolution requires clean sample preparation and storage, constant temperature and low relative humidity. The spring constant, or hardness, of the tapping cantilever was also a contributing factor. DNA is a soft biological material with a strong negative charge. The cantilever needs to tap across the topography of the DNA recognising the height and contrast of the sample. In conditions of high static or high relative humidity, it was more difficult to obtain high resolution images.

Cruciform and branched secondary structures were visible in both the AG8/12C and GG9/11G IL-6 promoter haplotype constructs but no quantifiable difference was observed between the haplotypes.
Chapter 7 Discussion

7.1 THP-1 cell line: a monocyte-macrophage model of the IL-6 inflammatory response

Macrophage-like THP-1 cells have been shown to be similar to primary monocyte-derived macrophages with 0.88 correlation in cytokine gene expression profiles (Kohro et al, 2004). The IL-6 response was determined as a marker of inflammation in differentiated and undifferentiated THP-1 cells.

Monocytic THP-1 cells were differentiated into macrophage-like cells using the phorbol ester PMA. PMA has been shown to activate the protein kinase C pathway of mitogen activated protein kinase (MAP) and extracellular signal related kinase (ERK) in differentiating HL-60 cells to macrophages (Schultz et al, 1997) and ERK in the Jurkat T cell line (Bradshaw et al, 1996). However, in THP-1 cells it has been shown to activate the ERK and c-Jun kinase but not the p38 MAP kinase (Carter et al, 2001).

There was little IL-1β-induced IL-6 gene expression in both the monocytic and macrophage-like THP-1 cells and the chromatin accessibility data (a measure of active transcription) showed that the IL-6 proximal promoter chromatin was less active when the cells were stimulated for 16 hours with IL-1β than at baseline or when stimulated with IFN-γ or LPS. Low expression of IL-6 was also observed in PMA-differentiated THP-1 cells as part of a study of human intestinal epithelial and smooth muscle cells (Ng et al, 2003). Interleukin-6 protein levels were not detectable in response to IL-1β.
stimulation for 24 hours. This may not be due to lower receptor number as PMA appears to induce the IL-1r in THP-1 cells (Spriggs et al, 1990).

In early reports, it was proposed that treating THP-1 monocyctic cells with 100nM PMA for 48 hours resulted in increased IL-1 receptor antagonist (IL-1ra) an anti-inflammatory cytokine, which decreased IL-1β binding (Bienkowski et al, 1990). It was later shown that soluble IL-1ra was degraded more slowly in monocyctic THP-1 cells treated with LPS, suggesting a role in the down-regulation of the pro-inflammatory cytokine IL-1β (Learn et al, 2000). Although the THP-1 cells in this study were not endotoxin tolerant (pre-treated with LPS), upregulation of IL-1ra in PMA differentiated THP-1 cells could explain the low response of THP-1 cells to IL-1β.

Cells of the monocyte-lineage including THP-1, U937 and primary monocytes, have been shown to constitutively express NF-kB (Frankenberger et al, 1994). Interleukin-1β signals through the interleukin-1 receptor associated kinase leading to the induction of signalling pathways including p38 MAP kinase and the activation of NF-kB. The action of IL-1ra is independent of the signalling pathway for IL-1β, that is there is no effect on the components of the signalling pathway (Evans et al, 2006). The inhibition of the IL-1β response was shown to be ATP dependent and cell specific. The experiments were performed in transfected HUVEC and HeLa cells and as macrophages are not easy to transfect it may not be possible to confirm this, however it does suggest a possible mechanism for the macrophage-like THP-1 response.
Stimulation of THP-1 cells for 16 hours with IFN-γ or LPS did not result in a significant difference in IL-6 promoter chromatin accessibility between monocytic and macrophage-like THP-1 cells but there was overall more chromatin accessibility in the IL-6 promoter in the macrophage-like cells. However, at 16 hours of stimulation with LPS, there was a significant difference in IL-6 gene expression in monocytic and macrophage-like THP-1 cells. At 24 hours of treatment with IFN-γ, there was a significant difference in IL-6 mRNA expression between monocytic and macrophage-like THP-1 cells. Thus, macrophage-like THP-1 cells responded as expected to IFN-γ and LPS stimulation. The THP-1 protein data were consistent with previous reports for IFN-γ and LPS stimulation (Biondillo et al., 1994).

