DIFFERENTIAL EFFECTS OF ANTI-TNF THERAPIES FOR INFLAMMATORY ARTHRITIDES ON IMMUNE RESPONSES TO MYCOBACTERIUM TUBERCULOSIS

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DECLARATION

I, Rachel Byng-Maddick, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Date:

ABSTRACT

Tumour necrosis factor (TNF) antagonists have revolutionised the management of rheumatoid arthritis (RA) and other inflammatory diseases. This success is partly tempered by substantially increased risk of granulomatous infectious diseases, particularly tuberculosis (TB). In this thesis I sought new insights into the mechanisms by which TNF blockade leads to an increased incidence of TB.

In a new analysis of data collected by the British Society of Rheumatology Biologics Registry, I confirmed that anti-TNF therapy leads to reactivation of latent TB infection, rather than increasing the risk of new TB infection, consistent with published literature.

I derived and validated four separate context-specific transcriptional modules representing TNF-inducible gene expression in macrophages, keratinocytes and whole blood. I used these modules to quantify TNF bioactivity in clinical samples from RA patients responding to anti-TNF therapies or treated with methotrexate only. As expected, anti-TNF therapy was associated with attenuated expression of the TNF modules in whole blood following ex vivo stimulation. However, anti-TNF therapy had no effect on TNF module expression and therefore TNF function, at the site of acute cell mediated immune responses in vivo, modelled by the tuberculin skin test. These data are consistent with a model in which anti-TNF therapies do not reach sufficient concentration within tissues to block TNF responses in an acute inflammatory challenge. Rather, my data suggest that anti-TNF therapies mediate their therapeutic and adverse effects by regulating TNF activity at foci of chronic inflammation or by alternative non-canonical pathways.

Finally I tested the hypothesis that anti-TNF therapy may inhibit cellular restriction of mycobacteria in human macrophage cultures. Using an in vitro model of human monocyte-derived macrophages, I established a new method to quantify fluorescent mycobacterial load both inside and outside cells, and showed that TNF blockade in this model did not have a significant impact on mycobacterial growth.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

1 ABBREVIATIONS

ABS   AB serum

ADA   Adalimumab

ALP   Alkaline phosphatase

AM   Alveolar macrophages

Anti-CCP   Anti-Cyclic citrullinated peptide

APC   Antigen presenting cell

AS   Autologous serum

BCG   Bacillus Calmette-Guérin

BSA   Bovine serum albumin

BSRBR   British Society of Rheumatology Biologics Registry

CCL   Chemokine (C-C motif) ligand

CD   Cluster of differentiation

CFA   Complete Freund's adjuvant

CFP10   Culture Filtrate Antigen 10

CFU   Colony forming units

CIA   Collagen induced arthritis

CTLA4   Cytotoxic T lymphocyte-associated antigen

CXCL   Chemokine (C-X-C motif) ligand

DAPI   4', 6-diamidino-2-phenylindole

DMARD   Disease modifying anti-rheumatic drug

DMSO   Dimethyl sulphoxide

ELISpot   Enzyme-linked immunospot assay

ELISA   Enzyme-linked immunosorbent assay

ESAT6   Early secretory antigenic target 6

ESX-1	ESAT6 secretion system
ETN	Etanercept
FACS	Fluoresence-activated flow cytometry
FcR	Fc gamma receptors
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haemotoxylin and eosin
HIV	Human immunodeficiency virus
HV	Healthy volunteers
IFNγ	Interferon gamma
IFX	Infliximab
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IKK	Inhibitor of NFκB complex
IL	Interleukin
INH	Isoniazid
iNOS	inducible Nitric Oxide Synthetase
IQR	Interquartile range
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTBI	Latent TB infection

Mab	Monoclonal antibody – refers to anti-TNF antibody therapies (Adalimumab and Infliximab)
MAP	Mitogen activated protein
M-CSF	Macrophage-colony stimulating factor
MDM	Monocyte derived macrophage
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MTX	Methotrexate
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NHP	Non-human primates
NOD	Nucleotide oligomerisation domain
NPH	Northwick Park Hospital
NTM	Non-tuberculous mycobacteria
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PHE	Public Health England
PPD	Purified protein derivative
PRR	Pattern recognition receptors
QFT	Quantiferon Gold in tube test
RA	Rheumatoid arthritis

RANKL	Receptor activator of NFκB ligand
RD	Region of difference
RF	Rheumatoid factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SLE	Systemic Lupus Erythematosus
SPPL	Signal peptide peptidase-like protease
STAT1	Signal transducer and activator of transcription 1
sTNF	Soluble tumour necrosis factor
TACE	TNF converting enzyme
TB	Tuberculosis
Th	T helper cell
TLR	Toll-like receptor
tmTNF	Transmembrane tumour necrosis factor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNFR-associated death domain
Treg	Regulatory T cell
TST	Tuberculin skin test
TT	Tetanus toxoid
UCL	University College London
UCLH	University College London Hospital

2   PUBLICATIONS

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3 INTRODUCTION

3.1 TUMOUR NECROSIS FACTOR

Tumour necrosis factor (TNF) is a multifunctional cytokine that plays important roles in diverse cellular events; together with the TNF receptor (TNFR) superfamily they are important regulators of immune cell proliferation, survival, differentiation and apoptosis [1-4]. TNF is mostly produced by activated macrophages, monocytes and dendritic cells, but also expressed by neutrophils, natural killer cells, endothelial cells, fibroblasts and activated lymphocytes [5, 6]. Activation of the toll-like receptor (TLR) and other innate sensors by viral, bacterial and fungal products can initiate TNF synthesis. However it is also constitutively transcribed at low levels in the secondary lymphoid organs such as lymph nodes, gut and synovium and produced following antigen recognition by T and B cells [7].

3.1.1 The structure and function of TNF

TNF is a 26kDa protein in monomeric form. It is generated as a transmembrane precursor protein – transmembrane TNF (tmTNF), which is arranged in stable homotrimers. It is proteolytically cleaved by metalloproteinases such as TNF-converting enzyme (TACE), also known as A Disintegrin And Metalloproteinase 17 (ADAM17), to its 17kDa trimeric soluble form (sTNF). The remaining membrane-spanning portion of tmTNF subsequently undergoes intramembrane proteolysis, which promotes the liberation of the intracellular domain for signalling to the nucleus [8]. Intramembrane-cleaving proteases include the signal peptide peptidase-like proteases (SPPLs) – SPP, SPL2a, SPPL2b, SPPL2c and SPPL3. It is likely that the function of these homologues may be to catalyse similar reactions in different cellular compartments [8] (see Figure 3.1).
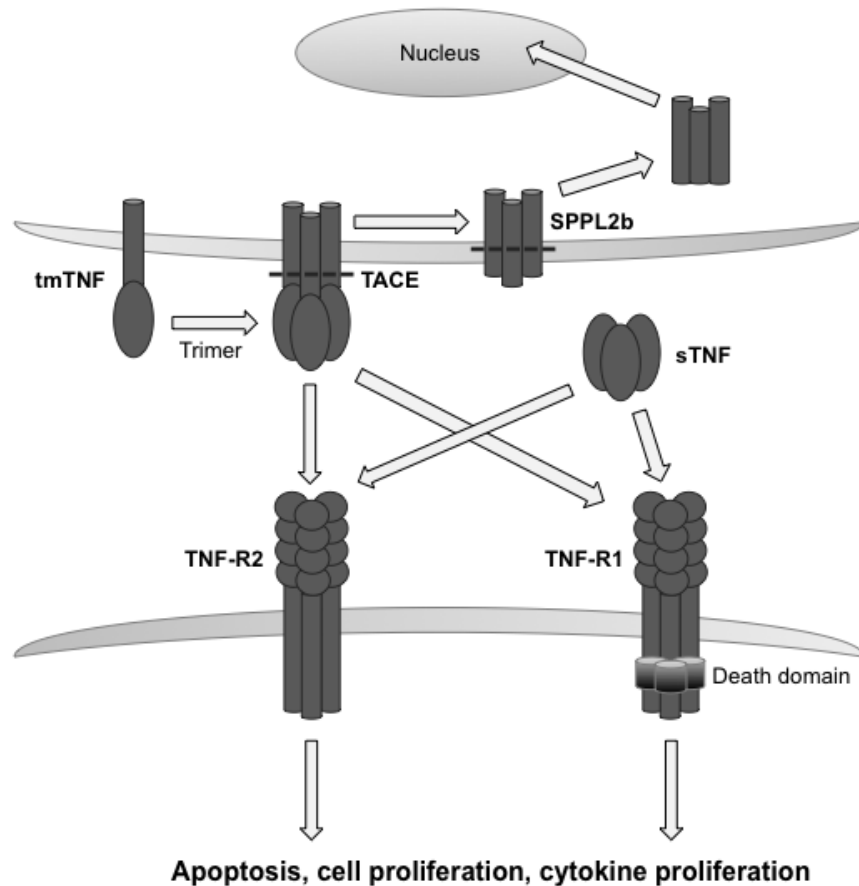
Figure 3.1 Biology of transmembrane TNF and soluble TNF

Transmembrane TNF (tmTNF) is a precursor form of soluble TNF (sTNF) that is expressed on TNF-producing cells as a homotrimer. After processing by TNF converting enzyme (TACE), sTNF is generated and binds to TNFR1 or TNFR2. tmTNF also binds to TNFR1 or TNFR2. Upon binding to TNF receptors, both tmTNF and sTNF mediate pleiotropic effects (apoptosis, cell proliferation and cytokine production). The remaining tmTNF (after cleavage with TACE) is further processed by signal peptide peptidase-like protease 2b (SPPL2b) and the intracellular domain is translocated into the nucleus where it mediates cytokine production.

