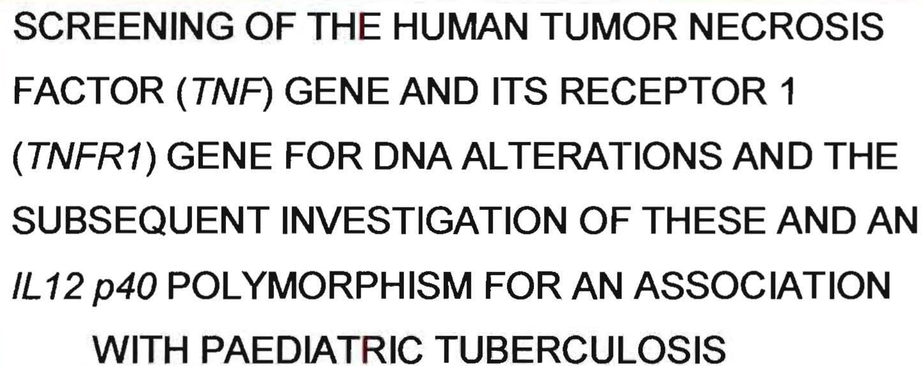

SCREENING OF THE HUMAN TUMOR NECROSIS FACTOR (*TNF*) GENE AND ITS RECEPTOR 1 (*TNFR1*) GENE FOR DNA ALTERATIONS AND THE SUBSEQUENT INVESTIGATION OF THESE AND AN *IL12 p40* POLYMORPHISM FOR AN ASSOCIATION WITH PAEDIATRIC TUBERCULOSIS

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Thesis Presented for the Degree of Master of Medicine in the Department of Paediatrics, University of Cape Town, 15 August 2004

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LIST OF SYMBOLS AND ABBREVIATIONS

A: adenine
APC's: antigen presenting cells
APS: ammonium persulphate
bp: basepair
BAL: bronchoalveolar lavage
BCG: bacille Calmette-Guérin
C: cytosine
CAD: coronary artery disease
CD: clusters of differentiation
CMI: cell mediated immunity
dH_2O: distilled water
DNA: deoxyribonucleic acid
dNTP's: deoxyribonucleotide triphosphates
G: guanine
HIV: human immunodeficiency virus
HLA: human leukocyte antigen
IL: interleukin
IFN: interferon
kB: kilobase
kD: kilodalton
KO: knockout
L: leucine
LPS: lipopolysaccharide
LT: lymphotoxin
MHC: major histocompatibility complex
NaOH: sodium hydroxide
NK: natural killer
P: proline
PAGE: polyacrylamide gel electrophoresis
PBMC: peripheral blood mononuclear cells
PDA: piperazine diacrylamide
RA: rheumatoid arthritis
SNP: single nucleotide polymorphism
SSCP: single stranded conformational polymorphism
T: thymine
TB: tuberculosis
TAE: Tris-acetate EDTA
TBE: Tris-borate EDTA
T1D: type 1 diabetes
Th1: helper T cells
TNF: tumor necrosis factor
TRAPS: TNF receptor-associated periodic syndromes
UTR: untranslated region

PREFACE

Infection with *Mycobacterium tuberculosis* is characterised by diverse outcomes; the majority of infected individuals remain well and yet others develop disease ranging from limited pulmonary tuberculosis to severe disseminated disease. The reasons for this diverse outcome are poorly understood, but host factors are thought to play an important role. In particular, a genetic component to susceptibility to tuberculosis has been proposed.

An important clue was the description of a group of Maltese children with an unusual susceptibility to progressive non-tuberculous mycobacterial infections. These patients showed defective tumor necrosis factor (TNF) production in response to endotoxin and a failure to upregulate TNF production in response to interferon gamma as well as diminished interferon gamma production during T-cell proliferation. They were found to lack expression of the interferon gamma receptor ligand-binding chain (IFN-γR1) on their cell surfaces due to a single point substitution resulting in a truncated protein. Since then other defects in the type 1 cytokine pathway leading to susceptibility to non-tuberculous mycobacteria, as well as to tuberculosis, have been described in rare isolated cases. From these findings, the hypothesis arose that less severe mutations in such pathways might individually, or in combination, lead to increased susceptibility to tuberculosis in the general population.

The following study forms part of a larger multi-centre collaboration, which aims to better understand the genetic basis of susceptibility to mycobacterial infection by addressing this hypothesis. The approach taken has been the recruitment and immuno-phenotyping of a large group of children with tuberculosis as well as control subjects. Candidate genes, of the type 1 cytokine pathways being investigated, include interferon gamma, interleukin-12 and their receptors and TNF. The focus of the study described in this thesis has been the screening of a sub-cohort of patients and control subjects

for DNA sequence alterations in the *TNF* and *TNFR1* genes. The individuals in this cohort were selected on the basis of their whole blood stimulation assays, where either high or low levels of TNF in response to non-specific stimulatory factors, were the determining criteria. It was assumed that these two phenotypic groupings would be enriched for gene variants contributing to the TNF responses recorded in the stimulation assays. Once identified, these polymorphisms would be screened for frequencies in the broader patient and control groupings and assessed for any association with susceptibility to tuberculosis. This study was considered important in attempting to explain which genes and their polymorphisms are involved in determining the high prevalence of tuberculosis in African populations.

ABSTRACT

Screening of the human tumor necrosis factor (*TNF*) gene and its receptor 1 (*TNFR1*) gene for DNA alterations and the subsequent investigation of these and an *IL12 p40* polymorphism for an association with paediatric tuberculosis

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Tuberculosis is a chronic infection caused by *Mycobacterium tuberculosis*. The cytokine, tumor necrosis factor (TNF), has been shown to be important in controlling tuberculosis infection in both the mouse model and in human disease. Variation in TNF production between individuals is thought to be genetically determined. Genetic deficiencies in this and related cytokine pathways have been shown to predispose to mycobacterial infection.

The study reported in this thesis has analysed the *TNF* gene and its type 1 receptor gene (*TNFR1*) in a selected cohort of 71 subjects, who produce high or low levels of tumor necrosis factor by *in vitro* assay. This was done in an attempt to determine a genetic basis for this difference. Children with a history of tuberculosis as well as healthy control children were included. The polymorphism screening method employed in this study was single stranded conformational polymorphism analysis. Once a polymorphism was detected, cycle sequencing was carried out. Ten polymorphisms involving nine single base substitutions and one single base insertion, including four novel changes, were identified in the *TNF* gene. One polymorphism occurred within a Sp1 binding site and another within an Ap2 binding site on the promoter. Ten polymorphisms, of which four were novel, were identified in the *TNFR1* gene. Of these ten, one polymorphism was a two-basepair deletion while the rest were single base substitutions. One polymorphism in the coding region resulted in the amino acid substitution of leucine for proline.

One previously identified polymorphism in the *interleukin-12 p40* gene, +1188 A→C, as well as the *TNF* –307 G→A polymorphism, were investigated for an association with paediatric tuberculosis. Genotyping of the *TNF* –307 G→A polymorphism, carried out using the Amplification Refractory Mutation System, was expanded to a cohort of 276 subjects, including 148 patients and 128 controls. Genotyping of the *interleukin-12 p40* +1188 A→C polymorphism was carried out by *TaqI* restriction enzyme analysis and expanded to a cohort of 281 subjects, including 156 patients and 125 controls.

Allele frequencies were compared between the control and patient cohorts by means of a Chi-squared test. No significant differences in the distribution of the *TNF* –307 or *interleukin-12 p40* +1188 polymorphisms were found between the patient and control subjects (P=0.8 and P=0.3, respectively). Neither of these polymorphisms is associated with paediatric tuberculosis in an African population. Time constraints prevented screening of the entire patient and control cohorts for associations of the novel polymorphisms identified by this study with paediatric tuberculosis. This, as well as the possible functional significance of these novel DNA alterations warrants further investigation.

August 2004

Chapter 1:
INTRODUCTION

1.1 TUBERCULOSIS

Tuberculosis (TB) is a chronic infection caused by *Mycobacterium tuberculosis*. Annually, about two million people worldwide die from TB infection while an estimated eight million new cases occur (Algood et al. 2003; Flynn 2004). TB is usually transmitted by inhalation of aerosolised bacilli produced during coughing by an infected individual, after which it may disseminate from the lungs throughout the body. Only about 10 percent of individuals who are infected develop clinical disease, with the remainder developing either sterilizing immunity or latent infection. Latent infection can progress to active TB if immunosuppression occurs (Mohan et al. 2001; Nunez Martinez 2001; Fenhalls et al. 2002; Algood et al. 2003). Immunosuppression could occur due to age, corticosteroids, malnutrition and human immunodeficiency virus (HIV) infection as well as other factors (Flynn 2004). Fortunately, people infected with latent TB are not contagious.

1.1.1 Paediatric tuberculosis

The World Health Organisation (WHO) has estimated that there are 1.2 million cases of TB in children every year, with about 450 000 dying annually from the disease. As many as 40% of all reported TB cases are paediatric in developing countries, with as many as 10% of all hospital admissions of children due to TB and 10% of paediatric deaths due to TB. It is therefore important and necessary to study and control paediatric TB. Childhood TB is a public health measure of recent transmission of *M. tuberculosis* from an infected adult. Many adults who develop reactivation and contagious TB probably acquired the infection during childhood (Starke 2003).

Paediatric TB varies from adult TB in many characteristics. Children are more prone to disseminated TB and meningeal disease than adults or adolescents

and the disease can be crippling in children (Smith et al. 1997; Starke 2003). Childhood TB results mostly from primary infection rather than from the reactivation of latent infection. It is a significant cause of mortality as children are more likely than adults to develop active TB after infection with *M. tuberculosis*. About 15-43% of children compared to 5% of adults develop TB after primary infection (Aubert-Pivert et al. 2000). TB infection in children is usually acquired from an infectious adult or adolescent living in the same household. About 5-10% of children infected with tuberculosis will go on to develop 'progressive primary TB', where the primary infection site enlarges and undergoes caseous necrosis, resulting in a cavity (Shewchuck and Reed 2002).

1.1.2 Overview of host defences against tuberculosis

Mycobacteria are inhaled in droplet nuclei past the protective mechanical barriers of the upper respiratory tract and reach the alveoli where the initial infection takes place (Smith et al. 1997). The alveolar macrophage is the primary host cell for *M. tuberculosis* and is the first line of defence against infection in the lung (Keane et al. 1997; Balcewicz-Sablinska et al. 1998). The mycobacteria are phagocytosed by these macrophages following which they persist and replicate within the macrophage, or are killed by these cells; the latter occurring through mechanisms that include reactive oxygen and nitrogen intermediates within lysosomal compartments, hydrolytic enzymes within the phagolysosome and activation of apoptotic pathways. If the bacteria manage to evade these mechanisms and continue to replicate, dissemination throughout the body via the lymphatics and the bloodstream could occur, thereby creating new sites of infection in other tissues. Macrophages also elaborate cytokines such as TNF, interleukin-12 (IL12) and chemokines in response to mycobacteria and these can act together to orchestrate the specific and non-specific host defences against tuberculosis. TNF is considered to be important in this initial host response; however it paradoxically appears to contribute to some of the pathological features seen in advanced tuberculosis infection (Smith et al. 1997).

The host defence against intracellular pathogens such as mycobacteria is dependent on effective cell-mediated immunity (CMI), which results in the local containment of pathogens inside well-organised granulomatous lesions in the lungs (Ottenhoff et al. 2002). The development of this antigen-specific host response is dependent on a co-ordinated interplay between T-cells, macrophages and other antigen presenting cells (APC's) such as dendritic cells. The T-cell-mediated immune response to microorganisms starts with the intracellular processing of protein and subsequent antigen presentation to T-cells by dendritic cells and macrophages in the context of major histocompatibility (MHC) molecules. APC's, especially the dendritic cells, migrate from the lung to the regional lymph node where they elaborate cytokines and present mycobacterial antigens to CD4+ T-cells (Smith et al. 1997).

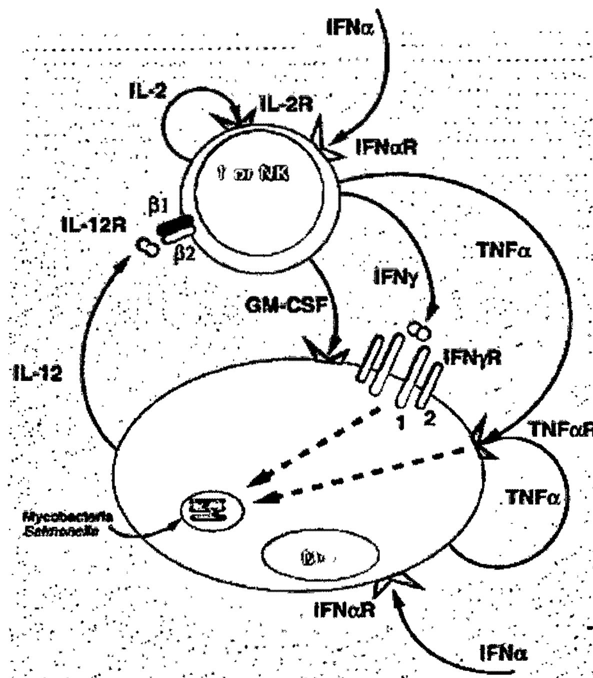

Figure 1.1. The cytokine interactions between the T or NK lymphocyte and the infected macrophage. Macrophages produce IL12, which leads to the T-cell production of TNF and IFNγ. IFNγ stimulates the macrophages to produce TNF and both TNF and IFNγ drive the production of IL12 (Holland 2000).

The T-cells then elaborate key effector cytokines such as interferon-gamma (IFNγ) and TNF (Smith et al. 1997) (fig. 1.1). This provides signals to attract further T-lymphocytes and monocytes to the site of infection where a granuloma is formed. The activation of infected macrophages by type 1 cytokines, such as IFNγ, is the principle effector mechanism of CMI, leading to killing of intracellular mycobacteria. Antigen-specific type 1 helper T-cells (Th1 cells) and natural killer cells (NK cells) produce the IFNγ protein which then binds to IFNγ-receptor R1/R2 complexes which are 90 kD and 62 kD respectively. These R1/R2 complexes are found at the macrophage surface (Ottenhoff et al. 2002). Their activation by IFNγ results in increased production of interleukin-1 (IL1) and TNF, enhanced antigen presentation and increased production of nitric oxide and reactive-oxygen intermediates (Newport et al. 1996).

Another cytokine, IL12, regulates the production of IFNγ by Th1 and NK cells. IL12 is a heterodimer consisting of 40 kD and 35 kD subunits (p40 and p35). It is produced by antigen-presenting cells, which include macrophages, monocytes and dendritic cells, after the activation of Toll-like receptors on these cells by bacterial ligands. IL12 is therefore a link between adaptive and innate immunity. IL12 binds to IL12 receptor β1 (100 kD) and β2 (130 kD) complexes at the surface of Th1 and NK cells (Ottenhoff et al. 2002).

Signalling through TNF receptor 1 (TNFR1) is thought to play an essential role in regulating granuloma formation (Roach et al. 2002). TNF is expressed at the site of human TB and is considered to be crucial in determining the course of the disease (Engele et al. 2002). Granulomas contain the bacilli in a low nutrient and oxygen environment, which prevents their spread (Algood et al. 2003). The process of granuloma formation is dependent on the activation of mycobacteria-reactive T-lymphocytes, especially $CD4^+$ and $CD8^+$ T-cells that secrete IFNγ (Roach et al. 2002). The granuloma increases in size as more and more mononuclear leukocytes, are recruited with various zones becoming apparent within the granuloma. As the infected macrophages die and the activated T-cells undergo apoptosis, the central region becomes necrotic,

otherwise known as caseous necrosis. Myeloid cells, multinucleated giant cells and lymphocytes surround this area of caseous necrosis. The lesion can go on to either calcify or liquefy. *M. tuberculosis* replicates exponentially within the liquefied necrotic lesions and if the lesion ruptures into an airway, the replicating bacteria can reseed other areas of the lung or the body or they can be expelled into the environment and infect another host (Fenhalls et al. 2002).

The protective immune response in humans against *M. tuberculosis* remains inadequately defined. IFNγ, together with TNF, activates anti-mycobacterial microbicidal mechanisms in murine macrophages (Bean et al. 1999; Mohan et al. 2001; Ottenhoff et al. 2002; Smith et al. 2002; Wagner et al. 2002). There are conflicting reports of a similar role for IFNγ in *Mycobacterium tuberculosis* infected human macrophages, although it can affect the growth of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) in human macrophages (Ottenhoff et al. 2002). It is not well understood how the bacilli can persist in the face of a strong immune response (Algood et al. 2003).

The high mortality rates in childhood TB may be due to the relative immaturity of early non-specific immune responses in children. The recruitment of activated cells to primary and post-primary sites of infection may be delayed in children due to defective chemotaxis of monocytes and macrophages. The development of an antigen-specific response may be delayed by inefficient presentation to naïve T-cells. This results in low levels of production of IFNγ and TNF, which may in turn limit the activation of macrophages and result in higher bacterial loads at the site of infection. Therefore, childhood TB is likely to involve defects in the immune defence against tuberculosis (Aubert-Pivert et al. 2000).

Cytokine profiles in bronchoalveolar lavage (BAL) samples from children infected with TB reveal that TNF mRNA's are more abundant in children with primary TB than in children with other pulmonary diseases. High levels of IL10 mRNA have been recorded in miliary TB. TNF is subject to down regulation

by IL10. This likely explains the poor granuloma formation and haematogenous dissemination occurring in miliary TB. The recurrent dissemination of *M. tuberculosis* in this form of TB is probably as a result of difficulty in containing the primary infection in granulomas (Aubert-Pivert et al. 2000).

1.1.3 Evidence of a genetic basis for susceptibility to tuberculosis

Evidence that genetic factors play a role in susceptibility to TB comes from twin studies, familial clustering of TB infection, racial differences in incidence, and animal models (Newport et al. 1996; Stein et al. 2003). Environmental conditions such as poverty, malnutrition, overcrowding and stress do not fully explain an increased susceptibility of some populations over others (Stein et al. 2003). Twin studies have provided convincing evidence that inherited susceptibility is a risk factor for tuberculosis with the degree of concordance for tuberculosis being higher for monozygotic than dizygotic twins (Comstock 1978; Cooke and Hill 2001; Jepson et al. 2001). A study carried out by Stead et al. (1990) revealed that both the incidence and prevalence of TB among residents in nursing homes and inmates in prisons in Arkansas were twice as high in blacks as in whites. This difference is usually attributed to social factors such as overcrowding and poverty; however this study looked at blacks and whites living in the same social conditions, i.e. in nursing homes and prisons and strongly suggests the existence of heritable differences between individuals and groups with respect to the immune defence system against TB.

A genome-wide search for tuberculosis-susceptibility genes in Africans, using linkage analysis and common ancestry using microsatellite (CAM) mapping, revealed evidence for these on chromosomes 15q and Xq. No single marker was strongly linked to disease suggesting that there is no single TB-susceptibility gene Africans (Bellamy et al. 2000). The Angelman syndrome gene, *UBE3A*, is found the region on chromosome 15 of suggestive linkage identified above. This gene encodes a ubiquitin ligase that has been found to be involved in the degradation of the T-lymphocyte Src kinase enzyme, Lck,

which in turn plays a role in transducing T-cell receptor signalling in T-cells. Therefore, alterations in *UBE3A* could affect the T-cell response to *M. tuberculosis* and thus further analysis to evaluate this gene as a major susceptibility gene for TB is warranted (Cervino et al. 2002).

Genetic deficiencies in the IFNγ receptor and IL12 and its receptor have pointed to critical roles for these cytokines in the protective immune response against infection by non-tuberculous, poorly pathogenic mycobacteria. (Newport et al. 1996; Engele et al. 2002; Ottenhoff et al. 2002). A number of different genes that play a role in the type 1 cytokine cascade have been investigated for coding sequence alterations that may increase susceptibility or resistance to TB infection. These include the genes for *IL12p40*, *IL12Rβ1*, *IFNγR1*, *IFNγR2* and *STAT1* (IFNγ-associated signal transducer and activator of transcription). The degree of impairment in function was found to correlate overall with the severity of the clinical, immunological and histopathological phenotypes analysed. Individuals with complete IFNγR deficiency have suffered from a severe and fatal outcome after infection with mycobacteria. Partial deficiency in IFNγR, partial deficiency in STAT1 or complete or partial deficiency in IL12Rβ1 have resulted in individuals developing milder less severe infections with possibly milder histopathological phenotypes. Deficiency in IL12Rβ1 is associated with a milder phenotype, possibly due to low levels of production of IFNγ, independent of IL12R. The contribution of particular genes could vary depending on a population's background, including previous infection pressure (Ottenhoff et al. 2002). For example, homozygosity for the IFNγ +874 T allele has been found to be associated with susceptibility to TB in Sicilians and in South Africans, but not in Malawians. Similarly, variants in mannose-binding lectin (MBL2) may be protective against meningeal TB in Cape Coloureds but this is not the case in The Gambia or Malawi. Therefore, genetic susceptibility to TB in Africans is polygenic (Fitness et al. 2004).

Definition of the disease phenotype has been a limiting factor in previous genetic studies as TB is a heterogeneous disease, which presents mostly as

pulmonary disease, but can affect nearly all organs. In addition, active disease may present at any time after infection, i.e. a person's current TB status does not reflect a long-term disease risk (Stein et al. 2003).

Since only a relatively small percentage of those infected with *M. tuberculosis* develop clinical disease, infection is necessary, but not enough, to cause clinical TB disease on its own. Although a number of studies such as those described above support genetic factors in human susceptibility to TB, no model for genetic susceptibility is accepted and this may in part be due to the heterogeneity of disease expression. Therefore, intermediate immune phenotypes of TB could be analysed as they may be more directly connected to gene expression. Intermediate traits for TB, including IFNγ, TNF and transforming growth factor β (TGFβ) secretion in stimulated peripheral blood mononuclear cells (PBMC's) were analysed in 177 index-case subjects and their household contacts in a Ugandan community. TNF was found to have the highest heritability (68%), which suggests a strong genetic component in susceptibility to TB. Therefore further study of TNF with respect to TB susceptibility is warranted (Stein et al. 2003). This provides a good reason for TNF as our choice as a candidate gene.

Susceptibility to mycobacterial infection is likely to be a complex, multifactorial and multigenic trait to which a number of genetic pathways contribute. The contribution of a particular genetic pathway to disease susceptibility could vary according to the stage of infection. For example, the genetic pathways that control susceptibility to primary infection with *M. tuberculosis* may not correlate entirely with those that control susceptibility to re-activation of latent TB. The classification of susceptibility-associated alleles in host genes will be beneficial in understanding the mechanisms of protection against TB infection as well as providing new targets for preventive or post-infection intervention (Ottenhoff et al. 2002).

1.2 TNF

TNF (previously known as TNFα or cachetin) is a pleiotropic inflammatory cytokine produced by a number of different cell types (Balcewicz-Sablinska et al. 1998; Knight and Kwiatkowski 1999; Keane et al. 2001; Roach et al. 2002). It is produced mainly by macrophages in response to stimuli that activate toll-like receptors and can also be expressed by activated T-cells, B-cells and NK cells (Ehlers 2003). Keratinocytes, astrocytes, macroglial cells, smooth muscle cells, intestinal paneth cells, mesangial cells and tumor cells also produce TNF (Makhatadze 1998). TNF, belonging to the TNF ligand family (Makhatadze 1998), is one of the first immunological mediators to appear when infection is encountered. TNF production does not usually necessitate previous immunological exposure; the macrophages of a non-immune individual that encounter a pathogen, release copious amounts of TNF within several hours (Knight and Kwiatkowski 1999).