Macrophages play a central role in the development of atherosclerosis, an inflammatory process, and are found in atherosclerotic plaques. Statins are used clinically for lipid-lowering but their pleiotropic effects, including an anti-inflammatory role, are being increasingly reported. Interleukin-6 mRNA expression was inhibited by statins in macrophage-like THP-1 cells. Atorvastatin and pravastatin are prescribed clinically to lower cholesterol by inhibiting endogenous cholesterol synthesis. The therapeutic dose of atorvastatin calcium is 10-80mg daily whilst the dose for pravastatin sodium is 10-40mg daily. This was calculated as 4-16μM and 6-23μM respectively. The concentrations of the statins used in vitro were 1μM, 5μM and 20μM, thus the in vitro concentrations were in line with the physiological therapeutic doses in vivo. There was no cytotoxic effect of the statins at higher concentrations as the levels of the housekeeping gene MLN51 remained
constant throughout the experiment. In this study, the statins were prepared in DMSO to ensure equivalent entry into the cells. This means that the reduction in IL-6 gene expression achieved by the two statins could be directly compared.

Although the two statins used in this study have the same mechanism of action on cholesterol metabolism, they differ in their chemical structure, which affects absorption and metabolism in vivo. Pravastatin is hydrophobic and would normally be bound to a carrier molecule for entry into the hepatocytes. Atorvastatin is lipophilic and enters the cells by passive diffusion through the cell membrane. The elimination half-lives in vivo also differ; pravastatin has a half-life of 1-3 hours whilst atorvastatin has a half-life of 14 hours (Pan et al, 1990; Cilla, Jr. et al, 1996).

Despite atorvastatin being approximately four times as potent as pravastatin with respect to LDL-cholesterol lowering (Pincus, 1998), no significant difference between pravastatin and atorvastatin on the reduction of IFN-γ stimulated IL-6 mRNA expression was observed in vitro. There was no observed affect of the statins on unstimulated IL-6 mRNA expression, which suggests that the mechanism of action could be through statin-induced repressors of IL-6 transcription.

Atorvastatin has been shown to act as a repressor of MHC-II mediated T cell activation only when stimulated with IFN-γ (Fehr et al, 2004). Treatment with atorvastatin has been shown to decrease the activation of the transcription
factor NF-κB, possibly by inhibiting protein isoprenylation, in primary rat vascular smooth muscle cells and in the U937 monocyte cell line in vitro (Ortego et al, 1999) and in human endothelial cells and vascular smooth muscle cells in vitro (Dichtl et al, 2003). This is consistent with the findings in primary adipocytes treated with cerivastatin (Van Harmelen et al, 2003).

In a study of primary adipocytes cultured from rabbits fed a high cholesterol diet and treated with atorvastatin for two weeks, interleukin-6 levels were measured by ELISA in cell culture supernatants. Atorvastatin resulted in decreased IL-6 concentration in a dose dependent manner up to 10μM (Zhao and Zhang, 2003). Simvastatin inhibited TNFα-induced IL-6 and IL-8 production in fibroblast-like cells from patients with rheumatoid arthritis (Yokota et al, 2006).

Treatment with pravastatin in in vivo clinical trials showed a reduction in cardiovascular events and a reduction in circulating levels of the cytokines TNF-α, IL-6 and IL-8 (Rosenson et al, 1999). The PROVE-IT Study and REVERSAL Trial (Salam, 2004) showed a reduction in CRP levels in patients prescribed either atorvastatin or pravastatin. Data from the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) Study Investigators (Schwartz et al, 2001) produced evidence of the effect of 80mg daily atorvastatin on plasma C-reactive protein, serum amyloid A and IL-6 in patients enrolled in this study (Kinlay et al, 2003; Kinlay et al, 2004).
In contrast, the effect of statins on macrophage-like THP-1 cells in vitro shown in this thesis suggests that the drugs not only act to reduce inflammatory markers at the level of the liver but may also have a local effect on reducing IL-6 gene expression in macrophages in the atherosclerotic lesion. By reducing inflammation in the lesion it may be possible to reduce or retard lesion development and thereby reduce recurrent ischaemic events, which may explain the MIRACL trial results and provide further evidence for the use of statins.