This figure is adapted from "Transmembrane TNFα: structure, function and interaction with anti-TNF agents" Horiuchi, T *et al* Rheumatology 2010; 49:1215-1228 [9]

Both tmTNF and sTNF are biologically active, exerting their effects via two structurally distinct transmembrane glycoprotein receptors on target cells – TNFR1 (also known as p55) expressed on almost all nucleated cells and TNFR2 (also known as p75), which has a restricted expression to cells of the immune system [10]. Following binding of these receptors, intracellular signalling pathways are initiated leading to gene transcription, which regulates cell proliferation, up-regulation of pro-inflammatory mediators, or apoptosis [1, 6, 11]. sTNF and tmTNF bind both receptors, although TNFR2 can only be fully activated by tmTNF [12]; thus sTNF preferentially binds TNFR1, whereas tmTNF binds to either TNFR1 or TNFR2 [10]. There is some degree of overlapping function and receptor crosstalk, depending on the activation state of the cell, among a host of other factors [13]. Although these receptors are specific for TNF, they are also able to bind lymphotoxin (LT) [7], which is another member of the TNF superfamily that plays an important role in lymphoid organogenesis and may also contribute to effector immune responses [14].

When TNF binds to the extracellular domain of TNFR1, a protein silencer which is bound to the intracellular portion of the receptor, is released [15]. This allows for binding of TNFR-associated death domain (TRADD) and other adaptor proteins on to TNFR1, which then activate key down-stream signalling pathways; Mitogen-activated protein (MAP) kinase signalling transduces a variety of cellular responses including proliferation, differentiation and cell death via the extracellular signal-regulated kinases (ERK), p38 MAP kinases and the cJun NH_2-terminal kinases (JNK) [16]. In addition, Nuclear Factor-κB (NF-κB), a family of transcription factors, is released following degradation of the IκB complex (IKK). NF-κB translocates to the nucleus to induce gene transcription for several pathways [15], including inflammation, cell survival, proliferation and differentiation and is the central mediator of the pro-inflammatory effects of TNF [17]. Following binding of TNF to TNFR1, the "classical pathway" of NF-κB activation occurs, with the p65-p50 heterodimer subunits being most important for transcription. Target genes include cytokines, chemokines, receptors regulating adhesion and migration of cells as well as angiogenesis [18]. TNFR1 activation is also involved in pro-apoptotic signalling via the Fas-associated death domain

(FADD) and caspase pathway. This assembles in the cytoplasm and initiates apoptosis, if the NF-κB signalling does not induce anti-apoptotic proteins [15]. Therefore binding to TNFR1 initiates signalling pathways involved in apoptosis, cell proliferation and activation, and production of pro-inflammatory mediators.

By contrast, TNFR2 signalling involves the activation of NF-κB via TNF-associated factors (TRAF) 1 and 2 and mediates MAP kinase activation to promote cell survival, proliferation and activation [10, 15]. TNFR2 may activate the "non-canonical pathway" of NF-κB, which triggers the p100 subunit to be processed and the generation of p52-containing heterodimers of this transcription factor [19]. The signalling pathways following binding to TNFR2 are less well characterised than TNFR1.

Analogous to the cytokine, both receptors can also be cleaved off the cell surface by the same metalloproteinase that cleaves tmTNF, TACE, and circulate in soluble forms [20]. When in circulation, TNFR are able to control TNF bioavailability and can act as 'decoy' (i.e. non-signalling) receptors for TNF [7, 21, 22].

In addition to binding to different TNFR, sTNF and tmTNF appear to have distinct biological properties. One important characteristic of tmTNF is its ability to act as a receptor that transmits outside-to-inside (reverse) signals back into the tmTNF-bearing cells. Binding of tmTNF to a TNFR or to anti-TNF antibodies, may induce anergy or apoptosis, or act as a costimulatory molecule to induce pro-inflammatory mediators in the tmTNF-bearing cell, depending on the cell type and conditions [23, 24].

3.1.2   The regulation of TNF expression

TNF clearly plays a critical role in inflammation and is a potent modulator of the early inflammatory response to a variety of physical, environmental, infectious and immunological stimuli. Multiple complex regulatory mechanisms control different stages of the TNF pathway, including gene transcription, mRNA turnover, translation and intracellular signalling [25]. The expression of TNF is highly regulated at the transcriptional level by a network of transcription factors, co-regulators and chromatin modifications [26]. The TNF gene promoter, third intron

and enhancer located immediately after the gene contain multiple transcription-factor binding sites that regulate transcription through binding with transcription factor families such as nuclear factor of activated T cells (NFAT), NFκB, E26 transformation-specific (Ets) transcription factor, interferon-regulating factor and CCAAT/enhancer-bing protein [25]. DNA methylation and covalent modification of histones (e.g. phosphorylation, ubiquitination and methylation) influence the chromatin conformation, representing the epigenetic level of the TNF locus regulation [27]. Negative regulation of TNF gene expression also occurs through induction of IκB, which can inhibit transcription of different genes including TNF in a gene-specific manner [28]. Post-transcriptional regulation of TNF activity may also occur via microRNAs (miRNA) binding to cytokine-encoding mRNA, which may trigger translational suppression or activation, or mRNA degradation, thereby influencing TNF production [29].

The TNF gene is activated in response to many stimuli that are characteristic of cellular activation, inflammation, infection and stress. Among these are pattern recognition receptor (PRR) ligands, such as TLR, ligands for antigen receptors (such as the T cell receptor or B cell receptor) and receptors for cytokines, including TNF itself [30] – for a full list, see

Table 3.1.

Controlling TNFR expression and activation may also regulate TNF. Receptor expression (at the mRNA and protein level) is influenced by a number of stimuli including TNF itself and IL1. Other factors, such as lipopolysaccharide (LPS), may regulate receptor levels by stimulating rapid internalisation of TNFRs.

In addition to controlling gene expression of TNF and TNFR, post-translational modification of TNF and TNFR by proteases such as TACE may alter bioavailability and activity. TACE is a transmembrane protein that is constitutively expressed, and its mRNA is found in most tissues [31]. The catalytic activity of TACE is induced by LPS, and downregulated by IL10 or tissue inhibitor of metalloproteinase-3, although the exact mechanism of TACE activation is still poorly understood [31].

Tight control of TNF expression in specific cell types and after specific stimuli is essential for cellular homeostasis and normal physiology in humans, as dysregulated TNF levels are found in multiple disease states including rheumatoid arthritis, Crohn's disease, psoriasis, cardiovascular disease, multiple sclerosis, septic shock and several different forms of cancer [32, 33].

Table 3.1 Inducers of TNF transcription

	Stimuli
PRR ligands	
TLR2	Peptidoglycan (gram positive bacteria), Atypical LPS (*P. gingivalis*)
TLR2/TLR6	Lipoteichoic acid (gram positive bacteria), Diacylated lipoproteins e.g. MALP-2, Zymosan
TLR3	Double-stranded RNA e.g. poly (I:C)
TLR4	LPS (gram negative bacteria), Synthetic lipid A, Taxol
TLR7	Loxoribine
TLR7/TLR8	Single-stranded RNA e.g. poly I, poly C, Imidazoquinoline compounds e.g. imiquimod
TLR9	Bacterial CpG-DNA
NOD2	Muramyl dipeptide
Antigen receptor ligands	
T cell receptor	Anti-CD3, Phytohaemagglutinin (PHA)
B cell receptor	Anti-IgG
Fc receptor ligands	
Mast cell receptor	IgE + antigen
NK cell receptor	Anti-CD16, immune complexes
Other stimuli	
Cytokines	IL1, IL2, IFNγ, GM-CSF, TNF
Mitogens	Concanavalin A, Phorbol 12-myristate 13-acetate (PMA)
Superantigens	Staphylococcocal toxic shock syndrome toxin-1, Staphylococcoal enterotoxin B
Phosphatase inhibitors	Okadaic acid, Calyculin A
Calcium ionophore	Ionomycin
Radiation	UV light, X rays
Osmotic stress	Raffinose
High glucose	
Silica particles	
Bacteria	*Mycobacterium tuberculosis, Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium, Escherichia coli*
Viruses	*Sendai* virus, Human cytomegalovirus, Vesicular stomatitis virus, Herpes simplex virus type II
Protozoans	*Plasmodium falciparum, Trypanosoma cruzi, Schistosoma mansoni*

Adapted from "Transcriptional Control of the TNF Gene" Falvo, J.V. et al Curr Dir Autoimmun 2010; 11: 27-60 [34]

3.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is the most common autoimmune inflammatory arthritis; UK prevalence is approximately 0.5-1% [35] and women are affected three times more commonly than men [36]. It is characterised by florid inflammation of the synovial membrane (synovitis) that leads to tissue destruction and joint damage through cartilage degradation and formation of subchondral bone erosions [37].

Patients with RA display characteristic signs and symptoms, including pain, stiffness and swelling of synovial joints, which are often associated with constitutional symptoms of fatigue, weight loss and low grade fever. Joint disease is usually symmetrical and involves small joints of the hands and feet. The natural progression of RA is typically a destructive deforming arthropathy ending with severe physical disability and multiple co-morbidities, which can be halted or prevented by disease modifying treatment. Diagnosis is classified according to the 2010 American College of Rheumatology / European League Against Rheumatism classification criteria [38]. Disease activity can be measured using the number of painful and swollen joints, the patient's perception of global pain (using a visual analogue score) and a measure of systemic inflammation (using the Erythrocyte Sedimentation Rate or C-Reactive Protein from a blood test).

Current treatment algorithms recommend methotrexate (MTX) as first line or standard therapy. Patients who have continued active inflammatory disease despite MTX are subsequently treated with other non-biological disease modifying drugs (including sulphasalazine, hydroxychloroquine or leflunomide), or biologic therapies such as anti- TNF therapy.