Human TNF has membrane-bound (26 kD) and secreted forms, which are both biologically active. The membrane-bound form is cleaved by a metalloprotease called disintegrin (TNF converting enzyme), to a secreted monomer (17 kD) of which three associate to form the biologically active extracellular form of TNF (Ruuls and Sedgwick 1999; Pfeffer 2003). TNF can induce a broad spectrum of biologic effects, including fever, tissue injury and alveolar macrophage activation (Balcewicz-Sablinska et al. 1998; Keane et al. 2001; Roach et al. 2002). TNF also plays a role in lipid metabolism, coagulation, insulin resistance (OMIM 191160) and apoptosis and in the endothelial trapping of circulating immune cells by upregulating adhesion molecules such as adhesion molecule 1 (ICAM-1) (Bean et al. 1999; Knight and Kwiatkowski 1999). It has therefore commonly been thought to mediate the deleterious effects of TB, which include fever, weight loss and tissue necrosis. TNF has been implicated in the pathogenesis of a number of human diseases including cancer, rheumatoid arthritis, diabetes, osteoporosis, multiple sclerosis, sepsis and inflammatory bowel diseases (Chen and Goeddel 2002).

stages of infection in order to control replication of bacilli within the major organs. The subcutaneous injection of TNF alone prevented the occurrence of necrosis in these major organs, but did not cure the inflammatory lesions present in these organs. This study therefore provides evidence that a lack of TNF leads to serious infection or death in mice infected with TB (Kaneko et al. 1999).

A more severe picture of non-containment and fatal disease occurs in the TNFR1 deficient mouse infected with BCG, therefore TNF signalling through its type 1 receptor is indispensable in the control of BCG infection. TNF-deficient mice had significantly delayed BCG clearance in the liver and spleen and its growth was uncontrolled in the lungs. There was a higher mortality rate of TNFR1-deficient mice compared to TNF deficient mice, which suggests that lymphotoxin alpha (LTα) signalling through TNFR1 could play a role in mycobacterial clearance. In addition, the TNF-deficient mice had reduced adhesion molecules such as ICAM-1 and CD11b and absent CD11c in granulomas studied in the liver when compared to wildtype mice, indicating that TNF is important for the expression of adhesion molecules. The formation of mycobactericidal granulomata has been shown to be ICAM-1 dependent. TNF deficient mice, reconstituted with wildtype bone marrow, were able to control mycobacterial growth to a similar extent as wildtype controls therefore TNF derived from hemopoietic cells rather than from mesenchymal origin is sufficient for a normal host response to infection with BCG (Jacobs et al. 2000 (a)).

Animal studies have provided evidence that the local pulmonary host response is a crucial part of the immune response to *M. tuberculosis*, even though the extrapulmonary immune response is important for eventual control of infection. To directly study the role of TNF in local host responses in the lung, Smith et al. (2002) generated and characterised transgenic mice with local pulmonary inhibition of TNF. They concluded that TNF inhibition in the lungs impairs the host response to *M. tuberculosis* infection and that the presence of TNF outside of the lungs cannot compensate for an

intrapulmonary defect in its activity. TNF facilitates bacterial clearance and appears to be an important modulator of lung inflammation. Inhibition of TNF activity in the lungs during the early stage of infection results in persistent inflammation and immune activation, due to an increase in the expression of proinflammatory cytokines such as IL1, IL6, IL18 and IFNγ, with an increase in mycobacterial tissue burden leading to exaggerated pathology and lung injury (Smith et al. 2002).

1.2.1.2 Evidence from the mouse model of 'reactivation tuberculosis'

Evidence that TNF is essential in preventing disease reactivation was provided in a study carried out by Mohan et al. (2001) in which they used persistent *M. tuberculosis* infection in mice as a model for latent TB. The neutralisation of TNF, 6 to 8 months post-infection with *M. tuberculosis* (laboratory mice normally live just over 2 years), was initiated by injecting MP6-XT22 (rat anti-murine TNF hybridoma) twice weekly for the duration of the experiment. Control mice received normal rat immunoglobulin G (IgG) following the same schedule. TNF neutralisation resulted in disease reactivation associated with an increase in bacterial numbers, which peaked at 20 days after MP6-XT22 treatment and not thereafter. The mice succumbed, with a mean survival time of 59 ± 22 days, after MP6-XT22 treatment. The TNF-neutralised mice displayed defective recruitment and/or migration of inflammatory cells, as indicated by much less apparent lymphoid aggregates in the MP6-XT22-treated mice 3 weeks post TNF neutralisation. In addition, the expression of nitric oxide synthase 2 (NOS2) was attenuated in the MP6-XT22 treated mice, compared to NOS2 levels in IgG-treated mice. These data suggest that attenuated NOS2 expression contributes to some extent to reactivation of infection in the TNF-neutralised mice (Mohan et al. 2001).

Evidence that TNF is important in granuloma formation is provided in this study as the lack of an organised granulomatous response appears to be the

common link to the unfavourable disease outcome associated with chronic persistence and reactivation of TB and TNF deficiency in acute TB. The results of this study suggest that TNF is critical in trafficking of immune cells to infectious foci, thereby promoting the formation and maintenance of well-organised granulomata, which control disease and prevent reactivation (Mohan et al. 2001).

1.2.1.3 Evidence from human tuberculosis

Evidence from studies in mice supporting a role for TNF in the defence against infection has been discussed in this thesis. Since no human subject has been recorded with a genetic deficiency of TNF, we rely on evidence from studies of the effect of anti-TNF treatment in humans. There are two drugs that specifically target TNF, namely infliximab and etanercept (Ehlers 2003).

Infliximab is a chimeric monoclonal antibody, which binds with high affinity to both monomeric and trimeric TNF, and is widely used in the treatment of rheumatoid arthritis (RA) and Crohn's disease (CD) (Keane et al. 2001; Wagner et al. 2002; Ehlers 2003). Infliximab therapy reduces inflammatory infiltrate and TNF production in the intestinal segments involved in CD (Nunez Martinez et al. 2001). The biological effect of infliximab persists for up to two months (Keane et al. 2001). Infliximab neutralises the inflammatory effect of TNF by binding to its soluble subunit and with transmembranous TNF so that it may not interact with its receptor (Nunez Martinez et al. 2001; Sandborn 2003). It forms stable complexes with soluble as well as transmembrane TNF and rarely releases them once bound (Ehlers 2003). It also causes lysis of TNF-producing cells via compliment fixation and antibody-dependent cytotoxicity and leads to T-cell apoptosis (Sandborn 2003).

There are now multiple reports of cases of TB following treatment with infliximab. The first case occurred during a clinical trial (Keane et al. 2001). The same authors subsequently investigated all reports of TB after infliximab therapy through the MedWatch spontaneous reporting system of the Food and Drug Administration. They found an increased rate of TB among patients

treated with infliximab when compared with background rates. Most of the 70 cases of tuberculosis associated with infliximab therapy reported in this study were from countries with low incidence rates of TB, less than 20 cases per 100 000 population per year. The background rate of TB in patients with RA in the US is 6.2 cases per 100 000 population per year and the estimated rate of TB among patients receiving infliximab for RA in the US is 24.4 cases per 100 000 population per year (Keane et al. 2001). The data obtained in the study by Keane et al. (2001) suggested an association with the use of anti-TNF therapy, such as infliximab or etanercept, and reactivation TB.

Of the 70 reports of TB after infliximab treatment, 56 percent of patients had extrapulmonary disease and 24 percent had disseminated disease. These forms of tuberculosis are usually associated with immunosuppression, such as in HIV infection. Generally only 18 percent of cases of TB not associated with immunosuppression manifest as extrapulmonary disease while disseminated disease usually accounts for less than 2 percent (Keane et al. 2001).

In other isolated reports, Lim et al. (2002) describe the reactivation of latent TB in a patient treated with infliximab for uveitis. The patient's T-cells were able to produce IFNγ and he had no other known immunodeficiency. The clinical course of disease described, was consistent with an association between infliximab and reactivation TB (Lim et al. 2002). Nunez Martinez et al. (2001) describe a patient who developed reactivation TB after infliximab therapy.

Most of the reported cases of TB in patients treated with infliximab occurred in countries with a low incidence of TB, as infliximab is expensive and rarely available in poor countries where TB is more frequent. It is likely that most of these patients had reactivation disease given the low background incidences of TB. It is feasible that patients receiving treatment with infliximab are susceptible to disease after primary infection and reinfection with *M. tuberculosis* (Keane et al. 2001).

Etanercept is a dimeric fusion protein consisting of the extracellular ligand domain of human TNFR2 and the Fc region of IgG1. It neutralises soluble TNF and LTα (infliximab cannot bind to LTα) (Ehlers 2003). Myers et al. (2002) describe a patient diagnosed with osteoarticular TB shortly after receiving treatment with etanercept (Myers et al. 2002). Etanercept has a more limited function than infliximab and neutralises soluble TNF but does not bind to transmembrane TNF (Sandborn 2003). It traps TNF/LTα at sites where they are abundant and then releases them at sites of lower concentrations (Ehlers 2003).

TNF alone does not activate antimycobacterial activity in murine or human macrophages, therefore the reactivation of TB in patients treated with infliximab is most likely not due to a loss of antimycobacterial activity, but rather reflects the disintegration of granulomata, thereby allowing dormant mycobacteria to convert into metabolically active and destructive pathogens (Engele et al. 2002).

1.2.2 The specific role of TNF in the host defence against TB

The evidence described above clearly shows the importance of TNF in controlling TB infection in both the mouse model and in human disease. There are a number of proposed mechanisms whereby TNF exerts this activity, including macrophage activation, granuloma formation, apoptosis and the activation of neutrophils.

1.2.2.1 Macrophage activation

Macrophages are stimulated upon infection with *M. tuberculosis*, and subsequently produce TNF and other cytokines to initiate an inflammatory response. T-cells are then recruited to the site of infection where they are needed for the creation of granulomas. TNF, together with IFNγ produced by T- cells, activates infected macrophages to produce reactive nitrogen

intermediates and nitric oxide synthase, compounds with mycobactericidal activity (Bean et al. 1999; Mohan et al. 2001; Smith et al. 2002; Wagner et al. 2002). Therefore, both IFNγ and TNF are likely to be significant in controlling mycobacterial infection and granuloma formation (Jacobs et al. 2000 (b)). However, the role of TNF is not as clear as that of IFNγ in human TB infection (Smith et al. 2002; Wagner et al. 2002).

TNF has opposite effects on the growth of virulent (H37Rv and *M. bovis*, pathogenic in mice) and attenuated (H37Ra, heat-killed *M. tuberculosis*, and BCG) mycobacteria. Virulent mycobacteria seem to have developed efficient evasion mechanisms for protection against the attack of the host immune system, while attenuated strains are susceptible to the activation of the host cell by TNF. Virulent strains can activate more alveolar macrophages for TNF production than attenuated strains. Exogenous TNF increases intracellular growth of virulent mycobacterial strains in alveolar macrophages and in contrast, limits proliferation of attenuated mycobacterial strains. Possible mechanisms as to how TNF interferes with the antibacterial effector mechanisms of human alveolar macrophages include deprivation of intracellular iron, modulation of the maturity of cells, regulation of chemokine/cytokine release and induction of apoptosis of infected cells (Engele et al. 2002).

1.2.2.2 Granuloma formation

Even early granulomas are not maintained when TNF signalling is absent, resulting in their disintegration, causing the death of mice infected with mycobacteria. Lung specimens from TB patients typically show well-formed granulomata, while the lung specimen from a TB patient that had taken infliximab showed a lack of granuloma formation with lymphoid inflammation (Keane et al. 2001). TNF probably regulates the inflammatory response by maintaining the viability of activated macrophages at infection sites. In the absence of TNFR1 signalling, hyperinflammatory T-cell mediated tissue

destruction becomes apparent, therefore TNFR1 is required for modulation of the T-cell response (Ehlers 2003) (table 1.1).

The pattern of cytokine production in the human lung granuloma was studied in five patients with pulmonary TB. All the patients produced TNF mRNA, with three of the patients displaying detectable levels of IFNγ and IL4 mRNA. Two patients were found to be negative for IL4 mRNA and positive for IFNγ mRNA. These latter two patients expressed lower levels of TNF mRNA and had more necrotic granulomas than the three patients who were positive for both IFNγ and IL4. Therefore it appears that TNF positively correlates with IL4 gene expression and negatively with caseous necrosis (Fenhalls et al. 2000).

Table 1.1 The effect of blocking TNF or LT during experimental mycobacterial infections in mice (Adapted from Ehlers (2003))

Mycobacterial strain	Experimental system	Effect on antibacterial protection	Effect on granuloma development
M tuberculosis	TNF-KO	Increased CFU	Delayed formation
M tuberculosis	TNFRp55KO	Increased CFU	Delayed formation, necrosis
M bovis BCG, *M tuberculosis*	tmTNF-TG	No or slightly increased CFU	None or marginal
M tuberculosis	anti-TNF mAb in chronic phase	Increased CFU	Disorganisation, diffuse infiltration
M bovis BCG	anti-TNF mAb	Increased CFU	Malformation, breakdown
M tuberculosis, *M bovis* BCG	sTNFRp55-TG	Increased CFU	Necrosis
M avium	TNFRp55KO	No or marginally increased CFU	Disintegration, disorganised mixed infiltrate
M tuberculosis	LTα-KO	Increased CFU	Delayed formation, structural defects
M tuberculosis	LTβRKO, LTβKO	Increased CFU	Delayed formation, delayed macrophage activation
M bovis BCG	LTβR-IgGFc	Increased CFU	Marginal
M bovis BCG	TNF-LTαKO	Increased CFU	Diffuse infiltrations

LT, lymphotoxin; tmTNF, transmembrane TNF; sTNF, soluble TNF; KO, knockout; TG, transgenic; IgGFc, fusion protein; CFU colony forming units

1.2.2.3 Apoptosis

M. tuberculosis infection induces TNF-dependent macrophage apoptosis (Balcewicz-Sablinska et al. 1998). Since TB is a chronic infection, apoptosis may favour the host by reducing the potential for macrophages to provide sanctuaries for mycobacteria. Further, during apoptosis, IL1β and nitric oxide are released, both of which play an important role in controlling mycobacteria. Conversely, the killing of alveolar macrophages could benefit the mycobacteria by reducing the number of phagocytes and possibly interfering

extravasation and migration of neutrophils to the site. The neutrophils phagocytose the tubercle bacilli in the presence of TNF secreted by the macrophages. These mechanisms are operative to sterilise limited challenges by mycobacteria but can be overwhelmed, with some individuals being able to resist infection longer than others (Kisich et al. 2002).

1.2.3 TNF signalling

The binding of a ligand, usually a microbial product, to a cell surface Toll receptor initiates the process of TNF production. This Toll receptor then stimulates a signal transduction pathway that activates NFκB transcription factors, which then enter nuclei and induce the transcription of genes associated with inflammation, including *TNF*. The production of TNF and other inflammatory cytokines acts to stimulate other inflammatory cells, which then release cytokines and consequently amplify the immune response (Suryaprasad and Prindiville 2003).

Human TNF is a polypeptide hormone that consists of three non-glycosylated subunits. The hormone is made initially as a prohormone that has 76 additional amino acid residues attached to the N-terminus, creating a hydrophobic domain that anchors the molecule to the cell surface (Makhatadze 1998). TNF is then proteolytically cleaved to remove these additional residues to produce a soluble 17 kD form (Makhatadze 1998; Hajeer and Hutchinson 2001).

TNF elicits its cellular responses through its interaction with two distinct receptors, the 55 kD, type 1 tumor necrosis receptor (TNFR1, p55, CD120a) and the 75 kD, type 2 tumor necrosis receptor (TNFR2, p75, CD120b) (Balcewicz-Sablinska et al. 1998; Papadakis and Targan 2000; Keane et al. 2001). Both these receptors bind TNF and LTα (previously known as TNFβ) specifically and with high affinity (OMIM 191190). TNFR1 and TNFR2 are initially cell surface membrane proteins, which can then be cleaved to become bioactive soluble molecules (Ruuls and Sedgwick 1999). TNF receptors are

part of the TNF-nerve growth factor (NGF) receptor family and are expressed on almost all somatic cells (Makhatadze 1998). TNFR1 is expressed on many cell types, while TNFR2 is expressed mainly on leukocytes and endothelial cells (Papadakis and Targan 2000). TNFR2 has a higher affinity for TNF, especially at lower concentrations of TNF (Hajeer and Hutchinson 2001).

The TNFR1 gene encodes a protein consisting of 455 amino acids. The signal transmembrane domain consists of about 23 amino acids, while the extracellular domain is made up of 171 residues and the cytoplasmic domain of 221 residues. The first 35 amino acids are hydrophobic and represent a signal sequence (Gray et al. 1990). The extracellular ligand-binding domain is composed of four repeats of cysteine-rich 40-amino acid motifs with each interrupted by an intron (Fuchs et al. 1992). The ligand-binding domain is from residues 77 to 114. There are two extracellular, cysteine-rich subdomains (CRD1 and CRD2) each consisting of three disulphide bonds and structurally conserved regions (Banner et al. 1993).

The TNFR1 and TNFR2 receptors are both shed from the cell surface in a soluble form, which aid in regulating TNF function. These soluble receptors neutralise TNF activity by competing with membrane-bound receptors and also stabilise TNF and prevent its degradation (Hajeer and Hutchinson 2001). The extracellular domains of the two receptors are very similar in structure, while their intracellular domains seem to be unrelated (OMIM 191190), which is consistent with the observation that the two receptors carry out different functions *in vivo* and activate different signalling cascades (Bazzoni and Beutler 1996).

The intracellular region of TNFR1 has a death domain (DD), consisting of about 70 amino acids, which is required for signalling apoptosis and activating NFκB (McDermott et al. 1999; Papadakis and Targan 2000). A 60 kD silencer of death domains (SODD) maintains TNFR1 in an inactive monomeric state by inhibiting the ligand-independent aggregation of TNFR1 cytoplasmic domains (Papadakis and Targan 2000). Both TNF and LTα bind as

homotrimers to TNFR1 and TNFR2, thereby eliciting their biological functions (Bopst et al. 2001). TNFR1 preferentially binds soluble TNF, while TNFR2 predominantly binds the membrane-associated form (Papadakis and Targan 2000).

TNF homotrimers bind to TNFR1's extracellular domain. Signalling is induced by the aggregation of the membrane receptors after the SODD dissociates from the cytoplasmic DD of the receptor. The adaptor protein TRADD (TNFR-associated death domain) is then recruited by the aggregated DD signalling complex (Papadakis and Targan 2000; Chen and Goeddel 2002). The TNFR1 signalling complex is formed when TRADD recruits FADD (Fas-associated death domain), TRAF (TNFR-associated factor 2) and RIP (receptor interacting protein), which then activates signalling cascades that lead to apoptosis, NFκB activation as well as JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) (fig. 1.2) (Papadakis and Targan 2000).

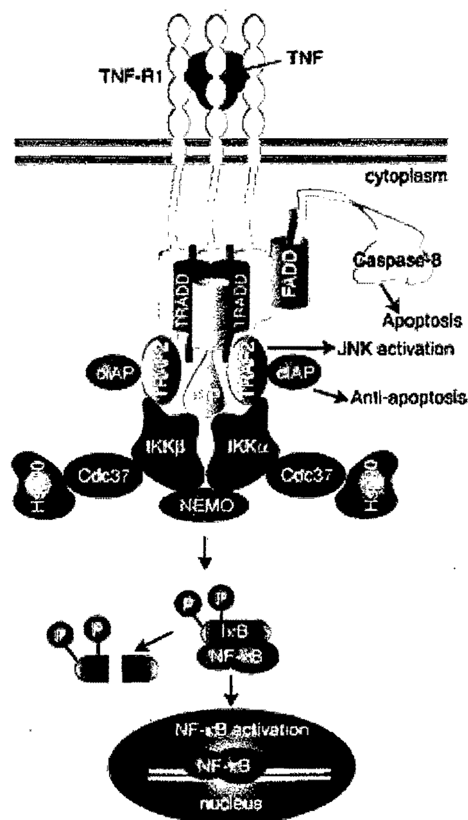

Figure 1.2. The TNF signal transduction pathway. TNF binds to TNFR1 with the resultant release of SODD and the formation of a receptor complex containing the

adaptor proteins TRAF2, FADD and RIP. These then recruit other proteins, which become activated and initiate downstream events including apoptosis, NFκB and JNK activation (Chen and Goeddel 2002).

TNF signalling through TNFR2 involves recruitment of TRAF2, TRAF1 and c-IAP's (cellular inhibitor of apoptosis proteins) to the intracellular domain of the receptor. This complex then mediates NFκB and JNK activation through TRAF2 (Papadakis and Targan 2000). KO mice for the TNFR1 gene are more susceptible to challenge with intracellular bacterial pathogens than TNFR2 knockouts or wildtype mice, which suggests that the proinflammatory effects of TNF are mediated predominantly through the p55 receptor (McDermott et al. 1999).

1.3 MUTATIONS AND POLYMORPHISMS IN THE *TNF* AND *TNFR1* GENES

1.3.1 *TNF*

The *TNF* gene encodes the TNF cytokine and is located within the highly polymorphic major histocompatibility complex (MHC) on the short arm of chromosome 6, (6p21.3). It is about 3 kilobase pairs in length, comprises 4 exons and 3 introns and codes for a precursor product of 230 amino acids and a mature product of 157 amino acids (Nedwin et al. 1985).

The synthesis of TNF is tightly controlled at many different levels due to its potentially harmful effects. This control is partially achieved by a number of negative feedback systems (Jongeneel 1995). Circulating TNF levels are regulated at a number of stages, including gene transcription, post-transcription control of mRNA stability, cleavage of the membrane form to produce the soluble form and receptor expression (Hajeer and Hutchinson 2001). Bacterial endotoxin and lipopolysaccharide (LPS) are strong inducers of *TNF* transcription. The *TNF* promoter is a 1.2 kilobase region that has a number of binding sites for transcription factors and other regulatory proteins.

These include NFκB binding sites (GGGGACTTTCC), a GC box or Sp1 binding site, a Y-box, a cAMP-responsive element-binding (CREB) protein binding site, AP-1, AP-2, Erg-1, H-APF-1, nuclear factor interleukin 6 (NF-IL6), Oct-2, nuclear factor of activated T cells (NF-AT) and Ets binding sites. There are four possible binding sites for NFκB. *TNF* expression is regulated by MHC class II ligands during transcription and translation (Makhatadze 1998; Takashiba et al. 1993; Ye et al. 2003). Evidence has shown that by blocking the action of NFκB alone, gene expression of *TNF* would be sufficiently inhibited (Ye et al. 2003). The activation of *TNF* by different transcription factors is dependent on the cell type involved as well as the nature of stimulation (Ye et al. 2003).

Studies in mice and subsequently in humans have confirmed the importance of promoter heterogeneity in the pathogenesis of disease (Suryaprasad and Prindiville 2003). There are a number of genetic variants in the human *TNF* promoter, including SNP's located at positions –1031, –863, –857, –851, –419, –376, –308, –238, –163, –49 and +70, +488 and +691 (intron 1) relative to the transcription start site as well as an insertion at position +70 and a deletion at position +691 (Hajeer and Hutchinson 2001; Bridges et al. 2002). The 5' region of *TNF* is therefore highly polymorphic, with the 3' region being less so. Local TNF levels may be under the control of specific polymorphisms, while circulating TNF levels may be under the control of a multifactorial regulatory process (Hajeer and Hutchinson 2001).

Do any of these promoter polymorphisms influence gene expression? Several groups have addressed this question. For example, electrophoretic mobility shift assay (EMSA) as well as reporter gene analysis of the –863 C→A polymorphism revealed that NFκB (both p65-p50 and p50-p50 dimers) bind to this site, with the C→A substitution inhibiting p50-p50 binding. This may decrease the enhancer effect of NFκB in TNF activation (Udalova et al. 2000). The *TNF* –376 G→A polymorphism is situated in a region of multiple DNA-protein interactions. The –376 A allele recruits OCT-1 to this region (Knight et al. 1999).