7.2 IL-6 gene expression in primary monocyte-derived macrophages from healthy individuals of known IL-6 promoter haplotype

A primary monocyte-derived macrophage model of inflammation was optimised for the quantitation of IL-6 gene expression under conditions of inflammatory challenge. Monocyte-derived macrophages were stimulated with three different inflammatory challenges to show if there was a common mechanism for the IL-6 promoter haplotype-specific gene activation. The results of the chromatin accessibility (CHART-PCR) assays and the gene expression experiments showed a stimulus-specific IL-6 response, but only stimulation with IL-1β produced a significant haplotype-specific mRNA response. The stimulus-specific data were consistent with different pathways of receptor-signalling. It is not clear whether the varied response of the population studied is due to stimulus-specific transcription factor binding, however there is a stimulus-specific difference in active chromatin across the proximal IL-6 promoter and this response varied according to the region of the proximal IL-6 promoter. This suggests that the regions of the IL-6
promoter co-operate to contribute to IL-6 gene expression and although the response to IFN-γ and LPS was not haplotype-specific, there is nonetheless an overall contribution of promoter organisation to IL-6 gene expression, which could be regulated by IL-6 promoter haplotype. The IL-6 protein concentration in cell culture supernatants was not significantly haplotype-specific, although cells from the +GG9/11G IL-6 promoter haplotype did produce slightly higher levels of IL-6 at baseline and when stimulated. Moreover, monocyte-derived macrophages from normal, healthy individuals were able to respond to the inflammatory challenges by producing IL-6, regardless of IL-6 promoter haplotype, which could explain why there is little consensus in the literature on the role of IL-6 promoter genotypes in disease.

IFN-γ and LPS have been shown to upregulate IL-6 mRNA expression within 60 minutes of stimulation in a calcium independent manner (Marriott et al, 1998). It has been previously reported that macrophages exhibit a differential response to LPS with or without priming with IFN-γ (Feng et al, 2004; Hayes et al, 1995). This synergistic effect was also seen in human retinal pigment epithelial cells stimulated with LPS and IFN-γ and resulted in a ten fold induction of IL-6 production (Nagineni et al, 1994). IFN-γ priming for LPS response resulted in the upregulation of toll-like receptor 4/MD-2 complexes and MyD88, activation of interleukin-1 receptor associated kinase, NF-κB activation and cytokine production in monocyte-derived macrophages (Bosisio et al, 2002). No significant increase in IL-6 mRNA expression was observed in the small study presented here, which could be due to the
duration of priming with IFN-γ being 12 or 24 hours compared to 40 hours (Bosisio et al, 2002).

A sequential test of association of each of the seven IL-6 promoter polymorphisms revealed that only the -373 9A/11T allele was associated with a significantly higher IL-6 response to IL-1β stimulation. This is consistent with the data from the comparison of the IL-6 proximal promoter haplotype (+GG9/11G) when stimulated with IL-1β.

Analysis of the IL-6 promoter using UCSC Genome Browser (http://genome.ucsc.edu/) showed that of the polymorphisms genotyped, only the promoter SNP -174 was within a highly conserved region in a comparison of 17 species (LOD score 220), although it was previously reported that the length of -373 AnTn region was highly conserved (Kelberman et al, 2004). The IL-6 -174 G/C SNP has been extensively studied with respect to disease association with little consensus on its role. The data in this thesis from normal, healthy volunteers did not show a significant role of the -174 SNP on its own. Moreover, a significant association in IL-6 mRNA expression was observed with the IL-6 -373 9/11 genotype.