3.2.1 Natural history of rheumatoid arthritis

In RA, the synovial membrane on the inner surface of the joint is characterised by a chronic infiltration of inflammatory cells and hypertrophy leading to synovitis. During the inflammatory process activated mononuclear cells (including T cells, B cells, plasma cells, dendritic cells, macrophages and mast cells) infiltrate the synovium, normally only a few cells thick, and proliferation of synovial fibroblasts

and angiogenesis occur [39, 40]. The synovial lining becomes hyperplastic, and the synovial membrane expands to form villi. Subsequently cartilage and bone destruction occur due to enhanced actions of prostaglandins, leukotrienes and matrix-degrading metalloproteinases (MMPs). The osteoclast-rich portion of the synovial membrane, or pannus, destroys bone, whereas enzymes secreted by neutrophils, synoviocytes and chondrocytes degrade cartilage [39].

RA synovial fluid has been shown to contain a wide range of effector molecules including pro-inflammatory cytokines (such as IL1β, IL6, TNF and IL18), chemokines (such as IL8, CXCL10, CCL2, CCL3 and CCL5), MMPs (such as MMP1, -3, -9 and -13) and metabolic proteins (such as Cox-1, Cox-2 and iNOS). These interact with one another in a complex manner that is thought to cause an escalating cycle of pro-inflammatory signals resulting in chronic and persistent inflammation [41, 42]. TNF is known to stimulate IL1 and IL6 production in synovial tissue, which further enhances the migration of inflammatory cells into the joint and stimulates MMP production in synovial fibroblasts and chondrocytes [43].

3.2.2   Immunopathogenesis of rheumatoid arthritis

Although the precise pathogenesis of RA remains unclear, T cells, B cells, macrophages, neutrophils and synovial fibroblasts are central to the mechanisms of joint inflammation and disease progression [39]. Overexpression of many inflammatory cytokines, including TNF, interleukins and proteinases play an important role in the inflammatory cascade of RA. This results in the formation and perpetuation of a chronic inflammatory synovitis leading to cartilage and bone destruction within the first year of disease.

RA is a heterogeneous disease with aetiology linked to genetic, environmental and stochastic triggers, leading to the loss of the immunological tolerance to self-antigens which is the first step towards developing autoimmune phenomena [44]. It is estimated that genetic factors (heritability) account for approximately 60% of the pathogenesis in studies from Northern Europe, which include class II major histocompatibility antigens/human leukocyte antigens (HLA-DR) and non-HLA genes including several single nucleotide polymorphisms [45]. The contribution of

HLA to heritability of RA has been estimated at 11-37% [45]. These are likely to increase susceptibility to RA, and may have some relevance to disease outcome and prognosis.

In genetically susceptible individuals, specific environmental factors may activate potentially pathogenic immune reactions, including antibody formation. Environmental factors that have been associated with the pathogenesis of RA include smoking [46, 47] and infections [48, 49] including Epstein-Barr virus, cytomegalovirus, proteus species, *Escherichia coli* and even *Mycobacterium tuberculosis* heat shock protein [50]. Other pathogenic bacteria such as *Porphyromonas gingivalis*, caused by periodontal disease [51, 52] and changes to the microbiome are also thought to contribute to disease pathogenesis and formation of antibodies [53, 54].

Approximately 50-70% of RA patients are seropositive for rheumatoid factor (RF), an autoantibody against the Fc portion of an immunoglobulin G (IgG) [55]. This is regarded as the classic autoantibody in RA, which is either immunoglobulin M (IgM) or less commonly an immunoglobulin A (IgA). Another group of autoantibodies specifically associated with RA bind the citrullinated isoform of proteins, and are known as anti-cyclic citrullinated peptide (anti-CCP) antibodies. The role of these antibodies is not entirely understood, but anti-CCP antibodies are thought to bind to citrullinated vimentin on the surface of osteoclasts and induce osteoclast differentiation via autocrine stimulation of TNF, leading to bone destruction [56]. It has been shown that patients with RA and high autoantibody titres have a notably worse prognosis with accelerated joint destruction, co-morbidities such as cardiovascular disease and other extra-articular manifestations, although their direct pathogenic role in humans is not yet clearly identified [57-59].

3.2.3 Immune dysregulation in rheumatoid arthritis

Disturbances of immune pathways, including macrophages, mast cells and natural killer cells contribute to the pathogenesis of RA [49]. Monocyte-derived cells massively infiltrate the synovial membrane where they are responsible for release of pro-inflammatory cytokines and chemokines that contribute to the destruction

of cartilage, bone and tissue within the joint [60-63]. A strong correlation has been seen with the number of macrophages in the synovium, disease activity and degree of erosion [64, 65]. These cells are highly activated, particularly during active inflammatory disease, and display a higher propensity towards the CD14^lowCD16^high phenotype [66, 67]. Moreover elevated levels of TNF and IL1 promote production of macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) from synovial fibroblasts and chondrocytes [68], which in turn influence the differentiation of macrophages. Indeed blockade of GM-CSF or M-CSF may inhibit the development of arthritis [69].

Circulating CD14$^+$ monocytes in RA patients also show signs of activation, with increased expression of CD14, Fcγ receptors (including CD16), CD54 and CD11b [66, 70, 71] and express increased levels of tmTNF [72]. These are thought to contribute to the pathogenesis of RA by producing potent amounts of TNF, IL6, IL1 and MMPs, leading to endothelial cell activation, acute phase reactions and cartilage damage.

It is thought that initially dendritic cells, macrophages and activated B cells act as antigen presenting cells to present autologous antigens to T cells [73]. This leads to the expansion of autoantigen-specific T cells in the joints and lymph nodes which secrete IL2 and IFNγ [74].

B cells also contribute to pathogenesis through the production of autoantibodies and cytokines which may further stimulate the production of pro-inflammatory cytokines including TNF through Fc receptors and complement activation [74]. Activation of B and T cells further stimulate macrophages to produce pro-inflammatory cytokines such as TNF, IL1 and IL6.

Although classically RA was thought to be a Th1 mediated disease, with IFNγ- and TNF-secreting cells acting in the synovium, potential plasticity of T cells and newer T helper subsets such as regulatory T cells and Th17, these are all now thought to play a vital role in disease pathogenesis [75].

There appears to be a relative lymphopaenia in RA, characterised by a reduction in number and function of naïve CD4$^+$ T cells, including repertoire contraction and

hyporesponsiveness to stimulation [76]. Phenotypically RA T cells in the synovium are predominantly effector memory cells with expression of specific chemokine receptors and integrins, suggesting that T cell subsets migrate to the area of inflammation [77].

Regulatory T cells (Treg) have been implicated in the pathogenesis of many autoimmune diseases including RA, systemic lupus erythematosus (SLE) and ANCA-associated vasculitis [78-80]. They are essential for self-tolerance, thus lack or dysfunction of these cells results in a breakdown of immunological tolerance and abnormal immune responses to self-antigens [81]. Treg are characterised by the stable expression of the lineage-specific transcription factor Forkhead Box P3 (FoxP3), which is pivotal for their function and homeostasis [82]. CD4$^+$ Treg are generated both in the thymus and periphery, the latter are typically known as induced Treg. They exert suppressive effects on CD4$^+$ and CD8$^+$ T cells, and also have some control of B cells, natural killer cells, dendritic cells and other antigen presenting cells. The exact mechanism of their regulatory effects is not known, although it is thought to be via regulatory cytokines IL10 and TGFβ, and cell-to-cell contact via cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and the membrane glycoprotein LAG3 [83, 84]. It has also been shown Treg can control B cell responses via a Fas dependent mechanism [85].

In active RA, Treg are unable to suppress Th1 responses including the production of IFNγ [86]. In addition Treg do not effectively suppress B cells in RA [85]. Moreover, B cells (unlike T cells) are resistant to the effects of Treg in RA indicating that restoration of Treg function may not be sufficient to restore tolerance. Potential mechanisms for defective Treg in RA include reduced CTLA4 expression [87, 88], as CTLA4-expressing Treg are able to reduce CD80 and CD86 expression on antigen presenting cells which may in turn inhibit T cell co-stimulation.

It is unclear whether the relative number of Treg in peripheral blood of RA patients is altered. Increased numbers of Treg are seen in RA synovium although they are greatly outnumbered by effector T cells [89, 90]. In animal models of

inflammatory disease, re-establishing the T effector to Treg cell ratio can control autoimmune responses.

T helper 17 (Th17) cells have also been implicated in the pathogenesis of RA, due to their expression of a number of pro-inflammatory cytokines including IL17. IL17 acts in synergy with TNF to induce chemokine and cytokine production from synovial fibroblasts, resulting in destruction of cartilage [91]. Treg and Th17 cells may develop from the same precursors under distinct cytokine conditions [92] and a subset of IL17-producing CD4$^+$FoxP3$^+$ Treg cells can be generated upon polarisation by cytokines such as IL6 [93]. In RA the balance between Th17 cells and Treg is shifted towards an increased number of Th17 cells with less functional Treg [94]. Both rely on TGFβ for their induction, but in the presence of other pro-inflammatory cytokines such as IL6, a Th17 response is favoured.

Treg are likely to play a significant role in preventing and/or reducing the pro-inflammatory state. Restoring the balance and efficacy of Treg may be important in the treatment of diseases such as RA.

3.2.4   The role of TNF in rheumatoid arthritis

Various immune modulators (cytokines and effector cells) and signalling pathways are involved in the pathophysiology of RA. However one key inflammatory cascade involves the overproduction and overexpression of TNF [95], leading to production of other pro-inflammatory cytokines including IL6 which drives persistent inflammation and tissue destruction [73]. TNF is a pleiotrophic pro-inflammatory cytokine that possesses multiple activities including the induction of other pro-inflammatory molecules, such as IL1, IL6, IL8 and the activation of macrophages, neutrophils and eosinophils [96].

TNF is found in high concentrations in synovial fluid and on the synovial membrane of affected joints where it is produced by activated monocytes and macrophages [95, 97-99]. TNFR expression also appears to be upregulated on the synovial membrane of RA joints, especially in areas adjacent to erosions and increased concentrations of shed receptors appears to correlate with disease activity [100, 101]. Activated NFκB transcription factors have also been found in

cultured synovial fibroblasts, human arthritic joints and the joints of animals with experimentally induced RA [17]. Raised levels of circulating TNF are also found in patients with RA [102, 103], although this may not correlate with clinical disease activity due to the transient nature of TNF expression [104].