TNF secretion is thought to be under the control of variable genetic elements within the MHC (Nadel et al. 1996). One such element has been described in the promoter region of *TNF*. This –308 G→A SNP has been the most studied one, however different studies have produced conflicting results as to whether the –308 A allele does influence *TNF* transcription or not (Hajeer and Hutchinson 2001). The –308 A allele has been shown to be a much stronger transcriptional activator than the more common –308 G allele using reporter gene assays (Wilson et al. 1997).

This bi-allelic, –308, polymorphism within the *TNF* promoter region has been associated with malaria severity in Gambian children, where those homozygous for the –308 A allele have shown almost a fourfold increase in risk of contracting cerebral malaria and a sevenfold increase in the likelihood of death or the development of serious neurological consequences. Possession of the –308 A allele may be associated with higher constitutive and inducible levels of transcription than that for the –308 G allele, thereby determining susceptibility to cerebral malaria (McGuire et al. 1994). The –308 A allele also appears to be associated with an increased severity of meningococcal disease in children, who have a genetic predisposition to secrete higher levels of TNF following invasion by meningococci. However it does not predispose individuals to acquiring invasive meningococcal disease (Nadel et al. 1996). Although much research has been done on the TNF gene, no definitive disease-related TNF polymorphism has been characterised (Ruuls and Sedgwick 1999).

1.3.2 *TNFR1*

The tumor necrosis factor receptor is part of a superfamily of receptors. The *TNFR1* gene encodes the 55 kD TNFR1 protein and is expressed in all nucleated cells. This gene is located on chromosome 12p13 and contains 10 exons (Fuchs et al. 1992; Bridges et al. 2002). The promoter region of this gene is poorly characterised, however there is an 809 base pair 5' region that

appears to be active in gene transcription. Promoter activity seems to be limited to a CT-rich region located from –207 to –385, which has high homology to the murine p55 TNFR1 promoter. The promoter region resembles that of a housekeeping gene as transcription is driven from multiple start points (*tsp*) and there are no TATA or CAAT box motifs. There are two closely located TCC repeat motifs upstream from the furthest upstream *tsp*, which are also present in the EGF (epidermal growth factor) receptor promoter. The sequence upstream from –385 has an inhibitory effect on promoter activity. The putative negative regulatory element is located between –663 and –809 (Kemper and Wallach 1993).

A number of genetic variants have been identified in this gene and in its promoter region, however their effects on transcription or protein function are largely unknown (Bridges et al. 2002). Three SNP's lie in the 5' promoter region, -609 G→T, -580 A→G and –383 A→C. There is an A→C SNP at position –383 relative to the translation start site. The –383 A→C SNP can be detected by *BglII* restriction enzyme digestion where the C allele abolishes the cutting site. A significantly increased frequency of the –383 C allele was seen in young-onset (25.6% heterozygotes) (P= 0.0056) and adult-onset diabetes (24% heterozygotes) (P=0.015) patients when compared with controls (11% heterozygotes). The C allele was also found to be associated with higher expression of *TNFR1* than the A allele *in vitro* (Nishimura et al. 2003).

The –609 T allele creates a *Bst4CI* restriction site (Allen et al. 2001). The allele frequencies of the three promoter polymorphisms were very different between healthy African Americans and healthy Caucasians; 0.13 versus 0.42 for –609 T, 0.49 versus 0 for –580 G and 0.14 versus 0 for –383 C (Bridges et al. 2002). No association of the –609 polymorphism with disease course or severity was found in multiple sclerosis (MS) (Weinshenker et al. 1999) or in coronary artery disease (CAD) (Allen et al. 2001).

A polymorphism in exon 1, P12P, has been identified by Loetscher et al. (1990). A novel missense substitution, resulting in an amino acid change of proline to leucine at codon 75 has been identified in a study of eighty-four African Americans and seventy-four Caucasians (Martin et al. 2003). A 100% of Caucasians were found to be wildtype (CCG, proline), while 85% of African Americans were wildtype, 15% were heterozygotes (C/T) and 1% were homozygous (CTG, leucine) for the substituted base (T) (Martin et al. 2003). Several missense mutations in the coding region of *TNFR1* have been identified in autoinflammatory syndromes (Bridges et al. 2002) (Table 1.2).

***TNFR1* gene mutations**

Mutations in the extracellular domain, that lead to decreased shedding and increased cell surface expression of TNFR1, have been found to be linked to dominantly inherited inflammatory syndromes (Hajeer and Hutchinson 2001). Six different missense mutations were identified in TNFR1 in seven families of varied ethnic backgrounds that suffered from periodic fever syndromes. The name TRAPS (TNF receptor-associated periodic syndromes) best describes this class of autoinflammatory disease. The mutations were present in the heterozygous state. Five of these mutations disrupted conserved extracellular disulphide bonds (McDermott et al. 1999).

Table 1.2 Reported mutations of the *TNFR1* gene

DNA alteration	Published
H22Y, exon 2	Aksentijevich et al. (2001)
C30R, exon 2	McDermott et al. (1999)
C30S, exon 2	Dodé et al. (2000)
C33Y, exon 2	McDermott et al. (1999)
C33G, exon 2	Aksentijevich et al. (2001)
P46L, exon 3	Aksentijevich et al. (2001)
T50M, exon 3	McDermott et al. (1999)
C52F, exon 3	McDermott et al. (1999)

C88R, exon 4	McDermott et al. (1999)
C88Y, exon 4	McDermott et al. (1999)
Splice site mutation, G→A, intron 3	Aksentijevich et al. (2001)
R92Q, exon 4	Aksentijevich et al. (2001)
R92P, exon 4	Aganna et al. (2001)

The P46L and R92Q mutations were identified in patients as well as at a low frequency in controls implying that both these mutations are probably low penetrance mutations rather than benign polymorphisms. The observation of multiple mutations at specific residues suggests a critical role for these sites. (Aksentijevich et al. 2001).

The R92P mutation was found to segregate with TRAPS in a two-generation, 6-member, Dutch family. This mutation was also found in two unaffected members of this family, which suggests that it has reduced penetrance (Aganna et al. 2001). A polymorphism in intron 6 was found to have no association with disease when studied in CAD by restriction digestion with *BstEII* (Allen et al. 2001). All of the 16 TRAPS mutations identified so far are clustered within the first two cysteine-rich extracellular subdomains (CRD1 and CRD2) (Aksentijevich et al. 2001).

1.4 IL12

One of the *interleukin-12 p40* gene polymorphisms, 3'UTR A→C, has also been examined in this thesis. The reason being that it was recently shown to be in association with type 1 diabetes (T1D). Interleukin-12 (IL12p70) (5q31-33) is a proinflammatory cytokine composed of two disulphide-bonded polypeptide chains, 35 kD and 40 kD, which are encoded by the *IL12A* and *IL12B* genes respectively. IL12p40 may form homodimers and is expressed mainly in dendritic cells, macrophages and monocytes while IL12p35 is expressed constitutively in many cell types. IL12 plays an important role in the defence against intracellular microorganisms by providing the stimulus for

CD4+ T-cells and NK cells to differentiate towards Th1 cells, which are associated with cell-mediated immunity and secrete IFNγ. IL12 is therefore likely to be important in the protective immunity against *Mycobacterium tuberculosis* infection (Huang et al. 2000; Ma et al. 2003).

Due to the significant role that the p40 subunit has in the immune system, it is likely that genetic variants in the gene that encodes this cytokine may be key to causing immunoregulatory abnormalities. IL12 deficient individuals would probably occur rarely, however less severe DNA alterations in the subunit genes could interact with other DNA variants to predispose to resistance or susceptibility to infectious diseases or to autoimmunity (Huang et al. 2000). Mice KO experiments revealed that IL12 p35 -/- and p40 -/- mice are susceptible to *M. tuberculosis* infection. Inherited impairment of immunity to mycobacterial infection has been reported in individuals with rare founder mutations in the *IL12B* gene (Ma et al. 2003).

1.4.1 A 3'UTR polymorphism of the *IL12 p40* subunit

Huang et al. (2000) identified an A→C base change at position +1188, in the 3'UTR of *IL12p40*, which results in the creation of a *Taq1* site at this position. This polymorphism has been shown to be associated with susceptibility to multiple sclerosis (MS) and T1D. The 3'UTR C allele is preferentially transmitted to T1D patients and may therefore confer susceptibility or be in linkage disequilibrium with the disease-predisposing variant. The A allele may thus confer resistance to T1D. Higher IL12 levels were found in the relatives of T1D probands. This increased IL12 may promote Th1 cells and exacerbate autoimmune destruction of β-cells, resulting in T1D as seen in NOD (non-obese diabetic; spontaneously develop T1D) mice (Morahan et al. 2001) on which studies have shown that administration of an antagonist to IL12 before the onset of insulitis results in the reduction of spontaneous and accelerated T1D (Adorini 2001).

In a study by Ma et al. (2003), a total of 223 white individuals (106 with TB infection and 117 without TB infection) and 363 African Americans (186 with TB infection and 167 without TB infection) were genotyped at the IL12 p40 3' UTR. The C allele occurred at a lower frequency in white subjects (0.18) than in African American subjects (0.34) ($P<0.001$). This study failed to provide evidence for a significant association between this polymorphism and adult TB. The TDT (transmission disequilibrium test) analysis revealed that none of the 3' UTR alleles were preferentially transmitted to affected children, therefore it was concluded that the 3' UTR polymorphism has no effect, or a negligibly small effect on human susceptibility to TB. This polymorphism may therefore not be functional in *IL12B* expression as previously thought, however this study had limited power and perhaps a further study with a larger sample size is necessary.

1.5 AIMS IN SUMMARY

The aims of this thesis can be summarised as follows:

1. To screen DNA from a select group of individuals, from a paediatric TB cohort, who show high and low whole blood TNF levels in *in vitro* stimulation tests using LPS as the mitogen. The screen was limited to the genes for *TNF* and *TNFR1*, with sequence alterations being detected by the single stranded conformational polymorphism (SSCP) technique and DNA sequencing.
2. To screen the entire cohort of patient and control samples for selected polymorphisms identified by SSCP.
3. To screen the entire cohort of patients and controls for the 3'UTR polymorphism the *IL12p40* gene.

Chapter 2:
PATIENTS, MATERIALS AND METHODS

2.1 OVERVIEW

The subjects (n=71) examined in this study were drawn from a much larger patient and control cohort being assembled as part of a Wellcome Trust/Burroughs Wellcome (WT/BW) initiative. This program is now in the final year of its five-year initial funding and has the broad aim of establishing the genetic basis of resistance or susceptibility to *Mycobacterium tuberculosis* infection. It has concentrated on a candidate gene approach and has allocated different candidate genes for study to the different units involved in this multi-centre collaboration, including a unit here in Cape Town at Red Cross Children's Hospital, Imperial College, London and Stanford University.

One of the candidate genes decided upon for the Cape Town unit included *TNF* as well as its receptor 1 (*TNFR1*) gene. It was decided that this thesis would concentrate on DNA screening of a group of selected patient and control subjects whose LPS stimulated white cells yielded either high or low TNF levels (within the upper and lower tertiles respectively). The assumption here was that this cohort would be enriched for polymorphisms impacting on TNF gene and protein expression and would be large enough to uncover polymorphisms of major biological importance in the South African population. The molecular methodology selected for this study included gene amplification by PCR primer sets, designed to amplify the promoters, coding regions and the 3' untranslated regions of each of the two genes. The primers were designed so that they overlapped the exon/intron boundaries in order to detect possible splice site variants and any variants in flanking intronic sequence.

Following amplification, each PCR product was subjected to single stranded conformational polymorphism (SSCP) analysis using the Multiphor II

Electrophoresis system. The SSCP gels were treated with a silver stain in order to visualise DNA bands and determine any band shifts. Running conditions for the SSCP-PAGE were optimised to yield two bands for most PCR products. Approximately 43 PCR samples were run on each gel with band shifts being determined by comparative visual analysis. If a particular PCR fragment's banding pattern appeared to be different when compared to other apparently 'normal' banding patterns, which were more numerous on a typical gel, that sample was subjected to repeat PCR and electrophoresis. If the sample again showed the same altered banding pattern then the PCR product was further analysed by Big Dye® cycle sequencing. The resulting sequences were analysed using the NCBI BLAST search site where the sample sequences were compared against reference sequences in the database. Sequence variants that were novel, underwent repeat PCR and cycle sequencing for confirmation.

One SNP detected in the SSCP study and the *IL12* 3'UTR SNP were selected for further evaluation of a possible association with *M. tuberculosis* infection. These were the *TNF* –307 G→A polymorphism and the +1188 A→C polymorphism, respectively. The frequencies of these polymorphisms in the control and patient cohorts were compared by Chi-squared analysis in order to determine the presence, or lack, of an association.

2.2 SELECTION OF PATIENTS

An existing WT/BW funded programme at the Red Cross Children's Hospital has been establishing a cohort of black and mixed ancestry children with TB as well as age-matched healthy controls since 2001. This programme recruits children with active TB as well as children with a past history of TB infection. *In* vitro immune phenotyping is conducted and DNA stored. Children are 12 years of age or younger and selected for this study based on a clinical or laboratory diagnosis of TB and a negative test for HIV infection. Blood is taken during the acute stage of illness (in the case of patients) either before or within 14 days of TB treatment being initiated as well as 6 and 12 months

after recovery. Whole blood stimulation is carried out on patient and control bloods. The blood is diluted 1 in 10 with RPMI. This is then stimulated with LPS (0.5ng/ml) overnight for 18 hours at 37°C with 5% CO_2. TNF is then measured in culture supernatants by ELISA (Pharmingen Cytokine ELISA protocol, 2nd Ed, San Diego, CA, USA).

For this thesis a total of 71 individuals from the above TB cohort were screened for sequence changes in the *TNF* and *TNFR1* genes. A total of 40 cases (26 with a past history of TB, 14 with active TB) and 31 control subjects were selected. Selection was based on TNF responses to LPS, with the upper and lower tertiles being selected for each group. In the control group, the cut-off levels of TNF were >2500pg/ml (n=13) or <1000pg/ml (n=18). In the past history group, the levels were >2000pg/ml (n=12) or <1000pg/ml (n=14). Amongst those with active disease, cut-off levels were >3000pg/ml (n=3) or <750 pg/ml (n=11). The cases included those with TB meningitis, pulmonary TB, pleural TB and disseminated TB.

2.3 MOLECULAR METHODOLOGY

2.3.1 DNA isolation and storage

DNA samples from the patients and controls were drawn from the DNA stock of the WT/BW project. These DNA stocks were derived from EDTA blood samples with the DNA being isolated according to the QIAmp ® DNA Blood Midikit/Maxikit protocol (Qiagen, Germany). DNA stocks were kept at -20°C for long-term storage.

2.3.2 Polymerase chain reaction (PCR)

PCR primers were designed by Susan Sullivan of the Wellcome Trust TB research group (Imperial College, London) (table 2.1 and table 2.2) and where necessary by this laboratory. Design criteria included a product size of approximately 350bp, a length between 19-24 bases and a GC+AT Tm of 64°C. All the primers flanked the exon/intron boundaries in order to detect polymorphisms in the introns. An internal control, with dH_2O in place of DNA,

was included in every PCR run to rule out template or amplicon contamination.

An additional control DNA, not taken from the TB cohort, was used to optimise each PCR primer pair. It was amplified with each primer set during PCR optimisation where different *Taq* polymerases and magnesium concentrations were tested and once the desired PCR product was obtained (as seen by agarose gel electrophoresis), the same PCR conditions were applied to the 71 selected DNA samples for amplification. A number of different *Taq* polymerase preparations were utilised in this study. *Taq* polymerase (Promega) is normally used in the Cape Town research laboratory. Red Hot® DNA polymerase (ABgene) is a 'red' thermostable DNA polymerase. It contains Thermoprine Plus DNA polymerase, and contains an inert red dye to facilitate low volume pipetting and as an indicator for enzyme addition. This enzyme displays enhanced thermal stability at DNA denaturation temperatures. HotstarTaq® DNA polymerase (Qiagen) has been designed to provide 'hot-start' PCR for higher PCR specificity through the minimisation of non-specific amplification products, primer-dimers and background smears. It is well suited for amplification involving complex genomic or cDNA templates, very low-copy targets or multiplex primer pairs. It is inactive at ambient temperatures, which prevents the formation of misprimed products and primer-dimers at low temperatures. The enzyme is activated by a 95°C incubation step for 15 minutes. This polymerase often increases the yield of specific PCR product.

We experienced some difficulties with the initial primer stocks as some primer pairs failed to amplify. These primers were checked for degradation by electrophoresis on a 10% sequencing gel (Sambrook et al. 1989) with silver staining (Caetano-Anollés and Gresshoff 1994). Appendix A summarises the materials used for this troubleshooting procedure. The resulting gel is shown in fig. 2.1.

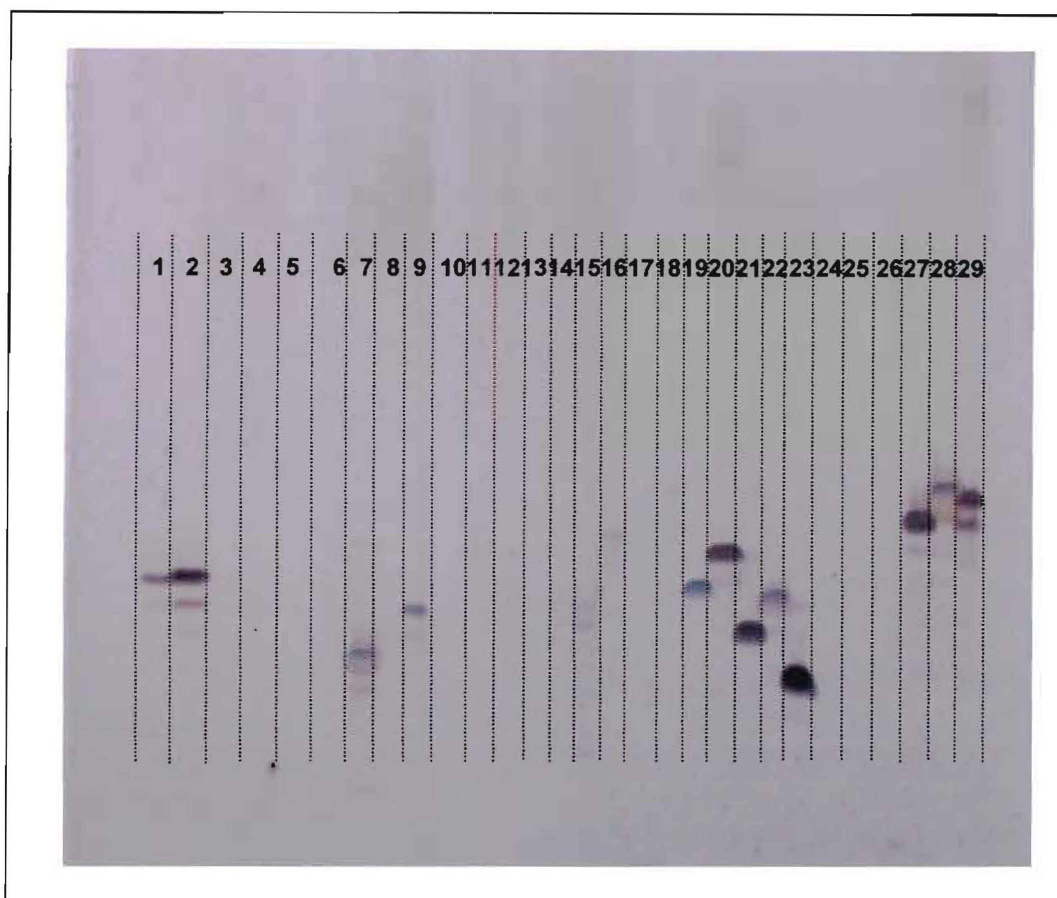

Figure 2.1. A 10% sequencing gel of primers that failed to produce the required PCR product. All primers were supplied at a concentration of 250μM. Some primers produced no bands at all (lanes 3, 4, 5, 6, 8, 11, 12, 13, 14, 17, 24, 25, 26) while others had multiple bands (1, 2, 7, 9, 15, 16, 27, 28, 29), indicating that degradation had taken place. Lane 10 had no sample loaded. Other primers produced bands with different intensities to their corresponding primer partner such as the pair in lanes 19 and 20. Primer bands were visualised by silver staining. What is interesting to note is that lanes 28 and 29 represent the forward and reverse primers, respectively, of a primer pair that amplified the target DNA, although there were multiple bands in these lanes and two main bands of different intensities. Perhaps some primer pairs are more sensitive to differing concentrations (although all stock concentrations prepared by the Wellcome Trust in the UK were assumed to be identical). Also, different levels of degradation may be tolerated until the primer no longer amplifies. Upon use of replacement primers, subsequent PCR worked successfully.

The primer sequences and the *Taq* polymerases utilised for the screening of the *TNF* gene are summarised in table 2.1.

Table 2.1 Primers for the amplification of the *TNF* gene, including the promoter, introns, exons and the 3'UTR

Gene region	Primer	Product size (bp)	Annealing temp. (Ta) (°C)	Polymerase
Promoter, part 1	p1f: TGAAGCCCAATAAACCTCTTTTC p1r: CTGTGGTCACATCTCCCCAG	353	59	HotstarTaq
Promoter, part 2	p2f: GGAATGGGTTACAGGAGACCT p2r: GTGAAATCACCCCCGGGAATT	353	59	Red Hot
Promoter, part 3	p3f: TCAGAATGAAAGAAGAGGGCCT p3r: CTGAACCCCGTCCCCATGC	370	59	Red Hot
Promoter, part 4	p4f: ATGGAGGCAATAGGTTTTGAGG p4r: AGGGAGCGTCTGCTGGCTG	353	59	HotstarTaq
Promoter, part 5	p5f: ATATAAAGGCAGTTGTTGGCACA p5r: CAGAAGAGCGTGGTGGCGC	353	59	HotstarTaq
Exon 1	e1f: AAGCTGCCAGGCAGGTTCTC e1r: GCACATCTTTCACCCATCCCA	352	59	HotstarTaq
Exon 2+3	e2,3f: CCTCGATGTTAACCATTCTCCT e2,3r: AGCCCTCCAAGTTCCAAGACA	354	59	HotstarTaq
Exon 4	e4if: CTGACTCTCCTCTCCCTCTC e4ir: GACCCCTCCCAGATAGATGG	350	59	HotstarTaq
Exon 4	e4iif: AGAGGGGGCTGAGGCCAAG e4iir: GCCACACATTCCTGAATCCCA	343	59	Red Hot
3'UTR	3'1f: AGCAACAAGACCACCACTTCG 3'1r: CGGTCTCCCAAATAAATACATTC	384	59	Red Hot
3'UTR	3'2f: CCCATGGAGCCAGCTCCCT 3'2r: TTAATTCCAAGAAGGAGACCATG	401	59	Red Hot + antibody

The sequence of the *TNF* promoter and the PCR primer sets for the amplification of overlapping fragments is given in fig. 2.2.