A previous report in an ex vivo model showed no difference between the IL-6 -597 G, -572 G, -174 G haplotype and other IL-6 promoter haplotypes stimulated with LPS. On closer inspection of the work however, it was clear that the authors were not working from a baseline monocyte-derived macrophage model. These results were observed in a whole blood assay of
IL-6 mRNA expression (Muller-Steinhardt et al, 2007) and this makes the result difficult to interpret in the context of the data presented in this thesis, although it does suggest that genotyping the -373 AnTn polymorphism in the IL-6 promoter may have shown haplotype-specific differences.

An AU rich region has been identified in the 3' UTR in human IL-6 mRNA (Neininger et al, 2002). AU rich elements (AREs) contribute to the localisation of mRNA, translation, and degradation, that is, they play a role in IL-6 mRNA stability. In mice, LPS induced IL-6 gene expression depends on MAPKAP kinase 2, and this was shown to be regulated through ARE translational control. Second messengers may increase mRNA stability; for example leukotriene B4, a lipid mediator of inflammation, increases the half life of IL-6 mRNA from one to three hours by a mechanism of increased transcription and stability (Rola-Pleszczynski and Stankova, 1992). IL-6 mRNA under basal conditions has a half life of 30 minutes and is destabilised by AUF1, a nuclear protein and ARE-specific mRNA destabilising factor (Paschoud et al, 2006). IL-6 mRNA in osteoclasts stimulated with IL-1β has a half life of 90 to 120 minutes whilst in the presence of the p38 MAP kinase inhibitor SB203580 this was reduced to 24 minutes (Patil et al, 2004), suggesting that p38 is required for IL-6 activation. In MC3T3-E1 osteoclast cells stably transfected with the IL-6 3'UTR, it was demonstrated that the main target of p38 MAP kinase activity was the 3' UTR of the IL-6 gene. In human fibroblast-like synoviocytes, IL-1β activation of MAP p38 kinase resulted in the stabilisation of IL-6 mRNA possibly through activation of AUF1 AU rich element binding protein in the 3' UTR (Miyazawa et al, 1998). These findings
suggest an additional mechanism of regulation of IL-6 expression in inflammatory conditions, where the IL-6 mRNA half life could be extended.

7.3 Mechanisms of normal IL-6 promoter activation

The chromatin accessibility assay is a quantitative 'snapshot' of chromatin remodelling events. Chromatin accessibility is determined by epigenetic modification, including histone acetylation, methylation, phosphorylation and ubiquitination (Im et al, 2004). Previous reports suggest that chromatin accessibility is cell-type specific. In monocytic cell lines stimulated with IL-1β chromatin remodelling events are required for inducible immediate-early response genes. This is a dynamic process, involving the recruitment of key transcriptional activators, including CK2, and leads to cytokine gene regulation (Liang et al, 2006). Using CHART-PCR chromatin remodelling was obvious in the IL-2 proximal promoter from 1.5 hours to 16 hours in murine EL-4 and primary CD4+ T cells stimulated with PMA (Rao et al, 2001). However, no chromatin remodelling was seen in the IL-2 promoter in CD4+CD25+Treg cells stimulated with PMA (Su et al, 2004), consistent with these cells not producing IL-2.

This cell type specific difference was observed between monocytic and macrophage-like THP-1 cells stimulated with IL-1β, IFN-γ or LPS in the three regions of the IL-6 promoter. There was greater chromatin remodelling in the IL-6 promoter in the macrophage-like cells than in the monocytic cells, which was consistent with higher IL-6 gene expression in the former.
In primary monocyte-derived macrophages from healthy individuals of known IL-6 promoter haplotype, chromatin remodelling was shown to be time-, stimulus and IL-6 promoter region-dependent. It was shown that chromatin remodelling in the IL-6 promoter is a dynamic process, occurring rapidly within 30 minutes of IL-1β stimulation of monocyte-derived macrophages. There were IL-6 promoter haplotype specific differences in chromatin accessibility, although not statistically significant, and this does suggest a possible mechanism of haplotype-specific IL-6 expression. This is the first report of detailed differential chromatin remodelling events across the IL-6 proximal promoter and provides evidence for the role of chromatin remodelling in the regulation of IL-6 gene activation in a monocyte-derived macrophage model of inflammation.