The role of TNF in inflammatory disease, such as RA is summarised in Figure 3.2.
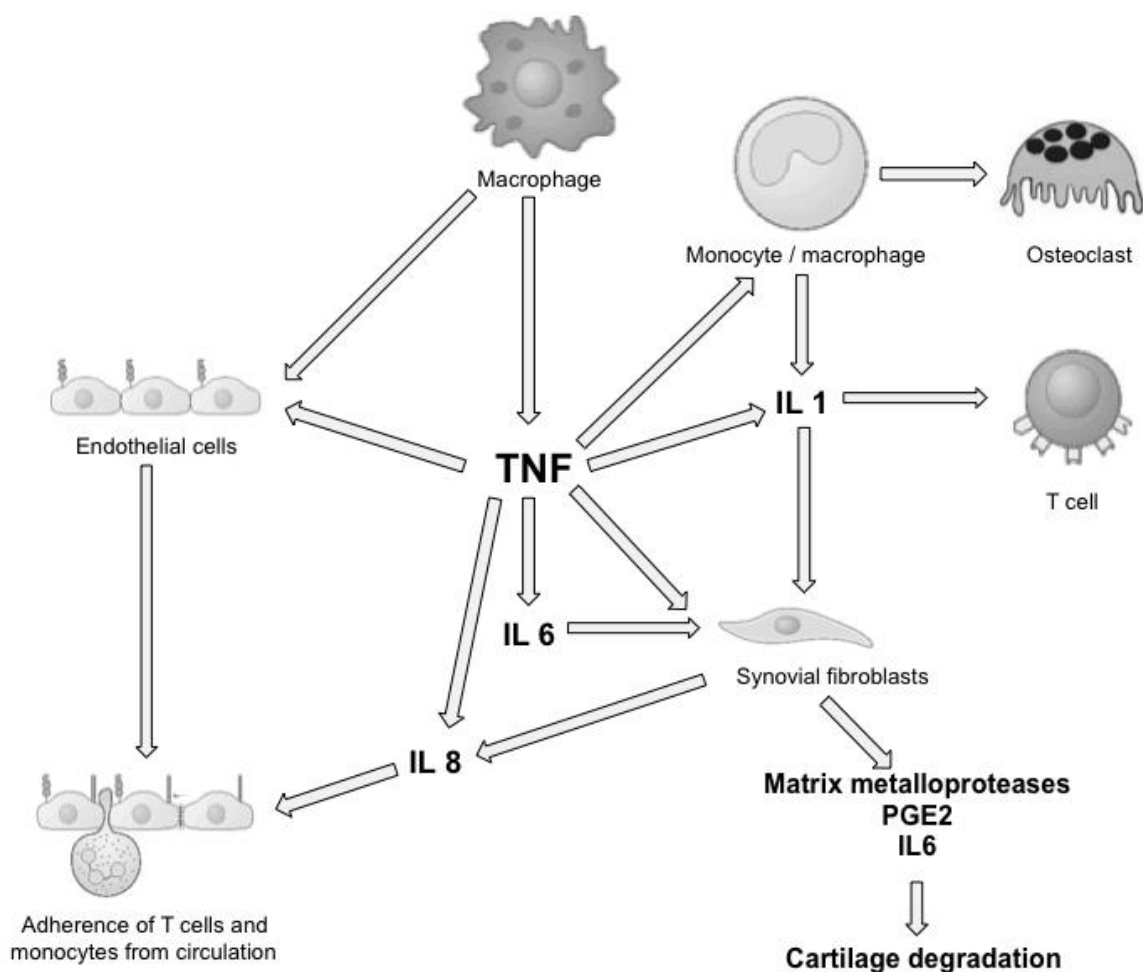
Figure 3.2 The role of TNF in rheumatoid arthritis

Tumour necrosis factor (TNF) plays a central role in the pathogenesis of rheumatoid arthritis. It is produced by activated synovial macrophages, as part of the inflammatory cascade and in turn leads to activation of other cells including monocytes, macrophages, T cells, synovial fibroblasts and endothelial cells.

This figure is adapted from Guzman, F. Mechanism of action, indications and adverse effects of: etanercept, infliximab and adalimumab. http://pharmacologycorner.com; Braun T, Zwerina J. Positive regulators of osteoclastogenesis and bone resorption in rheumatoid arthritis. Arthritis Res Ther 2011; 13:235 [105] and Fox, D.A. Cytokine blockade as a new strategy to treat rheumatoid arthritis: inhibition of tumor necrosis factor. Arch Intern Med. 2000;160:437-444 [106].

In the joint, TNF promotes the activation of synovial fibroblasts and consequently production of connective tissue growth factor. This promotes aberrant activation of osteoclasts and disturbs the homeostasis of the cartilage, ultimately resulting in destruction of the joint [5]. TNF primes for, or directly mediates, leucocyte activation, adhesion and migration, endothelial activation and angiogenesis, nociception, chemokine expression, stromal-cell activation, chrondrocyte activation and with receptor activator of NFκB ligand (RANKL), and contributes to the activation and effector function of osteoclasts [107]. It also increases endothelial layer permeability and expression of adhesion molecules and induces acute phase reactants and tissue-degrading enzymes produced by synoviocytes and chondrocytes in the joint [1].

Evidence for a pivotal role of TNF in the pathogenesis of inflammatory arthritis came from mice that had been genetically engineered to over-express human TNF, who developed a spontaneous inflammatory arthritis with synovial hyperplasia and destruction of cartilage and bone [108]. In humans, increased expression of TNF is found in many inflammatory diseases such as psoriasis, ankylosing spondylitis, juvenile idiopathic arthritis and inflammatory bowel disease. Inhibiting TNF in these diseases has substantially improved the outcome and clinical course of the disease [109].

Mouse models of collagen-induced arthritis (CIA) have been studied to further understand the autoimmune model and pathogenesis of RA. Immunisation of genetically susceptible strains of mice with an emulsion of complete Freund's adjuvant (CFA) and type II collagen induces an inflammatory arthritis with synovitis and erosions, histologically resembling RA [110]. Both T and B cell activation is important in this model of CIA. Cytokines of both Th1 (IFNγ secreting) and Th2 cells (IL4 secreting) are produced, and at disease onset a Th1 profile predominates. Passive addition of TNF, IL1 or transforming growth factor β (TGFβ) can enhance the expression of this autoimmune arthritis [111, 112]. Furthermore in this CIA model, neutralising TNF with a monoclonal antibody can ameliorate the inflammatory arthritis [112, 113]. It has been shown that early treatment with anti-TNF antibodies reduced cartilage destruction, consistent with

its anti-inflammatory effect, but at later stages with more established disease, no protection could be seen [114].

In addition, transgenic mice overexpressing human TNF rapidly and spontaneously develop severe chronic inflammatory polyarthritis, which is independent of effects from B or T cells [108, 115]. Subsequent treatment of these mice with anti-TNF antibody completely prevents disease, suggesting direct involvement of TNF in the pathogenesis of arthritis [108]. Furthermore in adult mice, sustained exposure to repeated injections of recombinant murine TNF led to attenuated T cell recall responses to specific antigens, including cytokine responses and proliferation [116]. This suggests prolonged TNF exposure alters signalling thresholds over time, leading to attenuated T cell receptor signalling [116]. This effect was particularly striking with respect to IL2 production, but suppressive effects for T cells differentiating in vivo or in vitro along with either Th1 or Th2 pathway were also noted [117].

3.2.5 Disease modifying treatment in rheumatoid arthritis

The objective of RA treatment is to control the clinical symptoms and slow or stop the radiographic progression and structural damage to improve function and quality of life. Traditional disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate (MTX), remain the basis of RA therapy, and should be initiated early in the course of the disease [118].

The DMARD era began in 1929 when gold compounds were used for the treatment of RA, after they had been used in the treatment of TB, based on the notion that RA and TB were linked [119] Robert Koch used gold cyanide to successfully kill mycobacteria in vitro, but results were not as promising in vivo. MTX – now first line therapy, was first used in the 1940s for the treatment of cancer, however in 1998 the Food and Drug Administration approved its use in the treatment of RA. The precise mechanism of MTX in inflammatory arthritis is unclear. It inhibits dihydrofolate reductase, which prevents de novo pyrimidine and purine synthesis, required for DNA and RNA syntheses. Consequently, there is inhibition of cellular proliferation of lymphocytes which are involved in the inflammatory process

[120]. T-cell activation and apoptosis are inhibited and expression of T-cell cytokines and adhesion molecules are altered [121].

In the last few decades, advances in the understanding of the pathogenesis of RA and the inflammatory cascade has led to the introduction of biologic DMARDs, a class of drugs that further improve clinical outcomes. These agents work by selective blockade of certain molecules of the inflammatory cascade, resulting in a significant reduction of inflammation. They are mostly used in patients who fail, or are unable to tolerate, traditional DMARDs, however MTX co-prescription is recommended when using most biological DMARDS [122, 123].

Available biologic therapies include TNF inhibitors (infliximab, etanercept, adalimumab, golimumab and certolizumab), which are currently the most commonly used biologic agents. Other licenced biologic therapies include anakinra (IL-1 receptor antagonist), abatacept (cytotoxic T-lymphocyte-associated antigen 4 immunoglobulin), rituximab (chimaeric anti-CD20 antibody) and tocilizumab (humanised IL-6 receptor antagonist).

Newer therapeutic targets for RA include small molecule inhibitors to janus kinase (broadly inhibits cytokine activity) and Syk (blocks activation of TNF-induced cytokine and MMP production from synoviocytes), and activation of regulatory T cells (BT-061) to restore immune homeostasis with quantitative and qualitative impairment described in autoimmune diseases [124-128].