-1274
CCCTCGATGAAGCCCAATAAACCTCTTTTCTCTGAAATGCTGTCTGCTTGTGTGTGTGTGTCTGGGA
GTGAGAACTTCCCAGTCTATCTAAGGAATGGAGGGAGGGACAGAGGGCTCAAAGGGAGCAAGAGC
TGTGGGGAGAACAAAAGGATAAGGGCTCAGAGAGCTTCAGGGATATGTGATGGACTCACCAGGTG
AGGCCGCCAGACTGCTGCAGGGGAAGCAAAGGAGAAGCTGAGAAGATGAAGGAAAAGTCAGGGT
-1013
CTGGAGGGGCGGGGGTCAGGGAGCTCCTGGGAGATATGGCCACATGTAGCGGCTCTGAGGAAT
GGGTTACAGGAGACCTCTGGGGAGATGTGACCACAGCAATGGGTAGGAGAATGTCCAGGGCTATG
AAAGTCGAGTATGGGGACCCCCCCTTAACGAAGACAGGGCCATGTAGAGGGCCCCAGGGAGTGA
AAGAGCCTCCAGGACCTCCAGGTATGGAATACAGGGGACGTTTAAGAAGATATGGCCACACACTG
GGGCCCTGAGAAGTGAGAGCTTCATGAAAAAAATCAGGGACCCCAGAGTTCCTTGGAAGCCAAGA
-690
CTGAAACCAGCATTATGAGTCTCCGGGTCAGAATGAAAGAAGAGGGCCTGCCCCAGTGGGGTCTG
TGAATTCCCGGGGGTGATTTCACTCCCCGGGGCTGTCCCAGGCTTGTCCCTGCTACCCGCACCCA
GCCTTTCCTGAGGCCTCAAGCCTGCCACCAAGCCCCCAGCTCCTTCTCCCCGCAGGGCCCAAACA
CAGGCCTCAGGACTCAACACAGCTTTTCCCTCCAACCCCGTTTTCTCTCCCTCAACGGACTCAGCT
TTCTGAAGCCCCTCCCAGTTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAAGTTAGAAGGAAAC
-362
AGACCACAGACCTGGTCCCCAAAAGAAATGGAGGCAATAGGTTTTGAGGGGCATGGGGACGGGGT
TCAGCCTCCAGGGTCCTACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTCGGAATCGGAGC
AGGGAGGATGGGGAGTGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAAATCCCCG
CCCCCGCGATGGAGAAGAAACCGAGACAGAAGGTGCAGGGCCCACTACCGCTTCCTCCAGATGA
-103
GCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGATTCTTTCCCCGCCCTCCTCTC
GCCCCAGGGACATATAAAGGCAGTTGTTGGCACACCC**A**+1GCCAGCAGACGCTCCCTCAGCAAGG
ACAGCAGAGGACCAGCTAAGAGGGAGAGAAGCAACTACAGACCCCCCCTGAAAACAACCCTCAGA
CGCCACATCCCCTGACAAGCTGCCAGGCAGGTTCTCTTCCTCTCACATACTGACCCACGGCTTCAC
CCTCTCTCCCCTGGAAAGGACACC**ATG**AGCACTGAAAGCATGATCCGGGACGTGGAGCTGGCCGA
GGAGGCGCTCCCCAAGAAGACAGGGGGGCCCCAGGGCTCCAGGCGGTGCTTGTTCCTCAGCCTC
TTCTCCTTCCTGATCGTGGCAGGCGCCACCACGCTCTTCTGCCTGCTGCAC

Figure 2.2. The DNA sequence of the *TNF* promoter (GenBank NT_009759). The sequences highlighted in blue refer to the forward and reverse primers for part 1, those in red refer to the primers for part 2, those in green to the primers for part 3, those in light blue to the primers for part 4, while those in yellow refer to the primers for part 5 of the promoter. The underlined and bolded A is the transcription start site and it is referred to as position +1. The underlined and bolded ATG is the translation start site.

All PCR amplifications were carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA). The final reaction volume was 25 μl in 0.5 ml tubes (tables 2.3, 2.4, 2.5 and 2.6). The primer sequences and the *Taq* polymerases utilised for screening the *TNFR1* gene are summarised in table 2.2.

Table 2.2 Primers for the amplification of the *TNFR1* gene, including the promoter, introns, exons and the 3'UTR

Gene region	Primer	Fragment Size (bp)	Annealing temp (Ta) (°C)	Polymerase
Promoter, part 1	p1f: CGATGATCTGTAAGGTGAGTTAT p1r: CCCTCGTTTTCTGCCCTGAC	353	59	Red Hot
Promoter, part 2	p2f: TGAGGAATGGCAGGCACCCA p2r: CTGGGTTCCAATTCAGAATGCT	352	59	HotstarTaq
Promoter, part 3	p3f: GTGTCCAAGACACATTCTTAGC p3r: AGAAAATTAAAGCAGAGAGGAGG	365	59	HotstarTaq
Exon 1	e1f: CCCCTCCCACCTTCTCTCC e1r: GAAGGTGCCTCGCCCACCA	344	59	HotstarTaq + Q-solution
Exon 2	e2f: CTAAGCTTCCCATCCCTCCC e2r: CTGAAGTCTCTAGGAGAGGAG	352	59	HotstarTaq
Exon 3	e3f: GTCTCTCCTGGGCTGGGATT e3r: TGCCCACACCCACCAGCCT	349	59	HotstarTaq + Q-solution
Exon 4	e4f: GTGGGTGCCTGTCTATGTGC e4r: TGGCCTCAGGAGAGCTGCG	349	59	HotstarTaq
Exon 5	e5f: ACCGTGCACCTCTCCTGTGA e5r: CCATCCAGTGCCCAGCAGC	350	59	HotstarTaq
Exon 6+7	e6,7f: TTCACCAGTGCCGTCTCTTCT e6,7r: CTCTCCCTCCCAAAGCCCC	381	59	Red Hot
Exon 8	e8f: AAGCTCTACTCCATTGGTGAGT e8r: GGAGTAACTCTCTCATTTCATCT	373	59	HotstarTaq
Exon 9	e9f: TGTTGCGCCACCTTCTCTCTT e9r: CCAGCCTCCTCGTCTCCAG	350	59	HotstarTaq
Exon 10	e10if: GACAGTGAGTTTCTCCCGCG e10ir: GCTCCAGCGTGGCCTCGC	370	56	Red Hot + Q-solution
Exon 10	e10iif: GCTGGCGACCTGGAGGCG e10iir: GCCCACGGCGCACCTCTC	370	59	Red Hot + Q-solution

The sequence of the *TNFR1* promoter and the PCR primer sets for the amplification of overlapping fragments is given in fig. 2.3.

-1210
ACCTAGGGGGTTGTAGTCGATGATCTGTAAGGTGAGTTATAATTGATGTATTGGAATATTTAGGAAA
AGGGCACTGGGAATATGCTAGGAACACCTTGATGGAGGTATCTTTATTTCCACGGCAGCTTCGTGG
ATACGTCTCATTGATTCTCATGGCATCACTTTCCCCATGTAGGTGGGCAGACATTGTTACCCCTGTT
TAATAAACAAGGAACCAACAGAGGCTTAGGAGAGGAGTTGCCTGATGTCGCATGATTGGTGGCAG
AGCCAGGATCAACAGTGGGGCAGGGTGGGGGGACCTGGCCAGGCAGAGAGACTGGATGAGACCT
GGGGTGAGGAATGGCAGGCACCCAGTCAGGGCAGAAAACGAGGGTTGGGACTTACTTTGAGTTTT
GGATTGGATCAGTAAATTCCCAAGAAAGAGGGAGACTAGGAGGCTAGTGAAGAACTCTGGAGTAAA
GGGGAGGATTACTAAGGGACATGGAGTACCTATCATGTGTCGGACGCTTATCTATATCTCTCCCAT
CTGAACAAATCCTTACAGGAACCCCAGGAGACAGGTTATCTCCACTCTGCAAATTGGAAAACAGAT
-618
CCAGACAGTTTCAGTTATGTGTCTGAGAAGTTCATTTATGTGTCCAAGACACATTCTTAGCTAAAAA
GCTAAGCATTCTGAATTGGAACCCAGAGAATTTGACTCCCAGACTCTGGATCTTTTCACTGCTGTGA
TCCATCTGGGAAAGGCTAGTGATGTGGGCAAGGGGCTTATTGCCCCTTGGTGTTTGGTTGGGAGT
GGTCGGATTGGTGGGTTGGGGGCACAAGGCAGCCAGATCTGGGACTCCTGTGCTTGTGACTGGA
CTACAAAGAGTTAAAGAACGTTGGGCCTCCTCCTCCCGCCTCCTGTGGCCTCCTCCTCCAGCTCTT
-289
CCTGTCCCGCTGTTGCAACACTGCCTCACTCTTCCCCTCCCACCTTCTCTCCCCTCCTCTCTGCTTT
AATTTTCTCAGAATTCTCTGGACTGAGGCTCCAGTTCTGGCCTTTGGGGTTCAAGATCACTGGGAC
CAGGCCGTGATCTCTATGCCCGAGTCTCAACCCTCAACTGTCACCCCAAGGCACTTGGGACGTCC
TGGACAGACCGAGTCCCGGGAAGCCCCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGG
GGGAGTGAGAGGCCATAGCTGTCTGGC**<u>ATG</u>**GGCCTCTCCACCGTGCCTGACCTGCTGCTGCCACT
GGTGAGACCAGGGACAAAGGGAAGAGTGGGCTGGTGGGCGAGGCACCTTC

Figure 2.3. The DNA sequence of the *TNFR1* promoter (GenBank NT_009731). The sequences highlighted in blue refer to the forward and reverse primers for part 1, those in red refer to the forward and reverse primers for part 2, those in green refer to the forward and reverse primers for part 3 of the promoter, while those in light blue refer to the forward and reverse primers for exon 1. The underlined and bolded ATG is the translation start site and it is referred to as position +1.

Different PCR mixes were utilised for the different *Taq* polymerases used. These are detailed in tables 2.3, 2.4, 2.5 and 2.6.

Table 2.3 Reaction mix for PCR amplification using *Taq* DNA polymerase *

Reagent	**Stock conc.**	**Volume**	**Conc./Amt.**
DNA	100 ng/μl	1 μl	100ng
Forward primer	10 μM	1.25 μl	0.5 μM
Reverse primer	10 μM	1.25 μl	0.5 μM
dNTP's (ABgene®, Surrey, UK)	2.5 mM	2 μl	200 μM
10X PCR buffer (Promega, Madison, WI, USA)	10X	2.5 μl	1X
Magnesium (Mg^{2+}) (Promega, Madison, WI, USA)	25 mM	1.5 μl	1.5 mM
Promega Taq (Promega, Madison, WI, USA)	5U/volume	0.13 μl	0.625U
dH_20		15.38 μl	
Total volume		**25 μl**	

* Promega, Madison, WI, USA

The amplification reaction was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) using the following reaction conditions:

1) initial denaturation - 94°C / 2 minutes for 1 cycle
2) 30 cycles: denaturation - 94°C / 30 seconds
   annealing - χ°C* / 30 seconds
   elongation - 72°C / 30 seconds
3) final step - 72°C / 5 minutes for 1 cycle

*χ°C = specific annealing temperature for each primer set

Table 2.4 Reaction mix for PCR amplification using Red Hot® DNA Polymerase *

Reagent	**Stock conc.**	**Volume**	**Conc./Amt.**
DNA	100 ng/μl	1 μl	100ng
Forward primer	10 μM	1.25 μl	0.5 μM
Reverse primer	10 μM	1.25 μl	0.5 μM
dNTP's (ABgene®, Surrey, UK)	2.5 mM	2 μl	200 μM
10X PCR buffer (ABgene®, Surrey, UK)	10X	2.5 μl	1X
Magnesium (Mg^{2+}) (ABgene®, Surrey, UK)	25 mM	1.5 μl	1.5 mM
Red Hot® Taq (ABgene®, Surrey, UK)	5U/volume	0.13 μl	0.625U
dH_20		15.38 μl	
Total volume		**25 μl**	

* ABgene®, Surrey, UK

The amplification reaction was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) using the following reaction conditions:

1) initial denaturation - 94°C / 2 minutes for 1 cycle
2) 28 cycles: denaturation - 94°C / 30 seconds
   annealing - χ°C* / 30 seconds
   elongation - 72°C / 30 seconds
3) final step - 72°C / 5 minutes for 1 cycle

*χ°C = specific annealing temperature for each primer set

Table 2.5 Reaction mix for PCR amplification using Red Hot® DNA Polymerase *1 and JumpStart™ Taq Antibody *2

Reagent	**Stock conc.**	**Volume**	**Conc./Amt.**
DNA	100 ng/μl	1 μl	100ng
Forward primer	10 μM	1.25 μl	0.5 μM
Reverse primer	10 μM	1.25 μl	0.5 μM
dNTP's (ABgene®, Surrey, UK)	2.5 mM	2 μl	200 μM
10X PCR buffer (ABgene®, Surrey, UK)	10X	2.5 μl	1X
Magnesium (Mg^{2+}) (ABgene®, Surrey, UK)	25 mM	1.5 μl	1.5 mM
Red Hot® Taq (ABgene®, Surrey, UK) and JumpStart™ Antibody (Sigma-Aldrich, GmbH, Germany)	5U/volume	0.75 μl	0.625U
dH_20		14.75 μl	
Total volume		**25 μl**	

*1 ABgene®, Surrey, UK
*2 Sigma-Aldrich, GmbH, Germany

The amplification reaction was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) using the following reaction conditions:

1) initial denaturation - 94°C / 2 minutes for 1 cycle
2) 28 cycles: denaturation - 94°C / 30 seconds
   annealing - χ°C* / 30 seconds
   elongation - 72°C / 30 seconds
3) final step - 72°C / 5 minutes for 1 cycle

*χ°C = specific annealing temperature for each primer set

Table 2.6 Reaction mix for PCR amplification using HotStarTaq® DNA Polymerase *

Reagent	**Stock conc.**	**Volume**	**Conc./Amt.**
DNA	100 ng/μl	1 μl	100ng
Forward primer	10 μM	1.25 μl	0.5 μM
Reverse primer	10 μM	1.25 μl	0.5 μM
dNTP's (ABgene®, Surrey, UK)	2.5 mM	2 μl	200 μM
10X PCR buffer (Qiagen, GmbH, Germany)	10X	2.5 μl	1X
HotstarTaq® (Qiagen, GmbH, Germany)	5U/volume	0.13 μl	0.625U
dH_20		16.88 μl	
Total volume		**25 μl**	

* Qiagen, GmbH, Germany

The amplification reaction was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) using the following reaction conditions:

1) initial denaturation - 95°C / 15 minutes for 1 cycle
2) 28 cycles: denaturation - 94°C / 30 seconds
   annealing - χ°C* / 30 seconds
   elongation - 72°C / 30 seconds
3) final step - 72°C / 5 minutes for 1 cycle

*χ°C = specific annealing temperature for each primer set

Agarose gel electrophoresis: The PCR products were separated by electrophoresis on 2% (w/v) agarose gels. Samples were mixed with loading dye (1 μl per 5μl PCR product) (appendix B) before adding to the wells. Gels

were electrophoresed in 1x Tris-acetate EDTA (TAE) running buffer (appendix B) and contained a molecular weight marker XIV (appendix B). Mini-gels (10 cm X 7 cm) were electrophoresed in small buffer tanks at 100V for approximately 30 minutes. Ethidium bromide solution (10 mg/ml) (Sigma, Poole, England) was added to the gel and gels were visualised using a UV transilluminator (UVP Inc. San Gabriel, CA, USA).

2.3.3 Single stranded conformational polymorphism (SSCP)

The method used for SSCP analysis was based on the technique developed by Orita et al. (1989) with modifications to allow for non-radioactive detection of the single-stranded nucleic acids by silver staining (Lohmann et al. 1992). SSCP is based on the hypothesis that the mobility of single-stranded DNA in a non-denaturing polyacrylamide gel is dependent on its size as well as its sequence-dependent conformation. SSCP analysis of the *TNF* gene and *TNFR1* gene products was carried out using a two buffer horizontal polyacrylamide gel electrophoresis based-system. The conformational changes of a single-stranded molecule induced by a polymorphism are sensitive to environmental influences such as the gel composition, temperature, ionic strength and additives such as glycerol (Liechti-Gallati et al. 1999).

The protocol for this technique presents standard SSCP conditions for mutation screening in different genes. Liechti-Gallati et al. (1999) who analysed 80 known cystic fibrosis mutations and 20 polymorphisms found that this system was able to detect at least 97% of all point mutations in the coding region of the *CFTR* gene. In addition, they identified five novel mutations in this gene and detected a base substitution in a fragment larger than 500 bp. The Division of Human Genetics, University of Cape Town, tested this protocol locally by studying known sequence variations in the Rhodopsin (RHO) gene and confirmed the 97% sensitivity recorded in previous studies (Roberts et al. 1999). The defined SSCP conditions for this protocol are a 12% nondenaturing polyacrylamide gel, 7% glycerol, two-phase buffer system and 90 minute electrophoresis at 12-15°C followed by silver staining. With this

protocol, denatured and partly re-annealed DNA fragments remain on the gel. This additionally allows the detection of heteroduplexes; SSCP and heteroduplex analysis can therefore be carried out simultaneously on the same gel, which increases sensitivity (Liechti-Gallati et al. 1999). The running temperature of the gel is easily controlled and manipulated by using a standard water cooler. PCR and SSCP can be carried out on 80 samples in less than 5 hours once optimal conditions have been determined. This Multiphor II system has improved the rate and sensitivity of mutation screening of the older SSCP techniques. In addition, visualisation is non-radioactive and the gels are quite small (118mm X 220mm X 3mm) and easy to handle (Roberts et al. 1999).

Figure 2.4 shows a typical SSCP gel using this technique. The band shifts are indicated on the gel (black arrows). Lane 1 shows an individual with two band shifts, indicated by the presence of a doublet in the single-stranded region of the gel and tight doublet in the double-stranded/heteroduplex region of the gel. In lane 2, these doublets are absent. Following this visual observation and confirmation, Big Dye® Terminator cycle sequencing is carried out to determine if this band shift is indeed a variant in the DNA sequence of that particular individual.

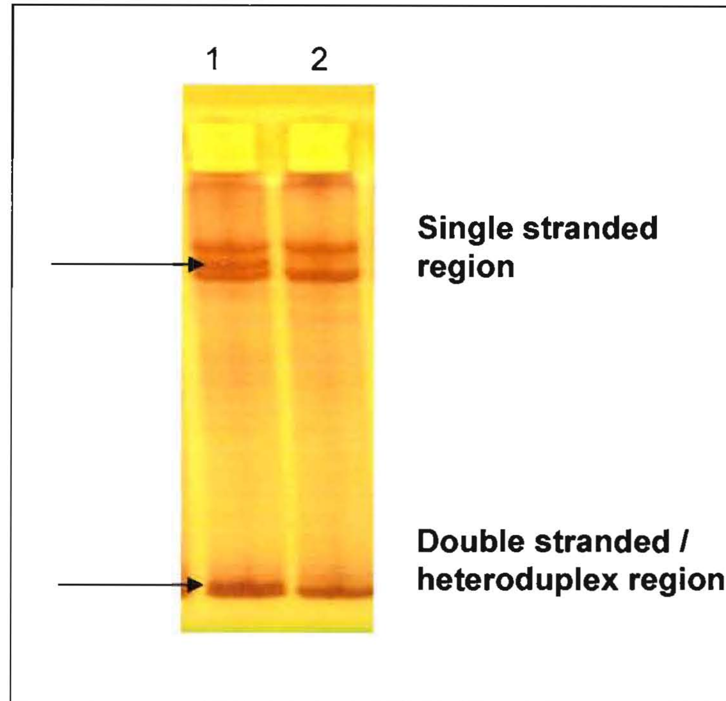

Figure 2.4. SSCP analysis of exon 3 of the *TNFR1* gene (PCR product; 349 bp). The silver stained polyacrylamide gel showing band shifts in both the single and double stranded DNA regions, lane 1; the normal banding pattern for this exon is shown in lane 2. The arrows indicate the positions of the band shifts in the SSCP gel.

As an internal study to ensure that the Multiphore II Electrophoresis system (Amersham Pharmacia Biotech) was working optimally for the purposes of this study, 66 DNA samples with 11 known mutations were obtained from the Wellcome Trust TB research group, Imperial College, London and were screened blinded. Table 2.7 summarises the PCR and SSCP conditions used to analyse these samples. All amplifications were preceded by a 2 minute denaturation step at 94°C and followed by a final elongation step at 72°C for 5 minutes. For each PCR cycle, 94°C and 72°C were used for the denaturation and elongation, respectively.

Table 2.7 PCR and SSCP conditions for validation of the Multiphore II electrophoresis system

Gene and region	Annealing temp. (°C)	*Taq* Polymerase	Cycle no.	Gel temp. (°C)
IFNγR1e5*[1]	59	Promega	28	12
IL12Rβ1e14*[2]	59	Red Hot	28	12
IL12Rβ1e15	59	Red Hot	28	12
IL12p40e4	59	Promega	25	15
IFNγR1e6	53	Promega	28	12
IFNγR1e7iii	59	Promega	28	12
IL12Rβ2e11	59	Red Hot + Ab	28	15
IFNγRβ2e2	59	Red Hot	28	12
IL12Rβ2e5	59	Promega	26	12
IFNγR1e1	65	Promega	25	15
IFNγR1p1	56	Red Hot	28	12

Examples: *[1] IFNγR1e5, Interferon gamma, receptor 1, exon 5
*[2] IL12Rβ1e14, Interleukin 12, receptor β1, exon 14

Two microlitres of the amplification product were added to 4 μl of SSCP loading dye (appendix C). Three microlitres of this mix was loaded onto a 0.5mm thick, 12% non-denaturing polyacrylamide gel (appendix C). Electrophoresis was carried out using a Multiphor II Electrophoresis system (Amersham Pharmacia Biotech) at 350V for approximately 120 minutes (10-15°C). Up to 46 samples could be run on one gel.

Silver staining: The SSCP and heteroduplex (HD) banding patterns were detected by silver staining (Lohmann et al. 1992). Appendix C summarises the materials used for silver staining. Following gel electrophoresis, the gel is washed with dH_2O and then covered in solution 1 (silver nitrate) and left at room temperature for 15 minutes. Thereafter, the gel is washed with dH_2O and then submerged in solution 2 (sodium hydroxide/formaldehyde) until the bands become visible. When the gel is sufficiently dark, and the bands intense, solution 2 is removed and the gel washed again with dH_2O. A sheet of blotting paper (Merck NT laboratory supplies (PTY) Ltd., Fedsure Park, Midrand, South Africa) is placed on the gel and the gel allowed to dry. The gel can be scanned into the computer using a standard flatbed scanner, with the

image saved for future analysis. The gel can be stored dry for future reference.

2.3.4 Sequencing analysis

PCR products showing confirmed aberrant banding patterns following SSCP analyses were subjected to the Big Dye® Terminator cycle sequencing for the incorporation of fluorophore-labelled dideoxynucleotides.

Purification of PCR products: PCR products to be analysed by direct sequencing were purified according to the protocol for the QIAquick™ PCR purification kit (QIAGEN, GmbH, Germany) to remove primers and dNTP's.

DNA sequencing: A half reaction of the Big Dye® Terminator v3.1 cycle sequencing kit (PE Applied Biosystems, Foster City, USA) was used in the sequence terminator reaction mix. Table 2.8 summarises the sequencing reaction mix, which was made up to a final volume of 10 μl.

Table 2.8 Sequence terminator reaction mix

Reagent	Volume
Forward and reverse primers (3.2 μM)	1μl
3-10 ng PCR product	1-5 μl
Termination mix (PE Applied Biosystems, Foster city, USA)	4 μl
dH_2O	Up to 10 μl
Final Volume	**10 μl**

The amplification reaction was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) using the following reaction conditions:

1) initial denaturation - 96°C / 10 seconds for 1 cycle
2) 25 cycles: denaturation - 96°C / 10 seconds

annealing - 52°C / 15 seconds

elongation - 60°C / 4 minutes

3) final step - 60°C / 4 minutes for 1 cycle

The PCR products were sent to the Central DNA Sequencer at the University of Stellenbosch for further purification and electrophoresis on the ABI 377 automated sequencer. All samples were sequenced in the forward and reverse directions using the same primers as those for PCR-SSCP analysis. Further analysis was performed using the NCBI BLAST search site (http://www.ncbi.nlm.nih.gov/blast/). The sequence data were compared to a reference sequence in the NCBI database and base mismatches indicated a single-base change. A frame-shift change, resulting from a possible deletion or insertion, was indicated by an unreadable sequence beyond the position of the insertion/deletion. The exact position of the DNA alteration was determined by referring to the GenBank sequence (TNF: NT_009759; TNFR1: NT_009731).