Five DNase I hypersensitive sites have previously been identified in the IL-6 promoter at position -1400, -600, -150, +200, +400. These were identified by DNase I footprinting using Southern blotting in breast cancer cell lines (Armenante et al, 1999). This method is not quantitative. The hypersensitive sites at -600 and -150 are within the IL-6 proximal promoter region 1 and region 3 respectively. In the present study, however, a DNase I hypersensitive region was identified from -340 to -470. The difference could be due to the numbering of the basepairs. In the present study, the labelling nomenclature is from the start of transcription (-1, indicated in Appendix I) a difference of 64 bases from the translational start. It is more likely that the early methods of identifying DNase I hypersensitivity could not accurately pinpoint the region. However, the results presented here, that the IL-6
proximal promoter has a region or regions of DNase I hypersensitivity, are consistent with the earlier data, although in a different cell type. DNase I hypersensitive sites are associated with promoter regulatory function and structure (Elgin, 1988). Nucleosomes are excluded from DNase I hypersensitive sites, which allows regulatory regions of genes to be accessible to transcription activation complexes. In this thesis, the finding of a DNase I hypersensitive site in region 2 of the IL-6 proximal promoter was made in primary cells derived from four individuals and not from a cell line. In spite of the biological differences between individuals, there was still a strong DNase I hypersensitive response in region 2 of the IL-6 promoter. Region 2 includes the variable AnTn polymorphic region at position -373 which was shown to be associated with a higher IL-6 mRNA response to IL-1β stimulation and suggests that this could be a key regulatory element in the IL-6 promoter.

The CHART-PCR approach was also used to quantitate DNase I hypersensitive regions in the mouse β-globin locus control region in foetal liver cells. The authors identified weak, strong and intermediate hypersensitive sites. These intermediate sites were flanking strong hypersensitive regions but were constrained by nucleosomes (McArthur et al, 2001). Nucleosomes have been shown in yeast cells to exhibit a DNA sequence preference, based on the ability of the sequence to loop into nucleosomes (Ioshikhes et al, 2006; Segal et al, 2006). This genome positioning of nucleosomes was shown to be functional and predictable, that is related to regions of gene regulation (Segal et al, 2006). There are low
numbers of nucleosomes around transcription start sites. Promoters with a TATA box within a nucleosome positioning sequence appear to be regulated by chromatin remodelling (Ioshikhes et al, 2006).

The IL-10 promoter was shown to have a DNase I hypersensitive site in macrophages but not T cells, that is macrophage-specific chromatin packaging (Saraiva et al, 2005). Chromatin accessibility was associated with a transient phosphorylation of histone protein H3 (Ser10) at specific sites in the IL-10 promoter and transcription factor binding (Zhang et al, 2006), necessary for gene expression. It would be useful to determine if this macrophage-specific chromatin response was seen in the IL-6 promoter and would have implications for the IL-6/IL-10 balance in regulating the inflammatory response in macrophages.

A region of thirteen AT repeats in yeast chromatin in the 3' region of the FBP1 gene was shown by T7 Endonuclease I digestion to have a cruciform structure. This region was part of a nuclease sensitive site flanked by nucleosomes (Del Olmo et al, 1993; Del Olmo and Perez-Ortín, 1993). The cruciform extrusion depended on the supercoiled state of DNA. Deleting the AT_{13} region abolished the hypersensitive site but did not alter the nucleosome position (Aranda et al, 1997).

Although cruciform folding was not predicted by MFOLD or Kinefold (Xayaphoummine et al, 2005) (http://kinefold.curie.fr/cgi-bin/form.pl), a version of web software for shorter sequences of DNA which predicted the
same folding profiles as MFOLD), digestion of the IL-6 promoter chromatin from monocyte-derived macrophages suggested the presence of cruciform secondary structure within the IL-6 proximal promoter. This has not been shown previously but is consistent with the DNase I hypersensitivity in region 2 of the IL-6 promoter.