Other less successful immune specific targets in the treatment of RA have included blockade of IL17A [129], B-lymphocyte stimulator (BLyS) and A PRoliferation-Inducing Ligand (APRIL) inhibitors – cytokines involved in regulation of B-cell maturation, proliferation, function and survival [130], and inhibition of p38 MAP Kinase – thought to play a key role in the regulation of pro-inflammatory cytokine production [131].

3.2.6 The development of anti-TNF therapy and its clinical applications

Anti-TNF agents have been successfully introduced for the treatment of several chronic inflammatory diseases, including RA, psoriasis, ankylosing spondylitis and inflammatory bowel disease. There are currently five licensed TNF inhibitors,

which have differences in their structure, function and administration routes (see Table 3.2). However they all share a common molecular mechanism – to competitively inhibit the binding of TNF to its cognate receptors.

The chimeric antibody infliximab (Remicade; Centocor Ortho Biotech) was the first TNF-directed drug to be approved in August 1998. This was followed by the approval of the TNFR2 Fc fusion protein etanercept (Enbrel; Amgen/Pfizer) in November 1998. The first fully human antibody, adalimumab (Humira; Abbott), was approved in December 2002, certolizumab pegol (Cimzia; UCB), a pegylated Fab fragment, was approved in April 2008 and another fully human antibody, golimumab (Simponi; Centocor Ortho Biotech), was approved in April 2009. Studies have shown that they are all highly effective in reducing joint damage and disability, and improving quality of life, especially when used in combination with methotrexate [132-134].

Interestingly a fusion protein formed with the TNFR1 (Onercept; Serono) was not shown to be as effective as etanercept (TNFR2 fusion protein) in psoriasis, psoriatic arthritis or RA and was associated with several cases of Systemic Inflammatory Response Syndrome (SIRS) during phase III trials [135]. No infectious aetiology was identified in these cases, suggesting that onercept had caused the SIRS reaction. It was therefore withdrawn from further clinical use.

Although all anti-TNF therapies are effective against RA, not all are efficacious in granulomatous diseases such as Crohn's disease, Granulomatosis with polyangiitis or sarcoidosis suggesting different structures confer different mechanisms of action [6, 41, 136]. It is likely that each drug binds to similar, but not identical epitopes on the TNF molecule, although these have not been clearly identified yet [137]. Etanercept occupies the receptor binding site on TNF preventing TNF-TNFR interaction [138]. Monoclonal antibodies adalimumab and infliximab also bind the TNF-TNFR interface – adalimumab binds extensively, whereas infliximab only partially binds to this site (although sterically hindering the ability of TNFR to form stable complexes with TNF) [139].

Etanercept also binds lymphotoxin, in addition to TNF, although it is not clear if the additional binding of lymphotoxin confers any therapeutic advantage [140].

Table 3.2 Structure and properties of anti-TNF therapies

	Structure	Half-life	Administration	Properties
Infliximab	Chimaeric IgG1 monoclonal antibody (human Fc and mouse Fab)	8-10 days	Intravenous injection every 4-8 weeks	Each infliximab molecule can bind 2 TNF molecules, and up to 3 infliximab molecules can bind to each TNF homotrimer Able to induce apoptosis via complement dependent lysis and antibody dependent cytotoxicity Binds monomer and trimer forms of sTNF Forms stable complexes with sTNF, can also bind tmTNF
Adalimumab	Fully humanised IgG1 monoclonal antibody	10-14 days	Subcutaneous injection every fortnight	Able to induce apoptosis via complement dependent lysis and antibody dependent cytotoxicity Binds sTNF and tmTNF Derived by phage display, that is specific for human TNF
Etanercept	Recombinant human fusion protein of 2 TNFR2 and Fc portion of human IgG1	3.5 days	Subcutaneous injection every week	Forms relatively unstable complexes with sTNF (with low affinity) Unclear whether can induce apoptosis Only able to bind trimer form of sTNF (not monomers) Forms a 1:1 complex with the TNF trimer Binds mainly sTNF and LT
Golimumab	Fully humanised IgG1 monoclonal antibody	12-15 days	Intravenous or subcutaneous injection monthly	Derived from TNF-immunised transgenic mice, engineered to express human IgG Able to induce apoptosis via complement dependent lysis and antibody dependent cytotoxicity Binds sTNF and tmTNF – binds sTNF more avidly than other monoclonal antibodies
Certolizumab	Fully humanised Fab fragment conjugated to polyethylene glycol (PEG)	14 days	Injected subcutaneously every fortnight	Because of absence of Fc portion, unable to induce complement dependent lysis or antibody dependent cytotoxicity

The exact mechanism of the anti-TNF therapies and modulation of immune regulation has not been clearly delineated. In patients with Crohn's disease, treatment with infliximab induces apoptosis of monocytes and activated T cells in vivo and in vitro, which is not seen with etanercept or certolizumab [141-143]. However both etanercept and infliximab induce apoptosis in monocytes and macrophages in synovial fluid and of monocytes in peripheral blood in vivo [144]. Moreover anti-TNF antibody therapy also reduces the number of infiltrating synovial granulocytes and macrophages in the joint as well as reduced expression of chemokines, IL8 and CCL2 [145]. In addition tmTNF is able to transmit signals back into the monocyte or macrophage, which may be activated by binding to its receptor or anti-TNF antibody therapy [146].

Infliximab also readily binds tmTNF; in vitro this may lead to cell lysis via complement-dependent cytolysis and antibody dependent cell-mediated cytotoxicity [147], whereas etanercept forms less stable complexes with tmTNF. This effect can be associated with transient leucopaenia, and could have more profound effects on cellular subsets. In a study of RA patients treated with adalimumab, peripheral blood showed a significant reduction in CD4$^+$ and CD8$^+$ producing IFNγ cells, four months after starting treatment [148]. However, this finding differs from previous studies, which have suggested that attenuated Th1 responses are restored by anti-TNF therapy [149]. Infliximab is also able to restore CD28 expression on activated CD4$^+$ T cells, which may lead to dampened sensitivity of co-stimulatory signals in T cell responses [150].

In RA, CD4$^+$CD25^{hi} FoxP3$^+$ Treg which usually comprise 5-10% of the CD4$^+$ T cell population, have impaired function and are outnumbered by pathogenic effector cells [86]. Following treatment with infliximab a novel population of Treg cells expressing low levels of CD62L were identified [151], mediating suppression of T effector cells via TGFβ and IL10, resulting in reduced IFNγ production [151]. In patients clinically responding to adalimumab, a higher percentage of FoxP3$^+$ cells have been seen, with restored regulatory function. These cells are able to suppress and resist conversion to Th17 cells by control of monocyte-derived IL6 production

[94]. However in patients responding to etanercept, Treg cell number and function remain similar to patients with active RA. A recent study has also demonstrated that when anti-TNF antibody therapy binds to tmTNF on monocytes, it leads to enhanced expression of tmTNF [152, 153], which subsequently drives expansion of regulatory T cells by binding to TNFR2 on their cell surface [153].

Anti-TNF therapy may also have an effect on T effector cells. It is thought that protein kinase B (PKB)/c-Akt hyperactivation in inflammatory T cells contributes to T effector unresponsiveness to suppression in juvenile idiopathic arthritis [154]. PKB/c-Akt activation positively regulates pro-inflammatory cytokine production such as TNF and IL6 [155], and in addition TNF can induce PKB/c-Akt activation [154]. Therefore using anti-TNF therapy to block this positive feedback loop may explain why etanercept is able to reverse the resistance of T effector cells to suppression, thereby re-establishing Treg mediated control of CD4$^+$ and CD8$^+$ T cells [156]. In addition, another study showed that 4-8 weeks after anti-TNF therapy, Th1 prevalence was higher than baseline in patients treated with etanercept and infliximab, whilst it was stable in the adalimumab group [157]. Th2 prevalence was higher in the anti-TNF antibody therapy groups, but remained stable in the etanercept group. Similarly Treg numbers increased, but Th17 cells remained unchanged [157]. This alteration in T cell subsets may lead to a more favourable balance of pro- and anti-inflammatory cytokines following anti-TNF therapy.

Despite their clinical success and limited understanding of the mechanisms of action, variable levels of soluble TNF receptor, serum or synovial TNF levels have been seen in patients subsequent to anti-TNF therapy [158-161], which do not necessarily correlate to a clinical response to treatment. This may be due to inadequate dosing of the drug or the presence of neutralising antibodies directed against anti-TNF antibody therapy [158]. However infliximab and etanercept have been shown to reduce circulating levels of other pro-inflammatory cytokines, such as IL6 [162, 163].

Increased vascularity and angiogenesis in the synovium of rheumatoid joints is associated with a large mononuclear cell infiltration and is thought to promote

delivery of cells and nutrients to the invading pannus [164]. TNF is known to increase vascular endothelial growth factor (VEGF) expression, and increase adhesion molecule expression on endothelial cells thus promoting angiogenesis, and stimulating the proliferation and migration of endothelial cells [145, 165]. However another role of anti-TNF therapy may be to reduce neovascularity within the joint, as infliximab is able to reduce inflammatory cell migration into RA joints [145], and certolizumab inhibits TNF dependent leucocyte adhesion and angiogenesis via inhibition of chemokine secretion and angiogenic adhesion molecule expression [165].

This suggests that TNF inhibition at the site of inflammation leads to the interruption of the cascade of inflammatory cytokines, which in turn is associated with the improvement in clinical status of these patients.

3.2.7 The use of gene expression profiling to assess response to therapy in RA

Gene expression profiling has been used extensively in RA. Initially it demonstrated that the transcriptome of synovial tissue could differentiate between RA and other diseases including SLE, ankylosing spondylitis or psoriatic arthritis and healthy controls [166, 167]. Groups of genes associated with RA have identified adaptive inflammatory response-related genes, bone and cartilage degradation enzymes and transcription factors, particularly the signal transducer and activator of transcription 1 (STAT1) pathway [168] and can differentiate between the high and low inflammation in the synovium [169].