2.4 GENOTYPING OF THE *TNF* -307 PROMOTER POLYMORPHISM USING ARMS-PCR

The G to A substitution at position –307 in the promoter region of *TNF* was detected by the ARMS (amplification refractory mutation system) method (Newton et al. 1989) that facilitates genotyping only by inspection of reaction mixtures after agarose gel electrophoresis. It clearly distinguishes heterozygotes from homozygotes for either allele at a particular locus. The terminal 3' nucleotide of a primer must be allele specific, the primer is therefore synthesised in two forms. The wildtype form is refractory to PCR on the variant template while the variant primer is refractory to PCR on the wildtype template. These terminal 3' mismatches destabilise the synthesis end of the primer and prevent elongation. To add further to the destabilisation, a mismatch was also introduced at the 3rd base from the 3' end.

The sequence of the forward primer used to amplify the wildtype allele was as follows:
5' AATAGGTTTTGAGGGGCAGGG 3' and the forward primer that amplified the variant allele was as follows: 5' CAATAGGTTTTGAGGGGCAGGA 3'. Both primers were designed to have a GC+AT Tm of 64°C. The PCR cocktail was as per table 2.6 with the same cycling conditions listed below the table. When the wildtype forward primer was used, only wildtype individuals (GG) and well as heterozygous (GA) individuals displayed a band in the agarose gel. When the variant forward primer was used, only those individuals who were heterozygous for the polymorphism or who were homozygous variant (AA) displayed a band in the agarose gel. The reverse primer, 5' AGGGAGCGTCTGCTGGCTG 3', is the same as that used during amplification of part 4 of the promoter of *TNF* (table 2.1). Figure 2.5 illustrates the ARMS primers used to genotype the –307 G→A polymorphism.

Figure 2.5. The ARMS primers used to genotype the –307 G→A polymorphism in the *TNF* gene. Primer wt refers to the forward primer that amplified the wildtype promoter sequence (-307G) while primer var refers to the forward primer that amplified the polymorphic promoter sequence (-307A). The introduced mismatch at the third base position is shown in bold type, while the terminal mismatch is underlined.

2.5 GENOTYPING OF THE *IL12 p40* 3'UTR POLYMORPHISM USING RESTRICTION ENZYME ANALYSIS

The *IL12 p40* 3'UTR comprises an A→C transversion at nucleotide position +1188 which creates a cutting site for *TaqI*. A 301 bp region of this 3'UTR, containing this polymorphic site was amplified using the forward primer 5'-ATTTGGAGGAAAAGTGGAAGA-3' and the reverse primer 5'-

AACATTCCATACATCCTGGC-3'. The amplicons were then digested by *TaqI* (New England Biolabs) at 65°C for 4 hours according to the manufacturer's instructions. Genotypes were assessed by agarose gel electrophoresis. Wildtype individuals (AA) displayed an undigested fragment of 301 bp, while carriers of the C allele presented a 162 bp and 139 bp double-band pattern in addition to the 301 bp fragment.

2.6 STATISTICAL ANALYSIS

To determine if the frequency of either of the two above-mentioned polymorphisms in the patient population was significantly different from the control population, a Chi-squared test was carried out. The Chi-test is calculated at a significance level of 95% i.e. a 5% probability that the null hypothesis (that there is no association between the polymorphism and the disease) is not true and 95% probability that it is true. A *P*-value of less than 0.05 is generally considered to be significant. If this value is greater than 5%, we usually accept the null hypothesis, i.e that there is no association. In order to calculate this *P*-value for the allele numbers, a 2x2 contingency table was constructed, http://vassun.vassar.edu/~lowry/odds2x2.html and http://members.aol.com/johnp71/ctab2x2.html. In order to calculate this *P*-value for the genotype numbers, a 2x3 contingency table was constructed, http://www.stat.sc.edu/~west/applets/contable.html. Yate's correction was applied for small numbers.

Chapter 3:
RESULTS

3.1 OVERVIEW

The 71 selected patient and control samples were analysed for sequence polymorphisms by SSCP analysis. Band shifts were detected upon visual observation. Samples were subjected to repeat PCR and SSCP-PAGE, followed by cycle sequencing. The results from this study are presented below.

3.2 SSCP SCREENING ANALYSIS

The internal validation study of the SSCP technique used to test DNA samples received from the UK collaboration resulted in ten out of eleven DNA polymorphisms being detected with one PCR failure. This positive outcome, together with published success rates of the technique (Liechti-Gallati et al. 1999) suggested the technique to be working and suitable for the purposes of this study.

TNF gene: Sequence variations in this gene were sought by screening all 4 exons as well as the immediate 5' and 3' flanking regions. The banding pattern and band shifts detected for this gene are shown in fig. 3.1 to 3.5.

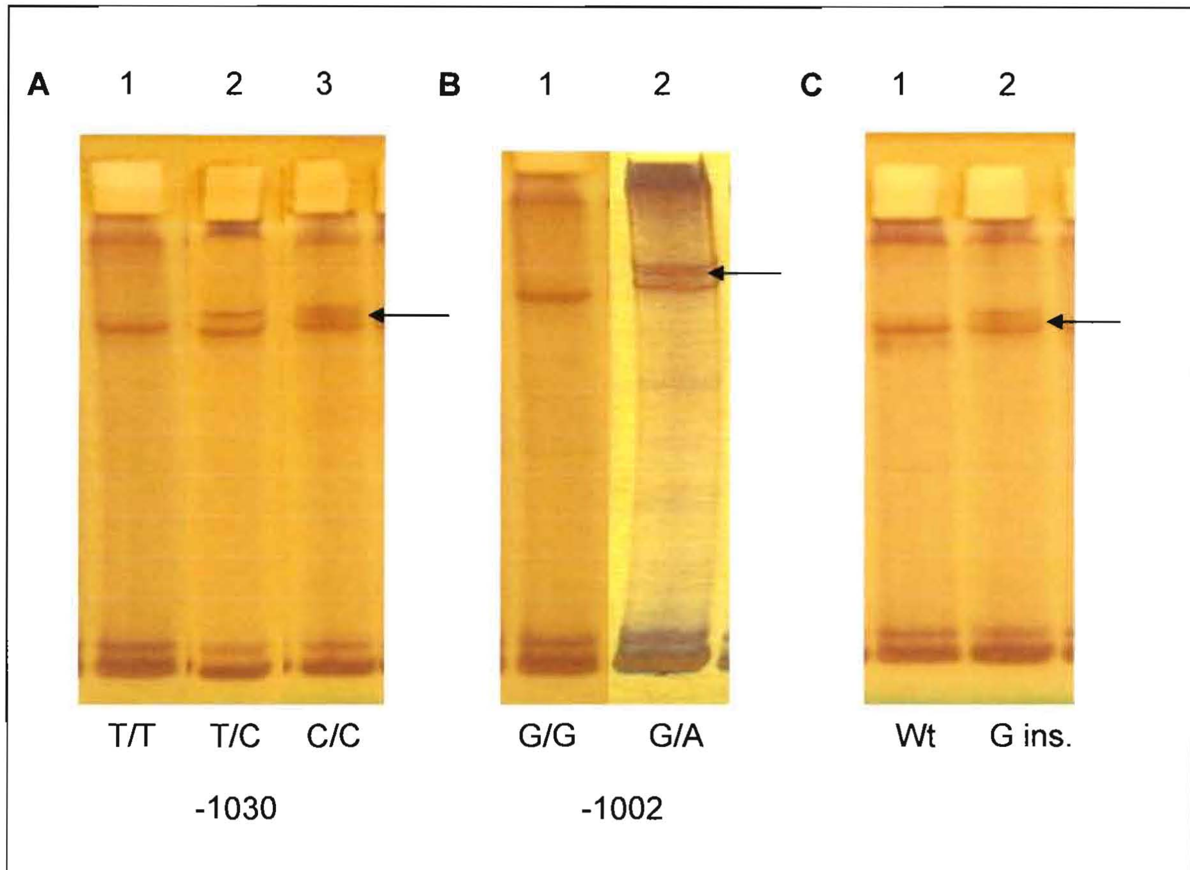

Figure 3.1. SSCP gel of the *TNF* promoter, part 1 (-1266 to –914; 353 bp). The band shifts of three different sequence alterations are shown. **A (-1030 T→C):** Lane 1 represents an individual who is wildtype (T/T); lane 2, a heterozygote (T/C); lane 3, a homozygote (C/C). The gel depicted here is a composite and any shifts other than those indicated are artefactual. **B (-1002 G→A):** Lane 1 represents an individual who is widtype (G/G); lane 2, a heterozygote (G/A). The gel depicted here is a composite and any shifts other than those indicated are artefactual. **C (G insertion):** Lane 1 represents an individual who is wildtype for this part of the promoter; lane 2, a heterozygote for a G insertion. The band shifts recorded are indicated by the black arrows. The bands may not appear clear due to the reproduction process.

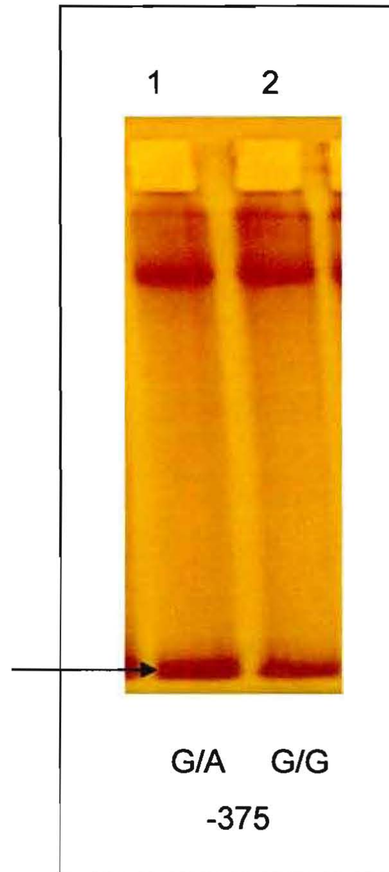

Figure 3.2. SSCP gel of the *TNF* promoter, part 3 (-663 to –294; 370 bp). Lane 1 represents an individual who is a heterozygote (G/A); lane 2, wildtype (G/G). The very tight doublet in the heteroduplex region recorded for the DNA sample in lane 1 is shown by the black arrow.

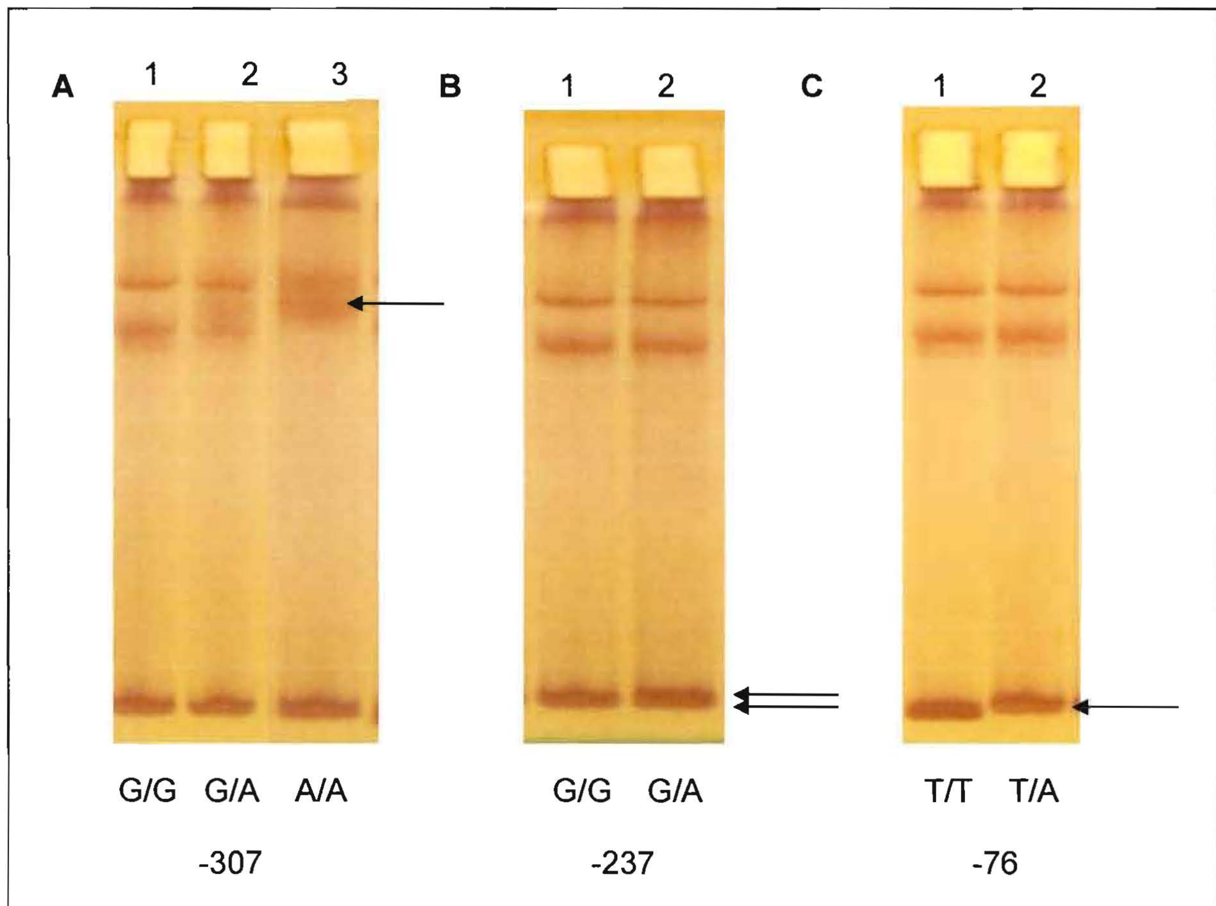

Figure 3.3. SSCP gel of the *TNF* promoter, part 4 (-335 to +18; 353 bp). The band shifts of three different sequence alterations are shown. **A (-307 G→A):** Lane 1 represents an individual who is wildtype (G/G); lane 2, a heterozygote (G/A) as indicated by the shadowing between the two bands; lane 3, a homozygote (A/A) as indicated by the lack of the lower band in the single stranded region of the gel and the dark shadowing. **B (-237 G→A):** Lane 1 represents an individual who is wildtype (G/G); lane 2, a heterozygote (G/A) indicated by a very tight doublet. **C (-76 T→A):** Lane 1 represents an individual who is wildtype (T/T); lane 2, a heterozygote (T/A). The band shifts recorded are indicated by the black arrows.

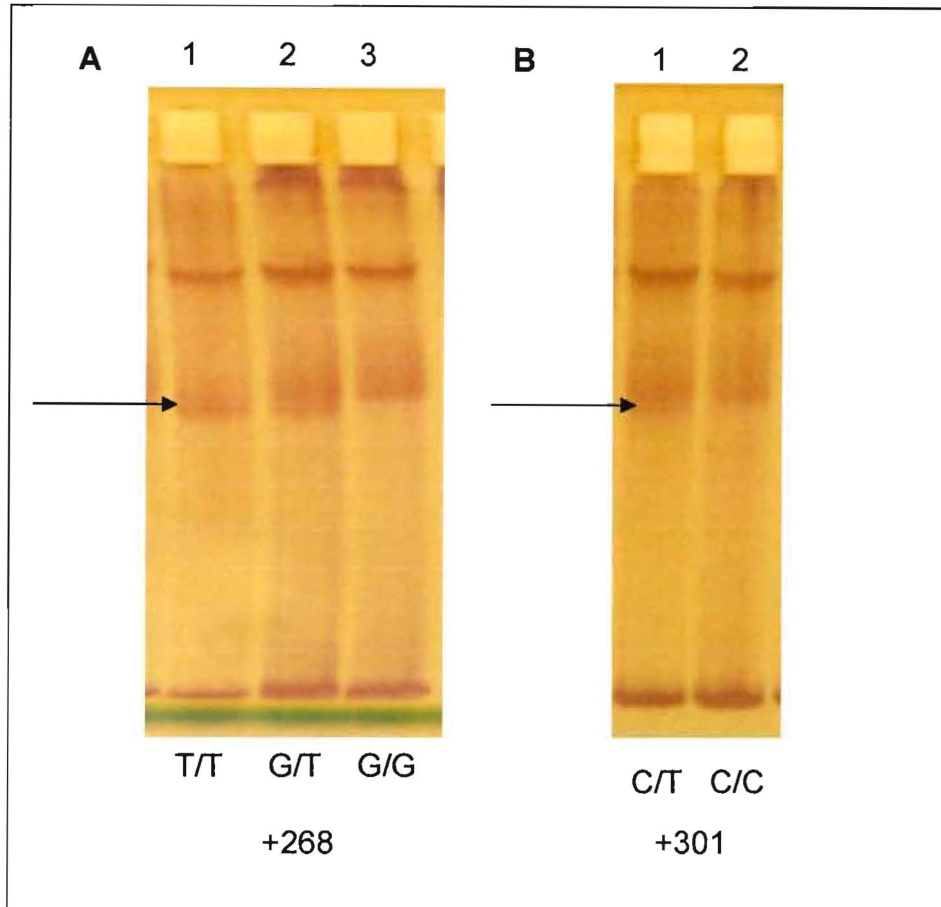

Figure 3.4. SSCP gel of the *TNF* promoter, part 5 (-25 to +327; 353 bp). The band shifts of two different sequence alterations are shown. **A (+268 G→T):** Lane 1 represents an individual who is a homozygote (T/T); lane 2, a heterozygote (G/T); lane 3, wildtype (G/G). This alteration represents a synonymous base change in the codon for arginine. **B (+301 C→T):** Lane 1 represents an individual who is a heterozygote (C/T); lane 2, wildtype (C/C). This alteration represents a synonymous base change in the codon for isoleucine. The band shifts recorded are indicated by the black arrows.

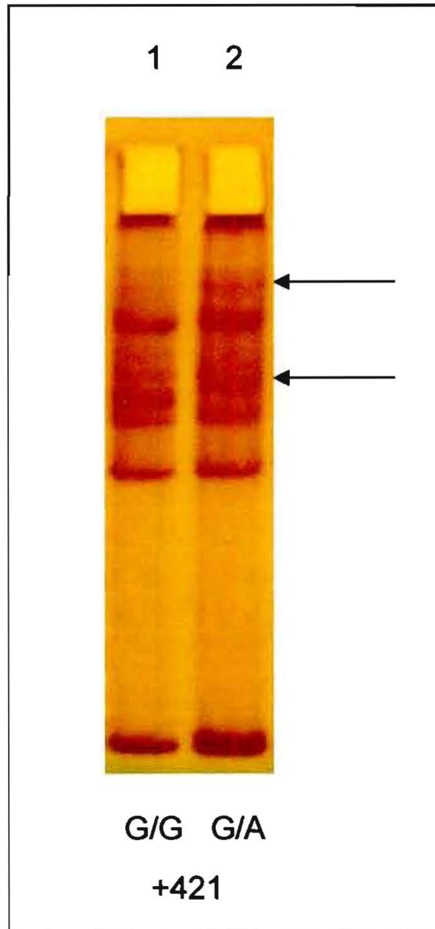

Figure 3.5. SSCP gel of the *TNF* exon 1 (+108 to +459; 352 bp). Lane 1 represents an individual who is wildtype (G/G); lane 2, a heterozygote (G/A). The band shifts recorded are indicated by the black arrows.

***TNFR1* gene:** Sequence variations in this gene were sought by screening all 10 exons, as well as the immediate 5' and 3' flanking regions. The banding pattern and band shifts detected for this gene are shown in fig. 3.6 to 3.11.

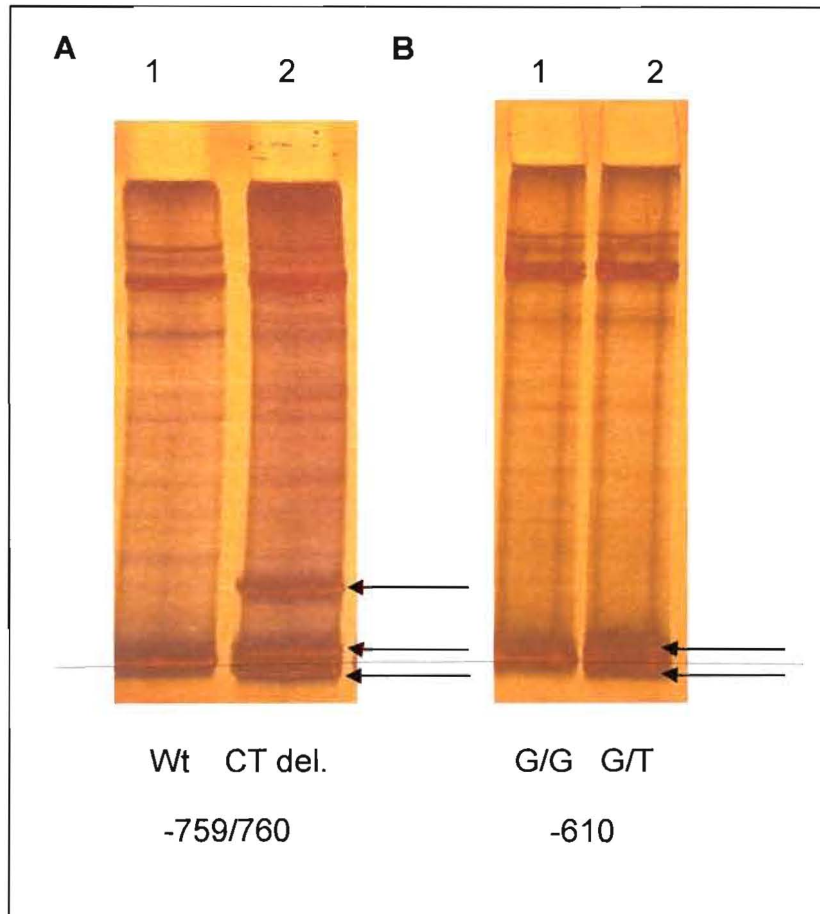

Figure 3.6. SSCP gel of the *TNFR1* promoter, part 2 (-877 to –526; 352 bp). The band shifts of two different sequence alterations are shown. **A (CT deletion):** Lane 1 represents an individual who is wildtype for this part of the promoter; lane 2, a heterozygote for a CT deletion. **B (-610 G→T):** Lane 1 represents an individual who is wildtype (G/G); lane 2, a heterozygote (G/T). The band shifts recorded are indicated by the black arrows, while the dashed line indicates the wildtype band.