Previous reports on cruciform extrusion have shown that negative supercoiling of the DNA double strand is required for cruciform or Holliday junction formation (Mikheikin et al, 2006). This was studied by atomic force microscopy in a supercoiled plasmid (Shlyakhtenko et al, 2000). Ruv A binds four-way junctions and the protein-DNA complexes were visualised on AFM. The images presented here show potential cruciform structures within supercoiled DNA based on the expected view. The numbers of cruciforms were not quantitated as there were insufficient fields in this pilot study, but the supercoiled structure was clearly visible.

Taken together these results suggest that a DNase I hypersensitive site in the IL-6 promoter region 2 is associated with cruciform structure and that region 2 may be flanked by constraining nucleosomes. The regions on either side of region 2 show intermediate DNase I sensitivity and are affected by the cruciform structure in region 2 (summarised in Figure 7.1). There is no evidence in the present study of haplotype-specific cruciform formation, however, it is a small study and is thus not conclusive.
Figure 7.1. A model of IL-6 promoter structure and function
Panel A shows the linear arrangement of the proximal IL-6 promoter including the transcription factor binding sites and locations of the single nucleotide polymorphisms and the variable AT tract in region 2. A DNase I hypersensitive site, associated with open chromatin, was demonstrated in region 2 of the IL-6 proximal promoter (panel B). It is suggested that nucleosomes on either side of the AT tract in region 2 constrain the DNA double helix, resulting in cruciform extrusion (panel C), which could affect transcription factor binding and gene expression.

Another mechanism to consider is the effect of the variable AnTn polymorphism on the curvature of the DNA helix. DNA curvature, the twisting
and writhing of DNA, affects transcription. Curvature of the DNA structure is a conserved feature of promoter organisation (Marilley and Pasero, 1996). A polyA-poly-T region in DNA was preferentially associated with DNA bending (Hagerman, 1986) and stacking of the A-T bases, as seen in the folding predictions of the IL-6 promoter haplotypes, is linked to helix formation (Dickerson, 1998). There are usually 10.4bp to a DNA helix (Wang, 1979), however it is possible, though not proved here, that the variable length AnTn (8A/12T, 9A/11T, 10A/10T, 10A/11T) region in the IL-6 promoter at position -373, is subject to variable curvature and could therefore affect the relative positioning of transcription factors on either side of the AT tract, and IL-6 gene expression.

This project studied the normal genetic variation and normal activation of IL-6 in healthy volunteers of known IL-6 promoter haplotype. IL-6 mRNA expression was induced in a haplotype-specific manner in response to IL-1β stimulation. Regulatory elements were identified in the IL-6 promoter. Haplotype-specific dysregulation of IL-6 and/or an imbalance with anti-inflammatory cytokines such as IL-10 could account for the varied disease associations reported.

7.4 Future work

The data presented here point to region specific chromatin remodelling, DNase I hypersensitivity and cruciform structure within the IL-6 promoter. The CHART-PCR regions could be further refined to provide a more detailed picture of the dynamic process of chromatin remodelling.
immunoprecipitation could be used to determine which transcription factors or complexes bind in each region of the promoter under different conditions of stimulation. This approach was used to study regulation of the IL-10 promoter in T cells and T helper cells (Kang and Im, 2005). A novel approach using anti-cruciform antibodies (Frappier et al, 1989) and QPCR could be used to immunoprecipitate and identify cruciform regions in the IL-6 promoter.

Transcription factor binding databases were used to determine if there was a consensus on transcription factors to look for with chromatin immunoprecipitation. MATInspector (http://www.genomatin.de) and TFSEARCH (http://www.cbrc.jp/htbin/nph-tfsearch) both identified a putative HNF-3 forkhead homologue 2 (Hfh-2) binding site in the region of the IL-6-373 AnTn region. This was not genotype-specific. PROMOTERSCAN (http://zeon.well.ox.ac.uk) and TFBIND (http://tfbind.hgc.jp/) identified the known transcription factors binding sites and additional potential sites with a lower weighting, such as HNF-4, with no predicted haplotype-specific difference. It could therefore be a complex of trans-activation factors which contributes to haplotype-specific IL-6 mRNA expression. For example, co-operativity between the NF-κB and NF-IL6 transcription factors in the regulation of the IL-6 promoter in human prostate cancer cells (Xiao et al, 2004a) and NF-κB co-operation between the c-jun and AP-1 sites in human multiple myeloma cells (Xiao et al, 2004b).