As approximately a third of patients do not have a clinical response to anti-TNF therapy, various groups have used gene expression profiling to predict those who will respond to treatment, using either monocytes [170], peripheral blood [171-174] or synovial tissue [175-178]. In responders to etanercept treatment one study identified a set of genes involved in NFκB and cAMP signalling after 72 hours (including NFKB1A, IL1B and TNFAIP3) [174]. Changes to the peripheral blood transcriptome were also seen four weeks after treatment with infliximab, with a downregulation of genes involved in inflammation, angiogenesis, T- and B-cell activation, which is thought to represent a dampening of TNF-associated genes

[172]. This was observed in all patients irrespective of clinical response to treatment [172], suggesting that rheumatoid disease may be heterogeneous, with some patients having a TNF-dependent disease and thus responding to anti-TNF therapy, whilst others may have disease driven by TNF-independent pathways. This is supported by evidence that RA patients not responding to two months of infliximab treatment may have increased Type I IFN responses [179], echoing alternative disease pathways.

Thus although we have some understanding of the mechanism of anti-TNF therapy and its effects on the immune system, further studies are required to understand why these drugs have different effects, but similar clinical efficacy in RA, and which of these effects are relevant to the pathogenesis of the disease.

3.2.8 The increased risk of infection associated with anti-TNF therapy

Clinical trials proved the efficacy of anti-TNF therapy for inflammatory arthritis [180-182], compared to standard disease modifying treatment, and showed they were relatively safe with minimal observed side-effects. However following more general use of TNF inhibitors on a wider population, concerns arose regarding potentially serious infections including tuberculosis (TB) [183], reports of multiple sclerosis [184], and malignancy [185].

TB was first recognised to be associated with anti-TNF therapy after identification from the Food and Drug Administration (FDA) Adverse Event Reporting System in the first year after the drug's approval [183]. Infliximab therapy was shown to have a tendency to cause reactivation of latent TB within 12 weeks of therapy, with the infection following an atypical course of more extra-pulmonary (50%) and disseminated disease (10%), compared to 15% and 1% in immunocompetent people. Other serious opportunistic infections included *Pneumocystis carinii* pneumonia, histoplasmosis, aspergillosis and severe Candida infections [183].

Although clinical trials are essential for demonstrating the efficacy of drugs and identifying more common adverse events, relatively small numbers of recruits and often a short duration of follow up limit their power to identify the full repertoire of adverse effects. Therefore national registries were established to monitor long-term safety of patients starting biologic therapies. A number of registries for

patients with RA have been established in Europe, the United States and Asia. Some registries have specifically looked at risk of TB in their cohort (see Table 3.3) and some have not (see Table 3.4).

Table 3.3 Registries reporting TB risk in patients with rheumatoid arthritis

Registry name	Number patients treated with anti-TNF therapy	Recruitment started	Biologic-exposed cohort	DMARD cohort	General population
ARTIS (Sweden)	10800	1999	n = 18 IR 39.4/100 000 patient years (p.y.) 95% CI 23.7-61.5	n = 32 IR 14.3/100 000 p.y. 95% CI 9.8-20.2	n = 43 IR 3.5/100 000 p.y. 95% CI 2.5-4.7
BIODASER (Spain)	5493	2000	IR 1113-1893/100 000 p.y. not included	IR 95/100 000 patients not included	IR 21/100 000 population not included
BSRBR (UK)	11757	2001	n = 40 IR 39-144/100 000 p.y. 95% CI 1.4-12.4	n = 0 IR 0	not compared
LORHEN (Italy)	1114	1999	n = 5 IR 1.9-3.7/100 000 p.y. 95% CI 0.00-7.34	not compared	not compared
RATIO (France)	1571	1997	n = 69 IR 116.7 / 100 000 p.y. 95% CI 10.6-222.9	not compared	not compared
SCQM (Switzerland)	2364	1997	n = 2 IR not assessed	not compared	not compared

Table 3.4 Registries not reporting on TB risk in patients with rheumatoid arthritis

Registry name	Country	Number of patients treated with anti-TNF therapy	Recruitment dates
ATTRA	Czech Republic	> 2000	2001
CORRONA	USA	8755	2002
DANBIO	Denmark	3056	2000
DREAM	Netherlands	546	2003
HRBT	Greece	715	2004
NOR-DMARD	Norway	4683	2000
RABBIT	Germany	5279	2001
REAL	Japan	1144	2005

Anti-TNF therapy was approved for use in RA in the UK in 2001, after which a national registry – the British Society for Rheumatology Biologics Registry (BSRBR) was established with the main aim of safety monitoring and observing potential toxicities during treatment with biologic therapies, particularly with regard to infection (including active TB), malignancy and other severe co-morbidity. These rates were compared to the observed rates in a contemporary cohort of RA patients treated with traditional, non-biologic DMARDs. The aim was to recruit all patients starting on anti-TNF therapy in the UK, thereby encompassing a broader range of patients both by age and co-morbidity than those recruited in clinical trials, thus more reflective of the UK RA population.

The registries have been useful in confirming the link between anti-TNF therapy and TB and have also shown that the risk of TB continues after stopping anti-TNF therapy [186, 187], there is a higher burden of extrapulmonary and disseminated TB [186-188] and that the risk of active TB is higher with infliximab and adalimumab (monoclonal antibodies) compared to etanercept (soluble TNF receptor) [186, 187, 189, 190].

In addition, reports from USA, Canada and Europe have estimated the risk of developing active TB in RA patients not receiving anti-TNF therapy is 2-4 fold higher than the general population [189, 191-194] and they also have a higher risk of malignancy [195]. However the risk of TB during anti-TNF therapy is 2.5-fold higher than biological-naïve RA patients and 7-17 fold higher than the general population [187, 191]. Most cases of TB in patients receiving immunosuppressive therapy have been attributed to reactivation of latent infection, rather than de novo infection with *Mycobacterium tuberculosis* [190, 196].

3.3   TUBERCULOSIS

TB is a global health problem with nine million cases of active TB per annum and one and a half million deaths occurring worldwide [197]. Infection with *Mycobacterium tuberculosis* (Mtb) may also result in latent TB infection (LTBI), which is estimated to have affected two billion people worldwide.

Mycobacteria are non-motile, non-sporulating, weakly gram-positive, acid-fast bacilli that appear microscopically as straight or slightly curved rods, 1-4µm in length and 0.3-0.6µm wide [198]. These bacteria express a unique long chain fatty acid structure that plays a critical role in structure and function of the cell wall [199].

3.3.1   The natural history of TB

Before the advent of anti-tuberculous therapy, TB was a major cause of death in both developing and developed countries. Although effective treatment has been available since the 1950s, more recently there has been an increase in multi-drug resistant and extensively drug-resistant mycobacteria. Diagnosis can be challenging and therefore some cases may not receive appropriate therapy in a timely manner, leading to deterioration of the clinical disease. It is estimated that if active pulmonary TB is untreated in otherwise immunocompetent individuals, the case fatality rate is approximately 70% for both males and females [200], although treatment significantly reduces this mortality rate. Increased risk of mortality is associated with HIV-1 infection, age more than 50 years, and diabetes mellitus [200].

After initial infection with Mtb, it is thought that there are four possible outcomes: immediate clearance of the organism, primary disease (immediate onset of active disease – thought to affect around 5% of individuals), latent infection or reactivation of disease (onset of active disease, many years following a period of latent infection).

It is not known what proportion of infected people successfully clear the infection. However the majority of people infected will develop LTBI defined as having immunological evidence of Mtb infection by tuberculin skin test or immune gamma

release assay, but with no clinical signs or symptoms and a normal chest x-ray [201].

Studies in individuals who are TST positive indicate a 10% lifetime risk of developing active disease, assumed to result from reactivation of the primary infection [202]. Risk of reactivation of LTBI is increased in people with HIV-1 co-infection and other causes of immunosuppression including age and corticosteroid therapy, as well as anti-TNF therapy. This indicates that an intact immune response is required to control latent infection.

Shaped by long-term co-evolution with its host, Mtb can survive in macrophages for years without being cleared by the host immune system [203]. Inside the hostile environment of the macrophage, Mtb co-ordinates its global gene expression patterns in a way that suits its intracellular survival [204]. The hypoxic environment produced by chronic persistent granuloma induces dormancy, where the mycobacteria undergo a non-replicative or low-replicative dormant stage [204, 205]. It is likely that replicating Mtb becomes dormant within infected macrophages having subverted their antimicrobial and antigen-presentation potential. Mtb in the dormant phase are unable to interfere with phagosomal pH, have a lower expression of proteins required for blocking phagosomal maturation, and do not inhibit the proteolytic efficiency of macrophages [206]. Dormant Mtb is unlikely to be able to infect tissue macrophages, and therefore resides within cells as an Mtb reservoir, although the exact physical location of bacilli during LTBI remains unclear [206].

Using ^{18}fluorodeoxyglucose (^{18}FDG) positron emission tomography in conjunction with computed tomography (PET-CT) imaging of granuloma in non-human primates, it has been possible to see the dynamic nature of each granuloma, which appear to function autonomously, even within the same host, presumably as a result of stochastic local events [207]. Within these models, mycobacterial killing efficiency has emerged as the key determinant of progressive disease or control [207].

3.3.2 The pathogenesis of TB

Mtb is a slow growing species, characterised by a 12-24 hour doubling period and prolonged culture on agar which can take up to 21 days. Mycobacteria are able to survive and multiply within host phagocytic cells (typically macrophages), suggesting they are able to modulate the normal progression of host immune responses [208].