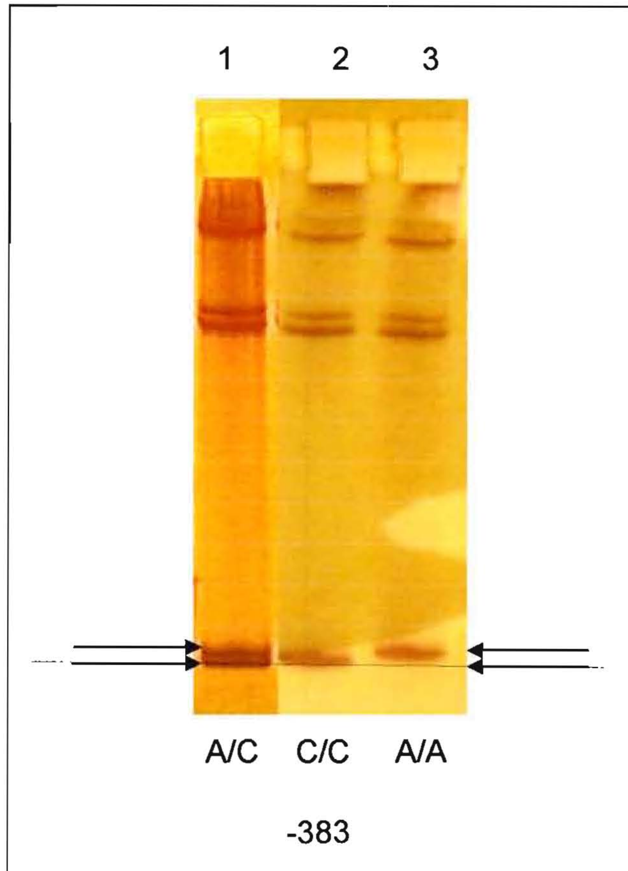

Figure 3.7. SSCP gel of the *TNFR1* promoter, part 3 (-579 to –215; 365 bp). Lane 1 represents an individual who is a heterozygote (A/C); lane 2, a homozygote (C/C); lane 3, wildtype (A/A). The band shifts recorded are indicated by the black arrows, while the dashed line indicates the wildtype band. The gel depicted here is a composite and any shifts other than those indicated are artefactual.

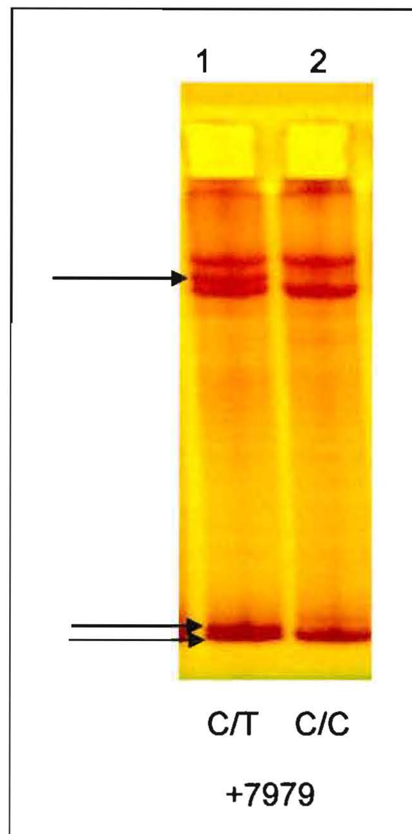

Figure 3.8. SSCP gel of the *TNFR1* exon 3 (+7814 to +8161; 349 bp). Lane 1 represents an individual who is a heterozygote (C/T); lane 2, wildtype (C/C). This alteration represents an amino acid change of a proline to leucine. The band shifts recorded are indicated by the black arrows.

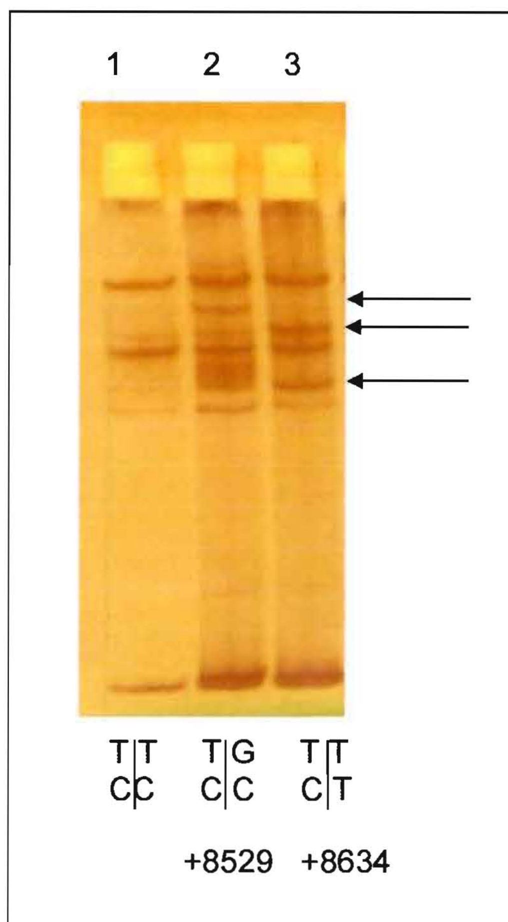

Figure 3.9. SSCP gel of the *TNFR1* intron 4 (+8432 to +8780; 350 bp). The band shifts of two different sequence alterations are shown. Lane 1 represents an individual who is wildtype (T/T) at position +8529 and wildtype (C/C) at position +8634; lane 2, a heterozygote (T/G) at position +8529; lane 3, a heterozygote (C/T) at position +8634. The band shifts recorded are indicated by the black arrows.

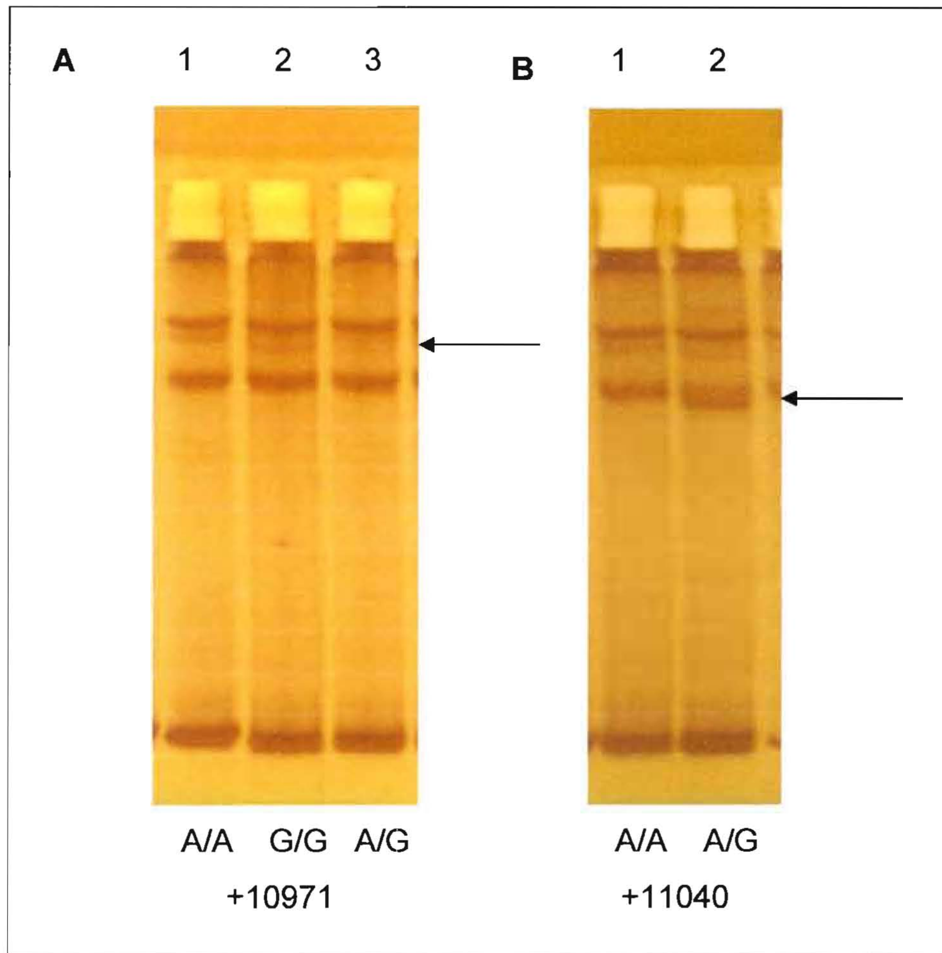

Figure 3.10. SSCP gel of the *TNFR1* intron 6 (+10862 to +11224; 381 bp). The band shifts of two different sequence alterations are shown. **A (+10971 A→G):** Lane 1 represents an individual who is wildtype (A/A); lane 2, a homozygote (G/G); lane 3, a heterozygote (A/G). **B (+11040 A→G):** Lane 1 represents an individual who is wildtype (A/A); lane 2, a heterozygote (A/G). The band shifts recorded are indicated by the black arrows.

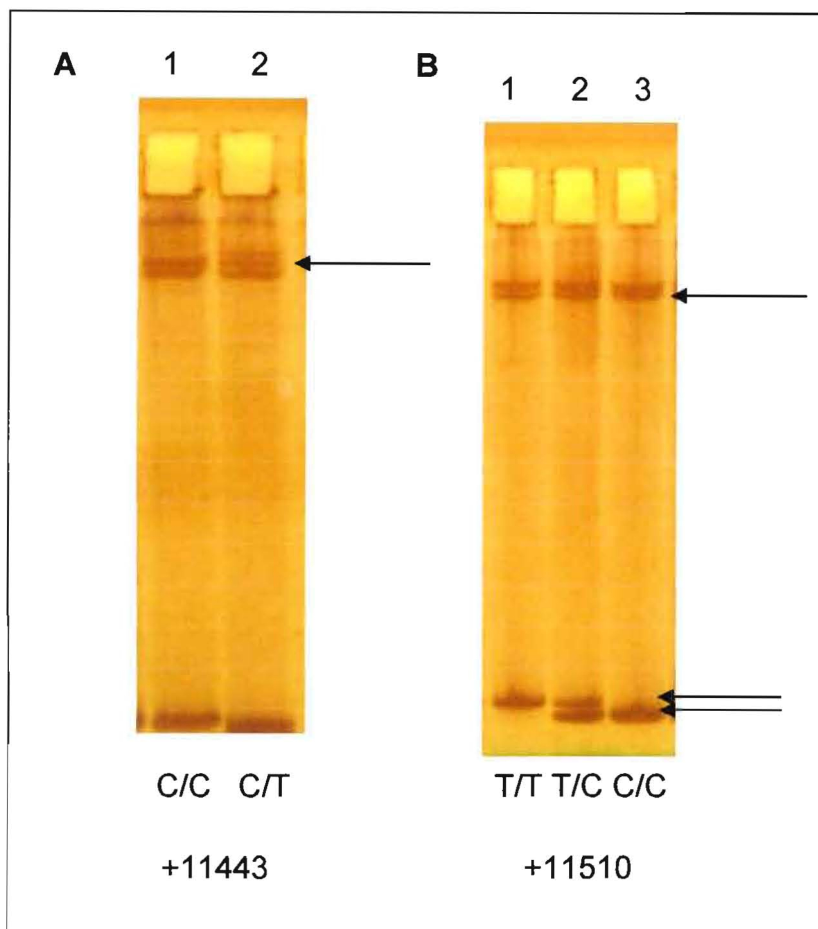

Figure 3.11. SSCP gel of the *TNFR1,* intron 7 (+11201 to +11573; 373 bp). The band shifts of two different sequence alterations are shown. **A (+11443 C→T):** Lane 1 represents an individual who is wildtype (C/C); lane 2, a heterozygote (C/T). **B (+11510 T→C):** Lane 1 represents an individual who is wildtype (T/T); lane 2, a heterozygote (T/C); lane 3, a homozygote (C/C). The band shifts recorded are indicated by the black arrows.

3.3 SEQUENCING ANALYSIS

A total of 20 band shifts were detected by PAGE-SSCP after which 61 samples were sequenced, each in the forward and reverse directions. This section gives the sequencing traces from only the novel SNP's and insertions or deletions detected. A homozygous base change in the DNA of a particular individual is indicated by a full-sized peak of a different fluorophore, while peak size reduction and dual fluorophores indicate heterozygosity. A deletion or insertion, which alters the reading frame, is indicated by an unreadable sequence in the sequencing trace beyond the position of the insertion or deletion. Figures 3.12 to 3.19 illustrate the traces obtained after sequencing of the novel sequence alterations.

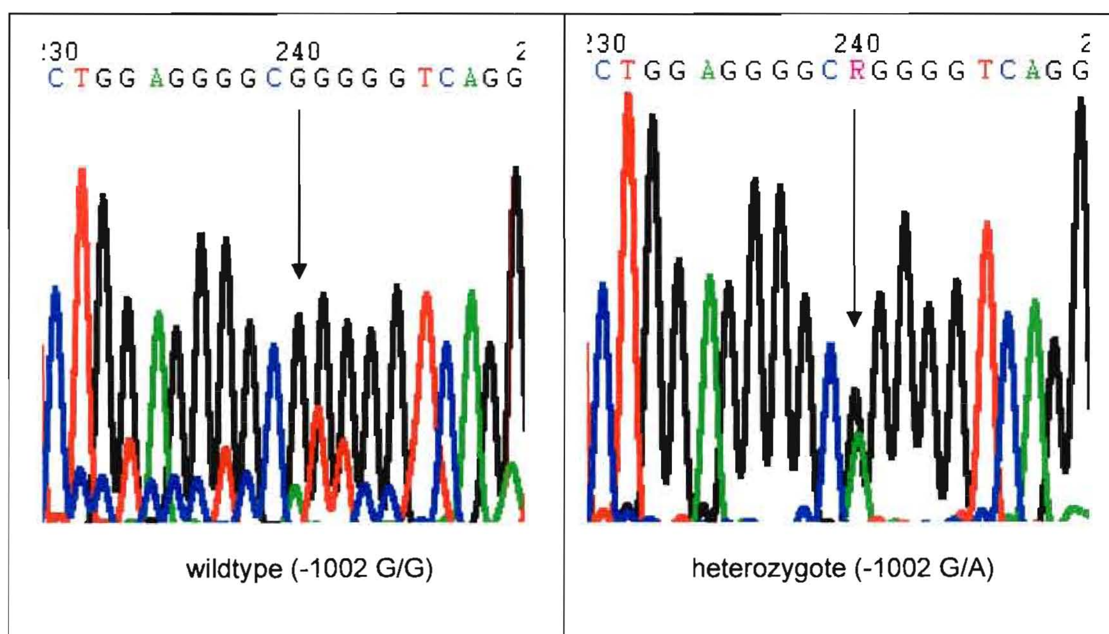

Figure 3.12. *TNF* promoter, part 1 (-1266 to –914): Sections of the forward sequencing traces showing the –1002 G→A transition from PCR products of an individual who is homozygous wildtype (G/G) and an individual who is heterozygous (G/A). Arrows indicate the position of the altered base.

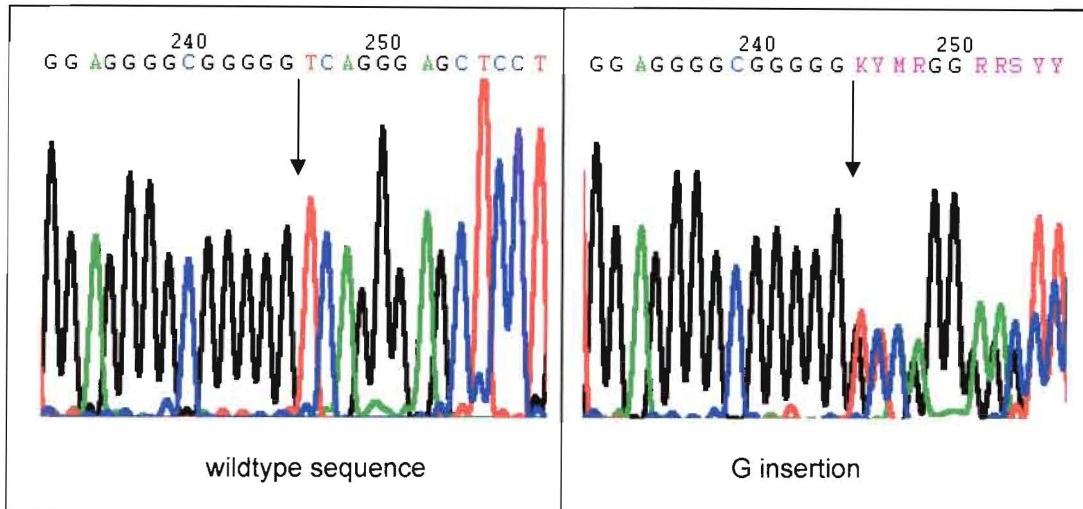

Figure 3.13. *TNF* promoter, part 1 (-1266 to –914): Sections of the forward sequencing traces showing the insertion of a G from PCR products of an individual who is wildtype and an individual who is heterozygous for a G insertion. Arrows indicate the position of the altered base.

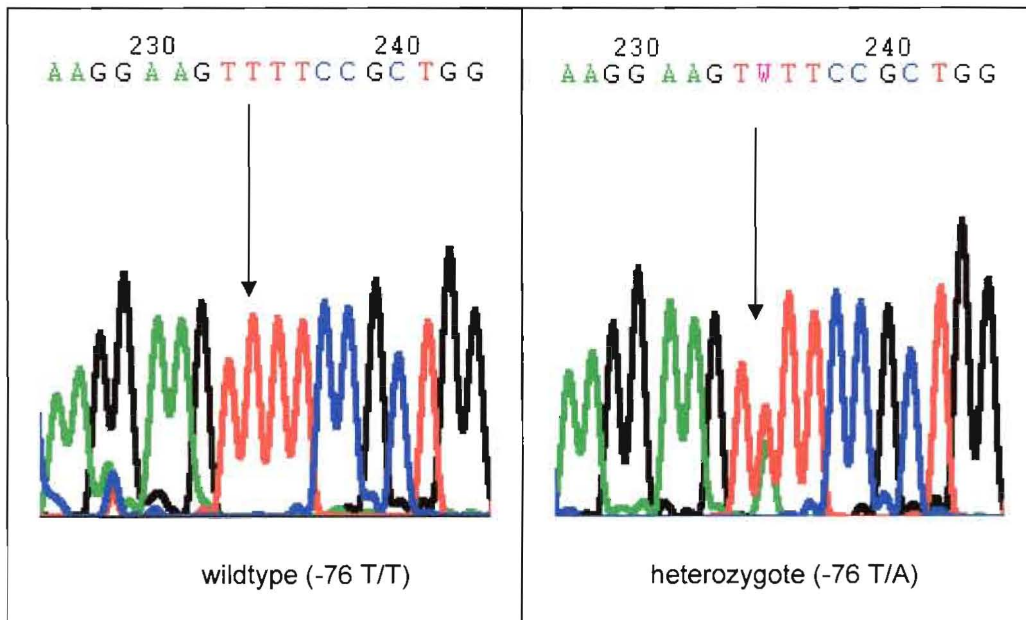

Figure 3.14. *TNF* promoter, part 4 (-335 to +18): Sections of the forward sequencing traces showing the –76 T→A transition from the PCR products of an individual who is wildtype (T/T) and an individual who is heterozygous (T/A). Arrows indicate the position of the altered base.

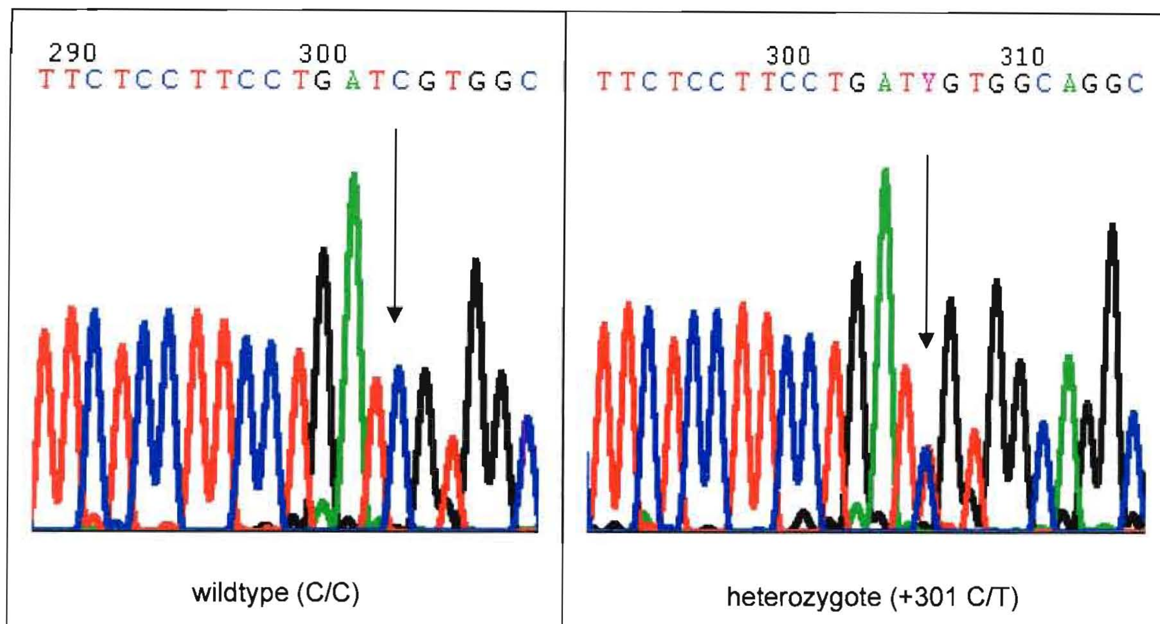

Figure 3.15. *TNF* promoter, part 5 (-25 to +327): Sections of the forward sequencing traces showing the +301 C→T transition from the PCR products of an individual who is wildtype (C/C) and an individual who is heterozygous (C/T). Arrows indicate the position of the altered base. This alteration represents a synonymous base change in the codon for isoleucine.

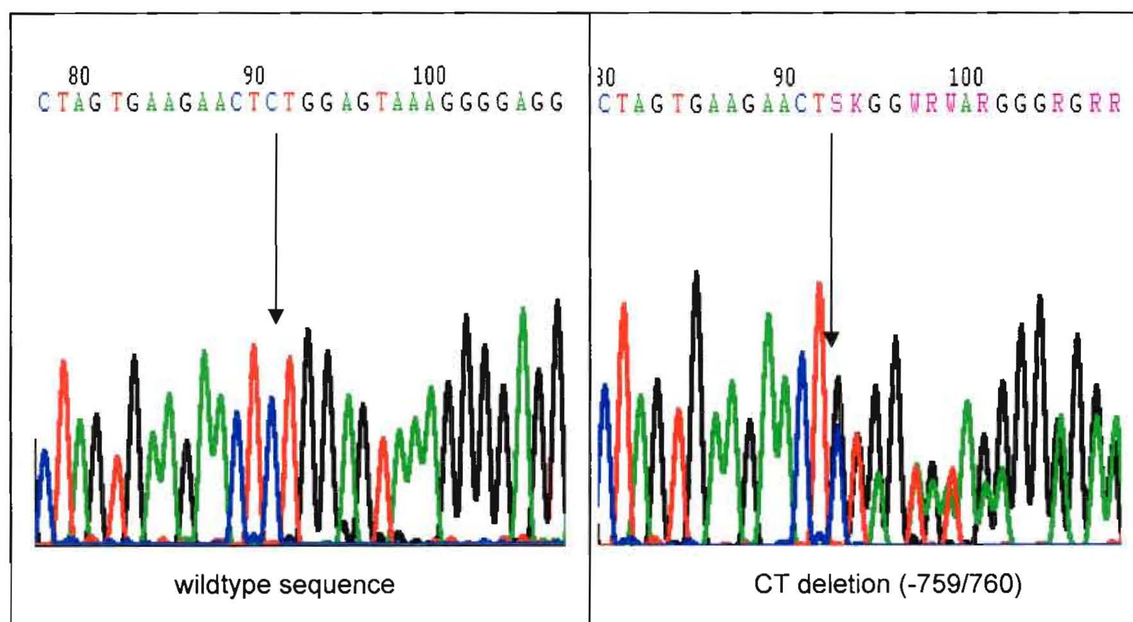

Figure 3.16. *TNFR1* promoter, part 2 (-877 to -526): Sections of the forward sequencing traces showing the –759/760 CT deletion from the PCR products of an individual who is wildtype and an individual who is heterozygous for a CT deletion. Arrows indicate the position of the altered base.