Further ex vivo studies could be undertaken to study the effects of inhibition of the receptor-signalling pathway components to identify which components
are activated and necessary for IL-6 transcription. Microarray studies would identify up-and down-regulated components of the process in this model, instead of the single output measured in this study.

In addition, this study only considers the role of chromatin remodelling in the proximal promoter, although the individuals have been genotyped for the distal promoter polymorphisms. It may provide additional information on the mechanisms of IL-6 promoter activation to consider the effect of all seven promoter polymorphisms on chromatin accessibility, although the present analysis of IL-6 mRNA expression confirmed that the -373 AnTn polymorphism was the only influence on IL-1β stimulated mRNA expression. It would also inform the study of IL-6 regulation to determine other sites of DNase I hypersensitivity across the IL-6 gene.

The AFM pilot study showed that the technique can be used to study DNA secondary structure at high resolution. By increasing the concentration of plasmid and therefore the number of molecules per field, the cruciform structures could be quantitated to determine if there is a haplotype-specific difference in IL-6 promoter cruciform. By choosing an appropriate negative control, that is, native plasmid, it could be shown that the cruciform structure occurs specifically within the IL-6 promoter construct.

A similar ex vivo study in primary macrophages from individuals with inflammatory conditions such as coronary artery disease or inflammatory
bowel disease could reveal dysregulation of the IL-6 promoter and provide a meaningful explanation for disease occurrence.
Appendix I Annotated Interleukin-6 gene and promoter

Legend
ATG/TAG start/stop codon
tata box
Exon 1 81bp
Exon 2 191bp
Exon 3 114bp
Exon 4 147bp
Exon 5 592bp
Bold CAPS = CDS
Unbold CAPS = EXONS
Polymorphisms
lower case = introns

Gene expression primers

-2049 cagccaggat caaacaagtg ggaagacgag aaaaaccttc ccaggtcagg -2000
-1999 ataacagagg atttggttga aataacaggg aatttaggtgc taccctctggg -1950
-1949 aaaaagggcc agggagagaa ggagacactt ttccctgcct gcctgtatgt -1900
-1899 cctatttgaa cattttatca tgaacacgaa cttccatattt aaaaaactct -1850
-1849 ttttattgaa aagataaact tgttgtgttgt aattgtgctac tcagttcaag -1800
-1799 tactttgaat ttattgaatt tatttttctt aaaaaagtag aatttgaataa -1750
-1749 aagcaagctc acattacata gacggatcac agtgcacggc tgcggagctg -1700
  rs2069824 T/C
-1699 ggacgagtgg ccctgctttca tgcaggaag aagaacattg ttcaggtgtg -1650
-1649 ccagttgct ctaagacagga gagcaactaa aatgaacacc tccagccatc -1600
-1599 ctccccctaat ttcatctttca caccaaagaa tccccagcgc gcaagagacc -1550
  rs2069825 [-/CT]
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  rs2069827 G/T
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502 ACCCTTTTCAG AACGAATGGGA CAAAACAAATT CGGTACATGCC TCGACGGCAT
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552 TCAGCGCCTTG AGAAAGGAGAGC AGATCTCAGCTGCGTCTGAGACGTTCTT
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602 CCGGTGCGCA TGCGCTCCCCT GGCCCGCTTGG GTGCGGGCGG GGGCTGGCGCT
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Appendix II Detailed IL-6 promoter haplotype folding predictions
### Appendix III Interleukin-6 genotyping results

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Appendix IV Poster abstracts and conference proceedings


Published abstracts from conference proceedings


Reference list


Birkenbihl RP, Kemper B (1998). Endonuclease VII has two DNA-binding sites each composed from one N- and one C-terminus provided by different subunits of the protein dimer. EMBO Journal 17(15):4527-34.


Evans I, Dower SK, Francis SE, Crossman DC, Wilson HL (2006). Action of intracellular IL-1Ra (Type 1) is independent of the IL-1 intracellular signalling pathway. Cytokine 33(5):274-80.