Mtb is transmitted from an individual with active TB coughing up fine aerosol droplets containing Mtb, to a recipient host. For an overview of the pathogenesis of TB, see Figure 3.3. Essentially inhaled mycobacteria penetrate the lower part of the lungs, which is well ventilated [209]. Here, they encounter resident alveolar macrophages (AM), pneumocytes and dendritic cells in the alveolar passages. Mtb is phagocytosed by macrophages and retained within a membrane-enclosed endocytic vesicles (phagosome) [210], which are programmed to degrade internalised material through rapid acidification and fusion with lysosomes, rich in proteolytic and lipolytic enzymes [211]. Further macrophages are recruited to the site of infected cells, forming a granuloma, which comprises an organised aggregate of differentiated macrophages and other immune cells, including T cells, B cells, dendritic cells, neutrophils, fibroblasts and extracellular matrix components [212, 213]. This initial granuloma, also known as a Ghon focus, may heal. In 90% of Mtb-exposed patients Mtb is subsequently controlled and active disease never develops [214].

Mycobacteria may be able to spread to the newly arriving macrophages, but as further cells arrive and the granuloma forms to contain bacteria, they develop a central area of necrosis that supports bacterial growth [212].

However pulmonary TB develops in the lung apices, thus the mycobacteria are able to translocate from the lung bases and lodge at the lung apex driving lung matrix destruction and cavitation [209]. Why and how the mycobacteria translocate is unknown, but cavitation leads to very high infectivity, thereby allowing it to propagate and spread to more individuals.
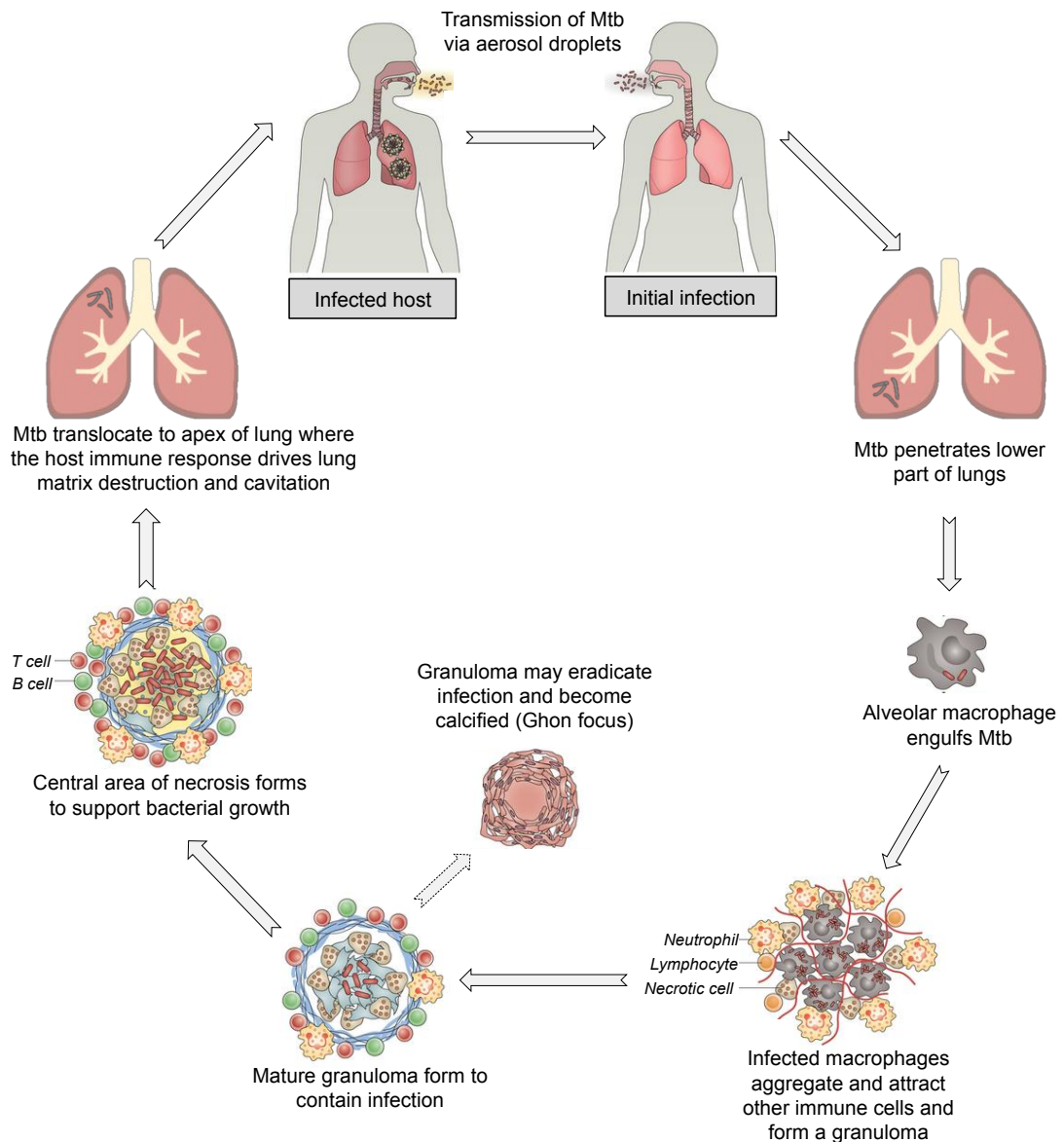
Figure 3.3 Pathogenesis of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb) is inhaled via aerosol droplets into the lung bases, where they encounter and are internalised through phagocytosis by alveolar macrophages (AM). Infected macrophages cluster and recruit other immune cells including T cells, B cells, activated macrophages and other leukocytes to form a granuloma with the Mtb-infected macrophages contained at the centre. The majority of infected individuals will remain in a 'latent' stage of infection, in which no clinical symptoms are present. A small of infected individuals will progress to active disease, where Mtb is released from granulomas eroded into the airways and translocates to the lung apex where further tissue destruction and cavitation may occur. When an individual with active TB coughs, they generate infectious droplets which propagate infection.

This figure is adapted from "In search of a new paradigm for protective immunity to TB. Nunes-Alves, C. et al. Nat Rev Micro 2014; 12: 289" [215] and "Tuberculosis: Time for a new Perspetive? Elkington, P.T. et al. J. Infect 2013; 66 (4): 299-302 [209].

The entry of Mtb into macrophages may occur via a variety of different receptors including complement receptors (CR1, CR3, CR4), mannose receptors (MR), the dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintergrin (DC-SIGN), surfactant protein receptors (SPR), scavenger receptors (SR) and Fc receptors (FcR) [216]. The ability of multiple receptor molecules to internalise Mtb is likely related to its complex cell surface structure [217], and the precise receptor involved in phagocytic entry into the cell may have an impact on the fate of Mtb once inside the macrophage [216]. For example ingestion of Mtb via FcR occurs by binding to IgG-coated targets, whereas complement receptors such as CR3 bind complement-opsonised targets, resulting in different mechanisms of uptake [218]. Subsequently different intracellular mechanisms occur – FcR ligation is accompanied by the activation of the respiratory burst (production of reactive oxygen species) and production of arachidonic acid metabolites and cytokines, such as TNF. However following binding to CR3 there is an absence of any of these pro-inflammatory signals [218].

Mtb also activates several PRRs: the TLR-2, -4 and -9, nucleotide oligomerisation domain (NOD)-like receptors and Dectin-1 on macrophages [219-221]. Binding of Mtb to these PRR leads to activation of the NFκB pathway via the MyD88 signalling pathway, resulting in the production of inflammatory cytokines and direct anti-microbial activity [222]. In addition, the NOD2 receptor may also activate the inflammasome [223]. Thus the NFκB signalling pathway can regulate innate immunity by controlling the expression of various immunomodulatory molecules (e.g. TNF, IL1β and IL12), and thus modulate the intracellular survival of invading bacteria [224]. Dectin-1 mediated recognition of Mtb induces Th1 and Th17 responses, and subsequent secretion of pro-inflammatory cytokines that promote bacterial killing and in addition promotes IL12p40 production by dendritic cells in response to Mtb infection [221].

Upon entry of Mtb into the macrophages, initial inflammatory responses are triggered which comprises secretion of various cytokines (including TNF, IL1β, IL6, IL12, IL18 and IL23) and chemokines. Subsequent phagosome acidification and fusion with lysosomes to form the phagolyosome, results in the killing of most

pathogens [225]. Activated macrophages also produce reactive nitrogen species (RNS) and reactive oxygen species (ROS), through activity of inducible nitric oxide synthetase (iNOS), which may promote effective killing of ingested bacteria in acidified compartments [226]. iNOS is a key enzyme required for nitric oxide production and Mtb control during infection of mouse macrophages, however the role of nitric oxide during infection of human macrophages remains unclear. Mtb may prevent phagosome-lyosome fusion thus preventing acidification and recruitment of lyosomal hydrolases [227], with a small proportion of bacteria breaking out of the phagosome to live in the cytosol [228], thereby subverting intracellular death and allowing for bacterial survival and growth.

3.3.3   The role of immune responses in protection against TB

Macrophages play a unique role in host response to mycobacterial infections, acting as both the primary effector cell for mycobacterial death and providing optimal conditions for intracellular mycobacterial growth and replication. However their role remains unclear as depletion of AM in mice, resulted in increased survival with lower mycobacterial growth compared to non-AM depleted mice, but fewer and more disorganised granulomas, suggesting that macrophages do not control mycobacterial growth alone [229, 230].

Another host defence mechanism used by macrophages is autophagy – the homeostatic process by which a cell degrades its own intracellular compartments, characterised by the emergence of an autophagosome. This captures defective organelles and large macromolecular aggregates and intracellular pathogens including Mtb, delivering them for lysosomal degradation and ultimately killing the ingested bacteria [231]. Autophagy limits bacterial growth, but also may dampen the host inflammatory response. Stimulation of mouse macrophages results in IFNγ production and induces autophagy, which is necessary for antimicrobial activity against Mtb [232]. Many of the autophagy-associated proteins are also involved in phagosome-lysosome fusion [233]. Induction of autophagy during mycobacterial infection increases acidification and subsequent maturation of mycobacteria-containing phagosomes, resulting in intracellular killing of Mtb [233]. However mycobacteria have also developed several strategies to subvert

autophagic killing – including ectopic production of the secretory proteins – early secretory antigenic target 6 (ESAT6) and culture filtrate antigen 10 (CFP10), secreted by the ESAT6 secretion system (ESX-1) system which inhibit autophagosome formation [234] and in addition, ESX-1 plays a direct role on inhibition of autophagy in dendritic cells [235]. ESAT6 and CFP10 are also thought to play a role in preventing phago-lysosomal fusion [236], although their main role is in Mtb virulence potentially via apoptosis inhibition and preventing IL12 and TNF production from Mtb-infected macrophages [237].