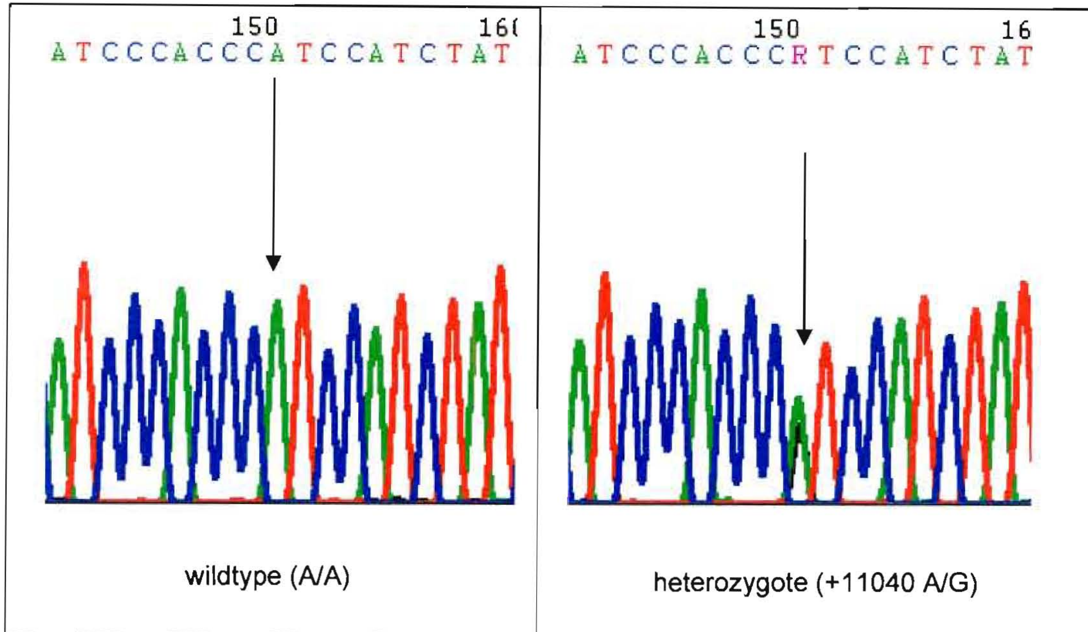

Figure 3.17. *TNFR1*, intron 6 (+10862 to +11224): Sections of the forward sequencing traces showing the +11040 A→G transition from the PCR products of an individual who is wildtype (A/A) and an individual who is heterozygous (A/G). Arrows indicate the position of the altered base.

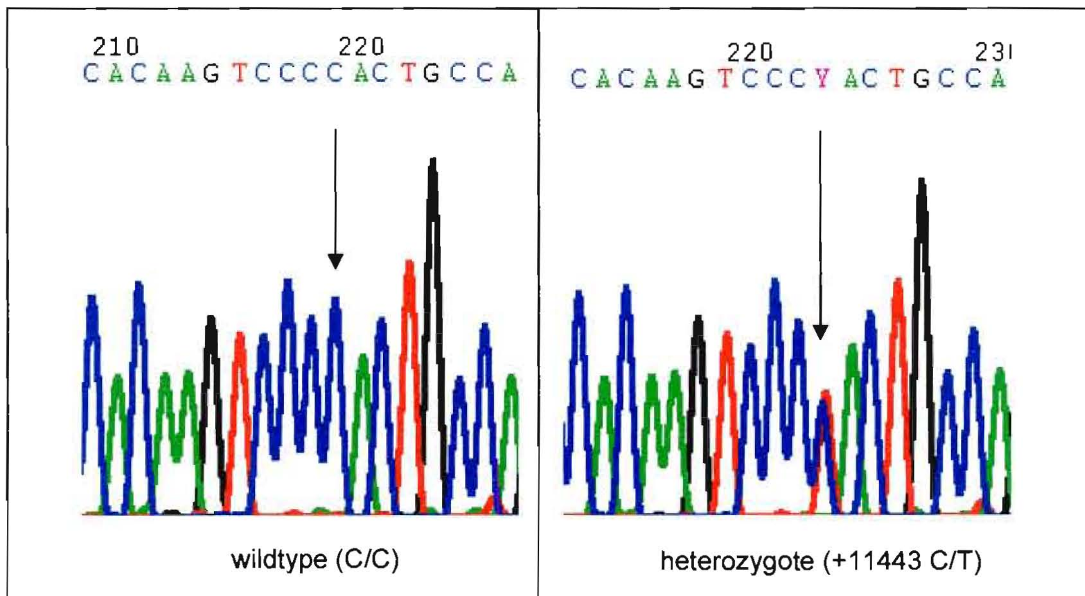

Figure 3.18. *TNFR1*, intron 7 (+11201 to +11573): Sections of the forward sequencing traces showing the +11443 C→T transition from the PCR products of an individual who is wildtype (C/C) and an individual who is heterozygous (C/T). Arrows indicate the position of the altered base.

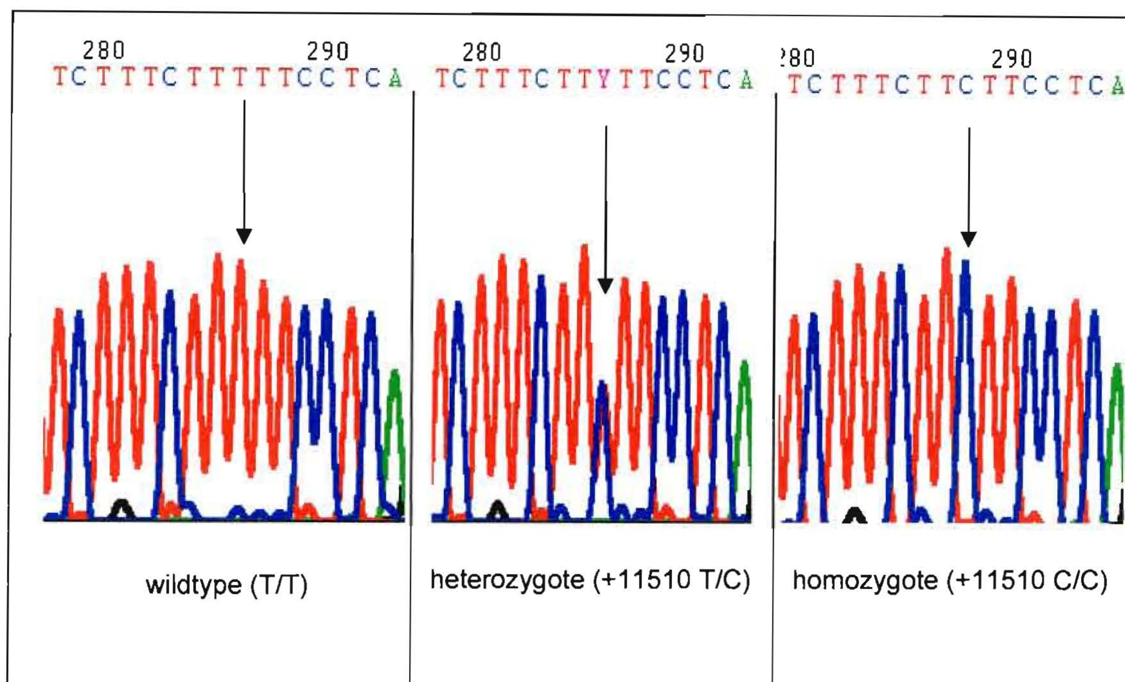

Figure 3.19. *TNFR1*, intron 7 (+11201 to +11573): Sections of the forward sequencing traces showing the T→C transition from the PCR products from an individual who is wildtype (T/T), an individual who is heterozygous (T/C) and an individual who is homozygous (C/C). Arrows indicate the position of the altered base.

Figure 3.20 summarises all the polymorphisms identified in the *TNF* gene in this study.

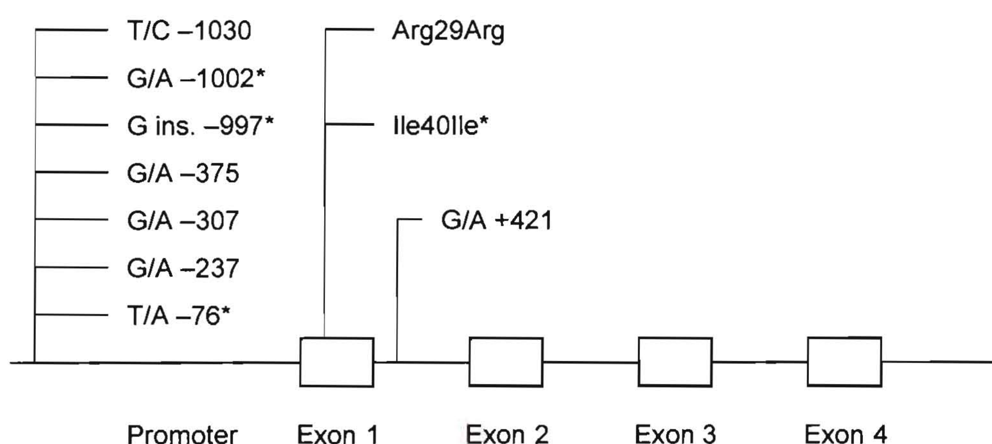

Figure 3.20. Summary diagram of the sequence alterations in the *TNF* gene detected by SSCP analysis and determined by sequencing. * novel sequence changes

Figure 3.21 illustrates the positions of the *TNF* promoter polymorphisms identified in this study.

```
CCCTCGATGAAGCCCAATAAACCTCTTTTCTCTGAAATGCTGTCTGCTTGTGTGTGTGTGTCTGGGA
GTGAGAACTTCCCAGTCTATCTAAGGAATGGAGGGAGGGACAGAGGGCTCAAAGGGAGCAAGAGC
TGTGGGGAGAACAAAAGGATAAGGGCTCAGAGAGCTTCAGGGATATGTGATGGACTCACCAGGTG
                                                        -1030
AGGCCGCCAGACTGCTGCAGGGGAAGCAAAGGAGAAGCTGAGAAGATGAAGGAAAAGTCAGGGT
              -1002   G insertion
CTGGAGGGGCGGGGGGTCAGGGAGCTCCTGGGAGATATGGCCACATGTAGCGGCTCTGAGGAAT
GGGTTACAGGAGACCTCTGGGGAGATGTGACCACAGCAATGGGTAGGAGAATGTCCAGGGCTATG
AAAGTCGAGTATGGGGACCCCCCCTTAACGAAGACAGGGCCATGTAGAGGGCCCCAGGGAGTGA
AAGAGCCTCCAGGACCTCCAGGTATGGAATACAGGGGACGTTTAAGAAGATATGGCCACACACTG
GGGCCCTGAGAAGTGAGAGCTTCATGAAAAAAATCAGGGACCCCAGAGTTCCTTGGAAGCCAAGA
CTGAAACCAGCATTATGAGTCTCCGGGTCAGAATGAAAGAAGAGGGCCTGCCCCAGTGGGGTCTG
TGAATTCCCGGGGGTGATTTCACTCCCCGGGGCTGTCCCAGGCTTGTCCCTGCTACCCGCACCCA
GCCTTTCCTGAGGCCTCAAGCCTGCCACCAAGCCCCCAGCTCCTTCTCCCCGCAGGGCCCAAACA
CAGGCCTCAGGACTCAACACAGCTTTTCCCTCCAACCCCGTTTTCTCTCCCTCAACGGACTCAGCT
                                                            -375
TTCTGAAGCCCCTCCCAGTTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAAGTTAGAAGGAAAC
                                                                -307
AGACCACAGACCTGGTCCCCAAAAGAAATGGAGGCAATAGGTTTTGAGGGGCATGGGGACGGGGT
                                                                     -237
TCAGCCTCCAGGGTCCTACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTCGGAATCGGAGC
AGGGAGGATGGGGAGTGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAAATCCCCG
CCCCCGCGATGGAGAAGAAACCGAGACAGAAGGTGCAGGGCCCACTACCGCTTCCTCCAGATGA
                                  -76
GCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGATTCTTTCCCCGCCCTCCTCTC
GCCCCAGGGACATATAAAGGCAGTTGTTGGCACACCCA+1GCCAGCAGACGCTCCCTCAGCAAGG
ACAGCAGAGGACCAGCTAAGAGGGAGAGAAGCAACTACAGACCCCCCCTGAAAACAACCCTCAGA
CGCCACATCCCCTGACAAGCTGCCAGGCAGGTTCTCTTCCTCTCACATACTGACCCACGGCTTCAC
CCTCTCTCCCCTGGAAAGGACACCATGAGCACTGAAAGCATGATCCGGGACGTGGAGCTGGCCGA
GGAGGCGCTCCCCAAGAAGACAGGGGGGCCCCAGGGCTCCAGGCGGTGCTTGTTCCTCAGCCTC
TTCTCCTTCCTGATCGTGGCAGGCGCCACCACGCTCTTCTGCCTGCTGCAC
```

Figure 3.21. Promoter sequence of the *TNF* gene. The letters indicated in red and labelled with a position number refer to the DNA alterations detected in this study. The underlined sequences indicate consensus sequences for transcription factors, Sp1 in which the –1002 polymorphism is found and Ap2, in which the –307 polymorphism is found.

Figure 3.22 summarises all the polymorphisms identified in the *TNFR1* gene in this study.

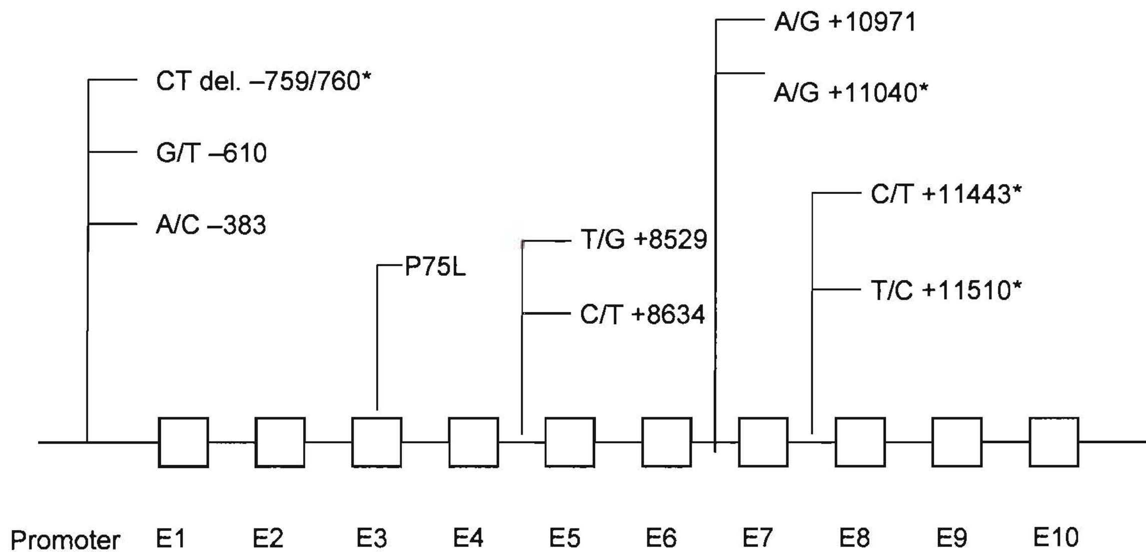

Figure 3.22. Summary diagram of the sequence alterations in the *TNFR1* gene detected by SSCP analysis and determined by sequencing. * novel sequence changes.

Figure 3.23 illustrates the positions of the *TNFR1* promoter polymorphisms identified in this study.

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ACCTAGGGGGTTGTAGTCGATGATCTGTAAGGTGAGTTATAATTGATGTATTGGAATATTTAGGAAA
AGGGCACTGGGAATATGCTAGGAACACCTTGATGGAGGTATCTTTATTTCCACGGCAGCTTCGTGG
ATACGTCTCATTGATTCTCATGGCATCACTTTCCCCATGTAGGTGGGCAGACATTGTTACCCCTGTT
TAATAAACAAGGAACCAACAGAGGCTTAGGAGAGGAGTTGCCTGATGTCGCATGATTGGTGGCAG
AGCCAGGATCAACAGTGGGGCAGGGTGGGGGGACCTGGCCAGGCAGAGAGACTGGATGAGACCT
GGGGTGAGGAATGGCAGGCACCCAGTCAGGGCAGAAAACGAGGGTTGGGACTTACTTTGAGTTTT
                                                           -759/760
GGATTGGATCAGTAAATTCCCAAGAAAGAGGGAGACTAGGAGGCTAGTGAAGAACTCTGGAGTAAA
GGGGAGGATTACTAAGGGACATGGAGTACCTATCATGTGTCGGACGCTTATCTATATCTCTCCCAT
CTGAACAAATCCTTACAGGAACCCCAGGAGACAGGTTATCTCCACTCTGCAAATTGGAAAACAGAT
        -610
CCAGACAGTTTCAGTTATGTGTCTGAGAAGTTCATTTATGTGTCCAAGACACATTCTTAGCTAAAAA
GCTAAGCATTCTGAATTGGAACCCAGAGAATTTGACTCCCAGACTCTGGATCTTTTCACTGCTGTGA
TCCATCTGGGAAAGGCTAGTGATGTGGGCAAGGGGCTTATTGCCCCTTGGTGTTTGGTTGGGAGT
                                               -383
GGTCGGATTGGTGGGTTGGGGGCACAAGGCAGCCAGATCTGGGACTCCTGTGCTTGTGACTGGA
CTACAAAGAGTTAAAGAACGTTGGGCCTCCTCCTCCCGCCTCCTGTGGCCTCCTCCTCCAGCTCTT
CCTGTCCCGCTGTTGCAACACTGCCTCACTCTTCCCCTCCCACCTTCTCTCCCCTCCTCTCTGCTTT
AATTTTCTCAGAATTCTCTGGACTGAGGCTCCAGTTCTGGCCTTTGGGGTTCAAGATCACTGGGAC
CAGGCCGTGATCTCTATGCCCGAGTCTCAACCCTCAACTGTCACCCCAAGGCACTTGGGACGTCC
TGGACAGACCGAGTCCCGGGAAGCCCCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGG
GGGAGTGAGAGGCCATAGCTGTCTGGC+1ATGGGCCTCTCCACCGTGCCTGACCTGCTGCTGCCA
CTGGTGAGACCAGGGACAAAGGGAAGAGTGGGCTGGTGGGCGAGGCACCTTC
```

Figure 3.23. Promoter sequence of the *TNFR1* gene. The letters indicated in red and labelled with a position number refer to the DNA alterations detected in this study.

In order to determine if any of the polymorphisms identified in this study were associated with high or low TNF levels, allele frequencies had to be compared between high and low TNF producers. The following table summarises the frequencies of the polymorphisms detected in the high and low producers of TNF and their *P*-values. There was no functional *in vitro* evaluation of TNFR1 available; allele numbers were therefore compared with TNF levels only.

Table 3.1 Allele frequencies of the *TNF* and *TNFR1* gene polymorphisms detected in this study compared to TNF production

Polymorphism	Frequency in high TNF producers	Frequency in low TNF producers	*P*-value
TNF –1030 T→C	T=0.75 (n=42); C=0.25 (n=14)	T=0.69 (n=59); C=0.31 (n=27)	0.6
TNF –307 G→A	G=0.86 (n=48); A=0.14 (n=8)	G=0.86 (n=74); A=0.14 (n=12)	0.8
TNF +268 G→T	G=0.91 (n=51); T=0.09 (n=5)	G=0.90 (n=77); T=0.10 (n=9)	1.0
TNFR1 –383 A→C	A=0.80 (n=45); C=0.20 (n=11)	A=0.87 (n=75); C=0.13 (n=11)	0.4
TNFR1 +10971 A→G	A=0.75 (n=42); G=0.25 (n=14)	A=0.76 (n=65); G=0.24 (n=21)	0.9
TNFR1 +11510 T→C	T=0.68 (n=38); C=0.32 (n=18)	T=0.77 (n=66); C=0.23 (n=20)	0.3

n=allele numbers

Allele numbers were compared using Chi-squared analysis. A *P*-value of less than 0.05 was considered to be statistically significant. Yate's correction was applied for small numbers. There does not appear to be any association between any of the above polymorphisms and TNF production. The other polymorphisms that do not feature in this table have sample sizes that are too small to calculate a *P*-value. A much larger study would need to be carried out to evaluate any association between TNF production and these rare polymorphisms.

3.4 GENOTYPING OF THE *TNF* –307 PROMOTER POLYMORPHISM

The G to A substitution at position –307 in the promoter region of *TNF* was detected by the ARMS-PCR method, which distinguishes heterozygotes from

homozygotes for either allele at a particular locus. Individuals who are amplified with the wildtype primer are shown in fig. 3.24, lanes 1, 3 and 5. Individuals who are amplified with the variant primer are shown in fig. 3.24, lanes 2, 4 and 6.

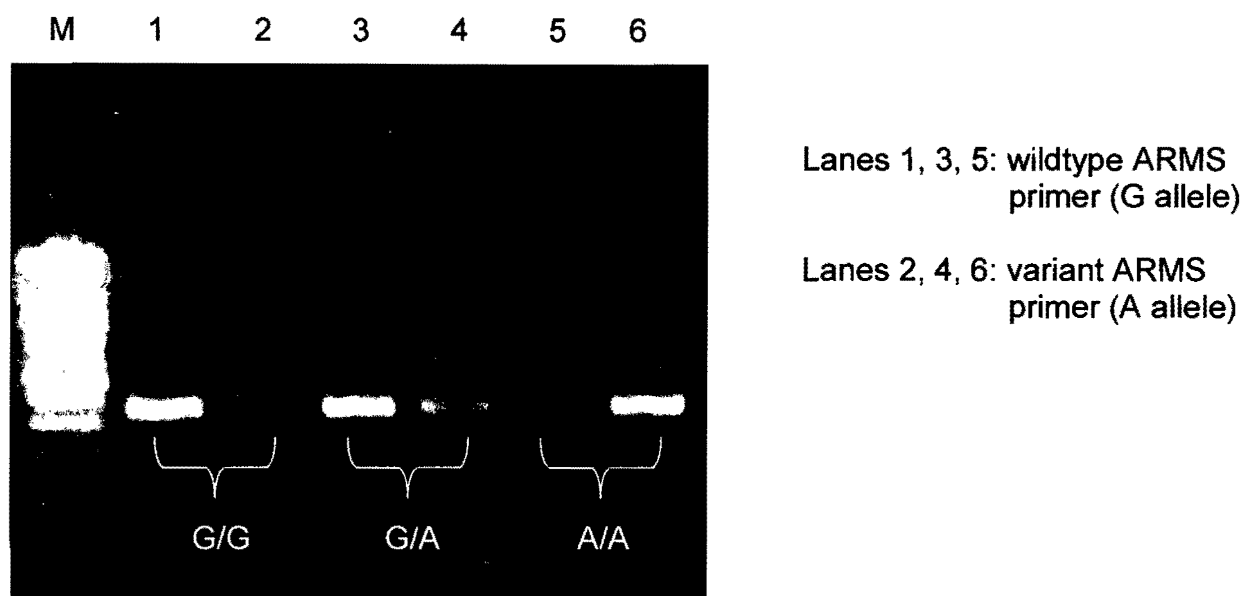

Figure 3.24. Genotyping of the –307 G→A polymorphism in the *TNF* promoter. A 2% (w/v) agarose gel showing a homozygote for the G allele (lanes 1+2), a heterozygote G/A (lanes 3+4) and a homozygote for the A allele (lanes 5+6). Lane M is the marker XIV (appendix B).

Over 500 childhood patients and controls were recruited and immunophenotyped as part of this multi-centre study, after which the DNA was extracted. Most of these subjects come from disadvantaged areas where TB infection is widespread. Due to sample degradation and volume limitation, only 276 individuals were genotyped for the *TNF* –307 G→A polymorphism. The demographics of the patient and control groups for this polymorphism are shown in table 3.2. All the observed frequencies were in Hardy-Weinberg equilibrium, which means that neither of the two loci is under selective pressure; the proportions of homozygotes and heterozygotes in each successive generation will thus remain the same.

Table 3.2 Genotype and allele frequencies in patients and controls for the -307 G→A polymorphism in the *TNF* promoter

Genotype and allele frequency	Healthy controls (N=128)	Patients (N=148)
GG	95 (0.74)	109 (0.74)
GA	31 (0.24)	35 (0.23)
AA	2 (0.02)	4 (0.03)
G/A	0.86/0.14	0.85/0.15

Allele and genotype numbers were compared using Chi-squared analysis. A *P*-value of less than 0.05 was considered to be statistically significant. There were no significant differences in the distribution of this polymorphism between the patient and control subjects when allele numbers (*P*=0.8) and when genotype numbers (*P*=0.8) were compared. Yate's correction was applied (Yate's Chi^2 = 0.027; degrees of freedom = 1).

3.5 GENOTYPING OF THE *IL12 p40* 3'UTR POLYMORPHISM

Two hundred and eighty one patient and control samples were genotyped for the *IL12* +1188 A→C polymorphism. Genotyping of the patient and control cohorts for this polymorphism was carried out by restriction enzyme analysis using the *TaqI* restriction enzyme. The digestion products were electrophoresed on 4% (w/v) agarose gels. An uncut fragment appears as a 301 bp band, while digested products appear as 162 bp and 139 bp bands.

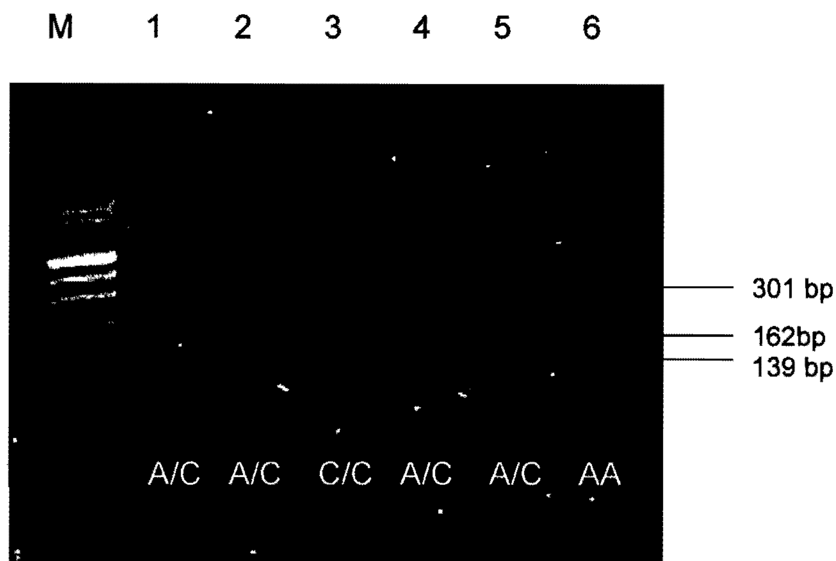

Figure 3.25. Genotyping of the +1188 A→C polymorphism in the *IL12* 3'UTR. A 4% (w/v) agarose gel showing hetrozygotes for the *IL12 p40* polymorphism (lanes, 1, 2, 4 and 5), a homozygote for the C allele (lane 3) and a homozygote for the A allele (lane 6). Lane M is the marker XIV (appendix B).

The demographics of the patient and control groups are shown in table 3.3. All the observed frequencies were in Hardy-Weinberg equilibrium.

Table 3.3 Genotype and allele frequencies in patients and controls for the *IL12* +1188 A→C polymorphism

Genotype and allele frequency	Healthy controls (N=125)	Patients (N=156)
AA	60 (0.48)	78 (0.50)
AC	46 (0.37)	65 (0.42)
CC	19 (0.15)	13 (0.08)
A/C	0.66/0.34	0.71/0.29

Allele and genotype numbers were compared using Chi-squared analysis. A *P*-value of less than 0.05 was considered to be statistically significant. There were no significant differences in the distribution of this polymorphism

between the patient and control subjects when allele numbers (*P*=0.3) and when genotype numbers were compared (*P*=0.2). Yate's correction was applied for small numbers (Yate's Chi^2 = 1.074; degrees of freedom = 1).

Chapter 4:
DISCUSSION

Mycobacterium tuberculosis infection is characterised by diverse outcomes; the majority of infected individuals remain well while others develop disease ranging from limited pulmonary tuberculosis to severe disseminated disease. Host factors are indicated to play an important role in these diverse outcomes, a role which has yet to be fully characterised. Genetic components of susceptibility to tuberculosis have been proposed with elements of the type 1 cytokine pathways being investigated. Players in these pathways include interferon gamma, interleukin-12, their receptors, and TNF and its receptor.

TNF gene polymorphisms have been implicated in the pathogenesis of a number of diseases, infectious and autoimmune, such as cerebral malaria (McGuire et al. 1994; Knight et al. 1999) and rheumatoid arthritis (Kaijzel et al. 2001). Given the strong evidence supporting a role for TNF in mediating protection and pathology in TB, from both murine models and human disease, it seemed appropriate to study our TB cohort for polymorphisms in the *TNF* gene and its receptor.

In an attempt to determine the specific influence of genetic variability in the *TNF* and *TNFR1* genes, this thesis focussed initially on a limited group of patients and controls, selected on the basis of whole blood TNF synthesis in response to non-specific stimulation tests. DNA samples from this selected cohort were screened for polymorphisms by SSCP analysis and cycle sequencing. In subsequent investigations a much larger cohort of TB patients and controls were screened for the frequencies of a polymorphism identified in this study, the *TNF* –307 G→A polymorphism, and an *IL12* 3'UTR A→C polymorphism, in order to evaluate any possible association with paediatric tuberculosis.

This study reports the identification of ten polymorphisms in the *TNF* gene and ten in the *TNFR1* gene, of these, twelve are known while eight are novel.

The six polymorphisms found in the *TNF* promoter, comprise five single nucleotide substitutions and a single G insertion in a run of five G's. For the remainder, two were found in exon 1 and one in intron 1. Three polymorphisms were detected in the *TNFR1* promoter, two of these being single nucleotide substitutions with the third being a dinucleotide deletion. For the remainder, most were intronic, two each in introns 4, 6 and 7. Only one exonic SNP was found in the *TNFR1* gene.

Eight novel polymorphisms were identified. These were the *TNF* –1002 G→A (AY799803), G insertion (AY799804), -76 T→A (AY799805), and +301 C→T (AY799806) promoter and exon polymorphisms, respectively. For *TNFR1* there was the dinucleotide promoter deletion, –759/760 CT (AY799807), and the intronic SNP's, +11040 A→G (AY799808), +11443 C→T (AY799809) and +11510 T→C (AY799810). The numbers in brackets refer to the GenBank numbers for these polymorphisms. PCR, SSCP and sequencing analysis were carried out twice on samples carrying these novel alterations to ensure that the changes were not the result of PCR or sequencing artefacts.

While this study only detected six polymorphisms in the *TNF* promoter, it is of interest that 11 are now known, and indicate a high degree of heterogeneity. There exists some confusion as regards the numbering of several of these polymorphisms, which has its roots in the miss-numbering of the original *TNF* promoter sequence. For example, what has previously been referred to as the *TNF* –308 polymorphism is actually 307 base pairs upstream of the transcription start site (Allen 1999).

Do these polymorphisms have functional significance?

Most studies that have investigated the functional significance of *TNF* promoter polymorphisms have focused on the –307 polymorphism, although the results are highly contradictory. Two studies that looked at the *in vitro* stimulation of TNF production by leukocytes from homozygous wildtype (-307 G/G) and heterozygous (-307 G/A) individuals reported higher TNF production by –307 G/A cells than –307 G/G cells, while four other studies reported no

significant effect, in similar investigations. The conflicting data from these six studies may be due to the use of different LPS concentrations in each as well as the small number of individuals with the –307 G/A genotype studied. This polymorphism has also been investigated by gene reporter assays, again with conflicting results; three reports of the –307A allele having an influence on *TNF* gene transcription and three reports of it not. Reasons given for the contradictions in these studies as well as in other reporter gene approaches include the use of different promoter regions, the type of cell used for transfection and the absence or presence of the 3'UTR. However, collectively the studies suggest that the –307A allele is associated with higher, and not lower, *TNF* gene expression (Allen 1999; Hajeer and Hutchinson 2001). In addition, the –307A allele is in linkage disequilibrium with the HLA-DR3 allele which has been associated with high TNF production, however the high TNF production associated with this DR3 allele may be due to its association with the –307A allele rather than the DR3 allele being responsible for higher TNF production (Allen 1999).

Studies that have investigated other *TNF* polymorphisms for an effect on gene transcription have also had contradictory results. One study on the *TNF* –237 G→A polymorphism has reported lower TNF production from LPS stimulation of whole blood from –237 G/A individuals than from –237 G/G individuals, while another study showed no difference. The *TNF* –375 G→A and –237 G→A polymorphisms have been shown to have no effect on transcriptional activity using a reporter gene assay (Allen 1999). The –375 G→A polymorphism has also been shown to be situated in a region of multiple DNA-protein interactions where the –376 A allele recruits OCT-1 to this region (Knight et al. 1999). In addition, there is support for linkage disequilibrium in this region of the promoter. In Caucasians, for example, the –375 A allele is in allelic association with the –307 G and –237 A alleles. A strong allelic association has also been shown between –237 G, -307 A and –375 G, and this association has been found to encode high TNF production *in vitro* (Hajeer and Hutchinson 2001).

The novel *TNF* –1002 G→A polymorphism occurs within the binding site for the transcription factor Sp1. It has been suggested that Sp1 may be involved in bringing distally bound proteins into the presence of the proximal promoter through DNA looping (Eichbaum et al. 1997). The *TNF* –307 G→A polymorphism occurs within a consensus sequence for an Ap2 transcription factor. However, the actual binding of Ap2 to this promoter element has not been shown (Wilson et al. 1997).

The *TNFR1* promoter is not well characterised and resembles that of a housekeeping gene. The –759/760 CT deletion identified in the *TNFR1* promoter is interesting as it occurs in a region that is thought to contain a negative putative regulatory element. The sequence upstream from –385, until the start of the promoter at position –809, has an inhibitory effect on promoter activity in reporter gene assays (Kemper and Wallach 1993). It may be significant that the CT deletion as well as the *TNFR1* –610 G→T polymorphism are located in this region and either could possibly influence the inhibitory property of this region. Promoter activity has been localised to a CT-rich region running from bases –207 to –385 and again, it may be of significance that the *TNFR1* –383 A→C polymorphism is situated in this region. Therefore, this –383 A→C polymorphism could increase *TNFR1* expression as was confirmed by Nishimura et al. (2003) who observed a significantly increased frequency of the –383 C allele in young-onset (25.6% heterozygotes) (P= 0.0056) and adult-onset diabetes (24% heterozygotes) (P=0.015) patients when compared with controls (11% heterozygotes). The *TNFR1* +7979 C→T polymorphism resulted in the substitution of proline for leucine in exon 3. Sequence variants that involve the removal of proline residues likely remove a distinct kink in the polypeptide chain and likely have an effect on the secondary and possibly tertiary structure of the protein.

Are the polymorphisms identified in this study associated with high or low TNF production?

It is important to evaluate the ability of DNA variants to influence TNF production, with regards to gene regulation and protein production. The *TNF*

–1030 T→C, -307 G→A and +268 G→T as well as the *TNFR1* –383 A→C, +10971 A→G and +11510 T→C (AY799810) polymorphisms were examined by Chi- squared analysis for a possible association with TNF expression. The study cohort comprised 71 individuals with 28 subjects having high TNF levels and the other 43 with low TNF levels, on whole blood stimulation. The polymorphisms mentioned above were the only ones that had sufficiently large enough sample sizes to evaluate. Less frequent polymorphisms identified in this study will need to be evaluated in the larger cohort. *P*-values calculated for the above polymorphisms failed to detect any significant association with high or low TNF production. However, it is interesting to note that two polymorphisms were only present in individuals with low TNF production, namely the *TNF* +301 C→T (AY799806) polymorphism and the *TNF* +421 G→A polymorphism although the number of individuals presenting with these two polymorphisms was very small (<5 individuals out of 71 for each polymorphism). These interesting leads warrant further investigation.

Association of the *TNF* –307 G→A and the *IL12* +1188 A→C polymorphism with *M. tuberculosis* infection

Major genes have been effectively found by linkage analysis; however these studies have limited power to detect modest gene effects. Direct tests of association with a candidate gene locus are more powerful at detecting modest gene effects and this approach has been used extensively with respect to genetic variation at the TNF locus, although familial inheritance patterns are not indicated in association studies. A large number of studies evaluating associations of TNF polymorphisms with a particular disease have found that apparent associations between TNF alterations and disease were confounded by linkage disequilibrium between TNF and HLA markers. Linkage disequilibrium also exists within the TNF locus. Association studies for this region frequently have a small sample size with insufficient power to detect a significant difference (Knight and Kwiatkowski 1999). In the broader context of mapping disease loci, linkage analysis is more powerful for the detection of high-risk alleles than association studies. Association analysis is however, more powerful for the identification of common alleles that result in

modest disease risks. It is easier to recruit large numbers of unrelated affected individuals for association studies than to recruit large numbers of pedigrees with multiple affected individuals, however, the region around a SNP that is shared in unrelated affected individuals is much smaller than the shared region for related individuals. Therefore, in order to be successful in mapping loci through association studies, much higher SNP densities are needed (Carlson et al. 2004).

This thesis selected two of the more interesting polymorphisms for an investigation of possible association with *M. tuberculosis* infection. These were the *TNF* –307 G→A polymorphism and the *IL12* +1188 A→C polymorphisms. At a significance level of 5%, there were no differences in the distribution of these two polymorphisms between the TB patient and control groups. The frequencies of the *TNF* –307G allele in the patient and control groups were 0.85 and 0.86 respectively. The frequencies of the *TNF* –307 A allele in the patient and control groups were 0.15 and 0.14 respectively. These frequencies correlate with those obtained in previous studies of other disorders (Chen et al. 1997; Patuzzo et al. 2000). The frequencies of the *IL12* A allele in the patient and control groups were 0.71 and 0.66 respectively. The frequencies of the *IL12* C allele in the patient and control groups were 0.29 and 0.34 respectively. These frequencies correlate with those obtained in a previous study of type 1 diabetes (Morahan et al. 2001). A factor that could have impacted on these results is the difficulty in obtaining an appropriate control group for association studies in paediatric TB, which is a problem due to the age spectrum of infection. An individual who at the time of this study appeared to be an unaffected control could still go on to develop TB at a later stage in life. This is particularly problematic when selecting childhood controls, since tuberculin skin test-positive healthy children (selected on the basis that they have encountered TB and yet remained healthy) are at risk of developing active disease.

Limitations

The methods employed in this study were effective in detecting a number of polymorphisms, however they are not without their limitations. Factors that could affect the sensitivity of SSCP analysis include the nature of the mutation, the sequence composition of the DNA fragment, the position of the mutation within the amplification product and the influence of the neighbouring bases. There is no theoretical model in SSCP for predicting the three-dimensional structure or mobility shift of single-stranded DNA under given conditions (Liechti-Gallati et al. 1999). The mobility shifted bands detected in this study varied from one to three in a single sample. The major banding patterns seen in the SSCP gels varied from two to five. This is due to the fact that both single and heteroduplex forms of the PCR products are seen on the same gel. Sequence alterations were found to affect both DNA conformers, most often individually but on occasion, band shifts were seen in both regions of the gel. In addition, the number of alterations found in this study is likely to be an underestimate of the changes in the cohort as the SSCP technique used is not 100% sensitive.

Another significant limitation in this study was the sample size screened for polymorphisms and more specifically, the sample sizes for the high and low TNF production groups. Time constraints as well as sample availability played a role in the choice of these sample sizes. The assumption here was that the selected cohort (n=71) would be enriched for polymorphisms impacting on *TNF* gene and protein expression and would be large enough to uncover polymorphisms of major biological importance in the South African population. It is likely that very rare polymorphisms or mutations were not detected as a result. Financial constraints prohibited the sequencing of all samples presenting with a band shift different to the wildtype, therefore only representative samples were sequenced. As a result, additional polymorphisms to those identified may have been missed as one banding pattern may have represented two different polymorphisms, although this would be expected to occur rarely.

Conclusions / future work

In spite of the limitations mentioned above, 20 polymorphisms were identified in the *TNF* and *TNFR1* genes, of which 4 in each gene were novel. What was also interesting to note was that almost half of the individuals investigated presented with a number of different polymorphisms, with one individual being heterozygous for eight of the rare alleles at the polymorphic loci identified in the two genes, while others were wildtype at the loci identified. This suggests that in the African population the *TNF* and *TNFR1* genes may be highly polymorphic and that perhaps it is the presence, or lack, of a number of these polymorphisms occurring together in a particular individual that predisposes him/her to tuberculosis infection.

In order to detect very rare polymorphisms, or in other words mutations, it is necessary to extend this study to the whole cohort. The whole paediatric TB cohort, consisting of over 500 patient and control samples, at the Red Cross Children's Hospital will need to be screened for polymorphisms in the *TNF* and *TNFR1* genes. In addition, the allele frequencies of these polymorphisms can then be compared between the control and patient groups in order to make any conclusions about possible association with TB disease. Functional assays like those mentioned in other studies that employ reporter gene constructs could be carried out to determine any possible functional significance of these polymorphisms. In addition, further investigation of the novel changes, such as the CT deletion in the *TNFR1* promoter, is necessary to understand their relevance to TB.

In conclusion, although the current thesis did not identify polymorphisms with any obvious association with either tuberculosis or TNF production, a number of novel polymorphisms were identified. These may interact with other loci to affect TNF responses and perhaps predispose to tuberculosis. The challenge now remains to identify such interactions.

Electronic-database information

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
(tumor necrosis factor [MIM 191160] and tumor necrosis factor receptor superfamily, member 1A [MIM 191190])

BLAST, http://www.ncbi.nlm.nih.gov/BLAST/

Webcutter 2.0 program http://www.firstmarket.com/cgi-bin/cutter/cut2.html (for restriction digestion)

NCBI, http://www.ncbi.nlm.nih.gov

Chi-test calculator (2x2 table), http://vassun.vassar.edu/~lowry/odds2x2.html
http://members.aol.com/johnp71/ctab2x2.html

Chi-test calculator (2x3 table),
http://www.stat.sc.edu/~west/applets/contable.html

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Appendix A: Primer troubleshooting

2.5.1 Tris-borate EDTA buffer (50x)

108g (0.89M) Tris (ICN Biomedicals Inc., Aurora, Ohio, USA)
55g (0.89M) Boric acid (ICN Biomedicals Inc., Aurora, Ohio, USA)
7.4g (0.02M) EDTA, pH 8.0 (BDH Laboratory supplies, Poole, England)
Make up to 1000ml with dH_2O

2.5.2 Sequencing gel (10%)

25 ml (30% w/v) acrylamide (BDH Laboratory supplies, Poole, England)
15 ml Tris Borate EDTA
31.5g Urea (Promega, Madison, WI, USA)
Add dH_2O to 75ml
Add 300 µl 10% APS (BDH Laboratory supplies, Poole, England)
Add 80 µl TEMED (Promega, Madison, WI, USA)

2.5.3 Silver staining solutions

Fixer solution: 150ml Ethanol (Merck KgaA, Darmstadt, Germany)
15ml Acetic acid (Merck KgaA, Darmstadt, Germany)
Add dH_2O to 1000ml

Pre-treatment solution: 22.5ml Nitric acid (Fluka chemika, Buchs, Switzerland)
Add dH_2O to 1500 ml

Silver solution: 3g Silver nitrate (Merck NT laboratory supplies (PTY) Ltd., Fedsure Park, Midrand, South Africa)
Add dH_2O to 1500ml

Developer: 45g Sodium carbonate (Merck KgaA, Darmstadt, Germany)
0.81ml 37% formaldehyde solution
Add dH_2O to 1500ml

Appendix B: agarose gel formulas

2.6.1 Agarose (Agarose, Roche Diagnostics, GmbH Mannheim, Germany) gel loading dye (6x)

0.25g (0.25% w/v) Bromophenol Blue (Merck KgaA, Darmstadt, Germany)

40g (40% w/v) sucrose (Merck chemicals and laboratory supplies (Pty) Ltd., Gauteng, SA)

4ml (20mM) EDTA, pH 8.0 (BDH Laboratory supplies, Poole, England)

Make up to 100ml with dH_2O

Store at 4°C

2.6.2 Tris-acetate EDTA buffer (50x)

242g Tris (Merck KgaA, Darmstadt, Germany)

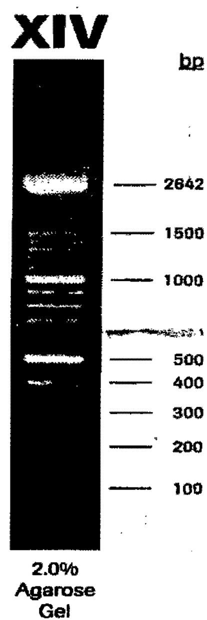
57.1ml glacial acetic acid (Merck KgaA, Darmstadt, Germany)

100ml 0.5M EDTA, pH 8.0 (BDH Laboratory supplies, Poole, England)

Make up to 1000ml with dH_2O

2.6.3 Marker XIV

2μl of agarose loading dye and 3μl dH_2O was added to 3μl of marker (Roche Diagnostics, GmbH, Germany)



Appendix C: SSCP and silver stain formulas

2.7.1 Acrylamide-piperazine diacrylamide (PDA) solution (40%; 99:1)

396g acrylamide (BDH laboratory supplies, Poole, England)

4g PDA (Bio-rad laboratories, Hercules, CA, USA)

Make up to 1000ml with dH_2O

2.7.2 Tris-formate buffer (0.75M)

90.8g Tris (Merck KgaA, Darmstadt, Germany)

Make up to 1000 ml with dH_2O, pH to 9.0 with formic acid (Merck KgaA, Darmstadt, Germany)

2.7.3 Tris-borate buffer (50x)

125.9g Tris (Merck KgaA, Darmstadt, Germany)

17.3g Boric acid (Merck KgaA, Darmstadt, Germany)

Dissolve in 950ml dH_2O, pH to 9.0.

Add 50μl (4% w/v) Bromophenol Blue (Merck KgaA, Darmstadt, Germany)

2.7.4 Glycerol stock (41%)

41ml glycerol (Saarchem (Pty) Ltd. Muldersdrift, South Africa)

59ml dH_2O

2.7.5 SSCP loading dye

47.5 ml (95%) formamide (RdH Laborchemikalien, GmbH and Co.)

0.16g (100mM) NaOH (Merck NT laboratory supplies (PTY) Ltd. Fedsure Park, Midrand, South Africa)

0.125g (0.25% w/v) Bromophenol Blue (Merck KgaA, Darmstadt, Germany)

0.125g (0.25% w/v) Xylene Cyanol (Saarchem (Pty) Ltd. Muldersdrift, South Africa)

Make up to 50 ml with dH_20

2.7.6 Non-denaturing polyacrylamide gel (12%)

5.3ml 40% acrylamide-PDA solution

8.5ml Tris-formate buffer

3ml 41% glycerol (Saarchem (Pty) Ltd. Muldersdrift, South Africa)

Add 200 μl APS (BDH Laboratory supplies, Poole, England)

Add 20 μl TEMED (Promega, Madison, WI, USA)

2.7.7 Silver staining solutions

Solution 1: 1g (0.1% w/v) silver nitrate (Merck NT laboratory supplies (PTY) Ltd., Fedsure Park, Midrand, South Africa)

Add dH_2O to 1000 ml

Solution 2: 15g (1.5% w/v) NaOH pellets (BDH laboratory supplies, Poole, England)

10ml (0.15%) formaldehyde (BDH laboratory supplies, Poole, England)

Add dH_2O to 1000 ml