The adaptive immune response is activated after mycobacteria are engulfed, and pro-inflammatory cytokines and chemokines recruit CD4^{+} and CD8^{+} T cells, natural killer cells and neutrophils to the site of infection. The recruited cells produce further cytokines and chemokines to recruit and activate more cells. Induction of the adaptive immune responses against Mtb is thought to be slow – around 3-8 weeks in humans and non-human primates, and infection may already be well established before a full response has been activated [238, 239]. This may be partly due to Mtb's ability to actively inhibit, subvert or otherwise modulate antigen presentation by major histocompatibility complex (MHC) class I, class II and CD1 molecules, thus avoiding immune recognition by T cells [240].

Cell-mediated immunity, the immune response involving activation of phagocytes, antigen-specific T cells and the release of various cytokines in response to antigen, is critical for control of Mtb infection. The importance of T cell immunity in the control of Mtb infection has been demonstrated in a number of animal studies; in mice, CD4^{+} T cells play an important role in immunity against Mtb infection [241-244] and Mtb infection following depletion of CD4^{+} T cells in Cynomolgus macaques resulted in severe exacerbation of primary TB with increased extrapulmonary spread and mycobacterial burden [245], although CD8^{+} T cells were able to compensate and rapidly differentiate into Th17-like/Th1-like cytotoxic effector cells [246]. In humans, co-infection of TB with HIV-1 is more likely to occur when CD4 numbers are reduced, although other altered inflammatory responses may also contribute to this.

Mtb-specific CD8^{+} T cells are induced in response to Mtb infection, and subsequently recognise Mtb-infected macrophages [247, 248]. Cytotoxic activity of CD8^{+} T cells includes at least two separate mechanisms: apoptosis via the Fas-FasL pathway and killing via perforin and granulysin [249]. In humans, CD8^{+} T cells can kill intracellular mycobacteria via the release of the antimicrobial peptide granulysin [250], however this molecule is not present in the mouse. The fact that no mouse analogue of granulysin exists, may in part explain why mice deficient in MHC Class I molecules (thus deficient in CD8^{+} T cells) have a higher burden of Mtb in the lungs and a modest decrease in survival [242], in contrast to CD4^{+} T cell-deficient or MHC Class II-deficient mice, who display more rapidly progressive bacterial growth and a sharp decline in survival [242].

The cytotoxic potential of CD8^{+} T cells to kill infected macrophages in vivo has been shown to be dependent on CD4^{+} T cells in the mouse models, suggesting that the susceptibility of CD4^{+} T cell knock-out mice to Mtb infection might be due in part to impaired CTL activity [251]. Mtb-specific CD8^{+} T cells are also involved in cytokine production, particularly IFNγ and TNF. Mechanisms that regulate relative cytokine or cytolytic activity of CD8^{+} T cells during Mtb infection are not fully understood.

When Th1 CD4^{+} T cells are presented with antigens in the context of MHC Class II, they produce pro-inflammatory cytokines such as IFNγ, TNF and IL2, which help to restrict mycobacterial growth [252]. In contrast Th2 CD4^{+} T cells induce an alternative immune response including production of IL4, IL5 and IL10. These together with Treg may dampen the host response and favour persistence of Mtb [253]. CD4^{+} and CD8^{+} T cells which simultaneously produce two or more cytokines, such as IFNγ, TNF and IL2 are generally considered to be superior effectors, compared to those only producing one cytokine, as they indicate a capacity for effector and proliferative functions [254]. Mtb-specific CD4^{+} T cells which produce IFNγ, TNF and IL2 are more commonly seen in LTBI than active disease, suggesting a protective immune function [255-257].

Production of IL12 by antigen presenting cells and IFNγ in a classical Th1 type response is important for mycobacterial control generally. This is reflected in

Mendelian susceptibility to mycobacterial disease in humans with genetic deficiencies affecting various parts of the IL12/IFNγ signalling axis [258-260]. In addition, IFNγ knockout models in mice show uncontrolled Mtb growth [261]. Indeed IFNγ is considered to be a key effector molecule because it directly augments intracellular killing of Mtb within macrophages via a nitric oxide-dependent mechanism [262]. Individuals with mutations in the IFNγ receptor and its key downstream signalling molecule (STAT1) are extremely susceptible to mycobacterial infections [263-265]. This infers a role for CD4+ and CD8+ T cells that generate antigen-specific IFNγ responses. Indeed mice deficient in TLR-2 or TLR-9 with high levels of IFNγ and TNF, with high infiltration of CD4+ and CD8+ T cells in lungs still succumb to Mtb infections indicating that the innate immune response has a more profound role than simply assisting adaptive immunity [266]. However when the acquired response is absent, bacteria may grow rapidly [267], indicating that both the innate and adaptive responses are both required for a robust host immune response to Mtb.

In addition, IL10 is a potent immunoregulatory cytokine in the host response to Mtb, produced by Th1, Th2 and Th17 cells, B cells, neutrophils, macrophages and some DC subsets [268]. Its role in Mtb infection may be viewed as aiding immune evasion or immune protective, as the absence of IL10 can enhance the immune response and early clearance of Mtb [269]. However IL10 may antagonise the development of Th1 responses through antigen presentation suppression and reduced IL12 production [270], and inhibit ROS / RNS–mediated Mtb killing [271-273], in addition to blocking antigen presentation via downregulation of the major histocompatibility molecules [271]. IL10 also blocks phagosome maturation and phagosome-lysosome fusion by a STAT3-dependent, p38 dependent mechanism, which facilitates Mtb survival and outgrowth [274]. Nonetheless it has been predicted through computational modelling that low levels of IL10 from activated macrophages is required for an efficient anti-microbial response, control of bacterial growth and for prevention of lung damage [275].

3.3.4 The role of TNF in TB

As outlined above, the immune response to Mtb is triggered by direct interactions of mycobacterial proteins and lipoproteins with TLR of alveolar macrophages leading to release of cytokines and chemokines including TNF and IL12 [222, 276].

TNF plays a role in macrophage activation and differentiation [277], apoptosis [278], chemokine expression [279, 280] and adhesion molecule expression [281]. TNF is assumed to be important in controlling both early and latent infection in non-human primates (NHP), given that NHP who received anti-TNF antibody therapy had uncontrolled and disseminated by eight weeks after Mtb infection, and a high rate of reactivation of active disease in NHP with LTBI [282].

The granuloma is a dynamic structure containing a variety of cell types. However there are thought to be unique migrational behaviours of cells, in particular effector T cells, which are highly motile, but tend to remain localised on the borders of the granuloma rather than entering or exiting the structure [283]. TNF is thought to be essential for the formation and maintenance of granulomas, by regulating the tight apposition of macrophages and lymphocytes [284].

Animal models have demonstrated that TNF deficiency increases susceptibility to granulomatous infections [277, 285]. Rabbits with active TB and receiving etanercept, displayed increased bacilliary load and larger granuloma with more extensive necrosis and cavity formation [286]. In TNF deficient mice, and mice treated with anti-TNF antibody, the absence or neutralisation of TNF decreases both the recruitment of inflammatory cells and disrupts the normal architecture of granuloma [284, 287, 288]; Presumed to be because TNF usually induces chemokines including CCL3, CCL4, CXCL2 and CCL2 and CCL5 and subsequent leucocyte recruitment to infected organs [284]. Blockade of TNF signalling by anti-TNF antibody in mice with established BCG granulomas, resulted in a loss of granuloma cellularity and protective function, leading to an increased bacillary load [287, 288]. Mice infected with Mtb, and treated with anti-TNF antibody before [277] and after infection [289] had disorganised granuloma, whereas latently-infected NHP treated with anti-TNF antibody had normal granuloma structure, although the granuloma were larger than those of wild-type NHP [282]. Moreover,

NHP treated with anti-TNF antibody did have increased bacterial burden, disease and extrapulmonary spread in keeping with reactivation of LTBI. This is similar to the zebrafish model of *Mycobacterium marinum* infection, in which normal and accelerated granuloma formation was observed in the absence of TNF, despite increased bacterial growth [290]. These data suggest that early granuloma formation is independent of TNF signalling, at least in NHP and zebrafish. Interestingly, no differences were seen in T cell number or cellular phenotype of the granuloma, or mycobacteria-induced production of IFNγ in NHP with acute infection, despite those being treated with anti-TNF therapy having more disseminated TB disease [282]. In addition, NHP with latent TB infection had a higher bacterial load and disease burden if treated with anti-TNF therapy, compared to control NHP [282]. Anti-TNF therapy in Mtb-infected NHP was associated with increased levels of IL12, reduced levels of CCL4 and increased chemokine receptor expression [282], suggesting that TNF plays a role in regulation of IL12 expression and regulation of critical cytokines and chemotactic factors that ultimately contribute to disseminated disease. Therefore in the NHP model, TNF affects the localisation of cells, and the integrity of cellular interactions within the granuloma, rather than the overall granuloma structure.

Mtb-infected murine bone-marrow derived macrophages treated with anti-TNF antibody therapy or from TNFR1 knockout mice, demonstrated that chemokines involved in chemotaxis of cells to the site of infection, including CXCL9, CXCL10, CXCL11, CCL5 and CCL2 were dependent on TNF [291]. However the lack of TNF did not completely stop chemokine expression, indicating that other cells or environmental factors may stimulate chemokine production [291]. TNF blockade in Mtb-infected NHP also lead to altered chemokine expression, impaired cellular recruitment and resulted in a higher proportion of extrapulmonary disease than is usually seen in these animals [282].

Human peripheral blood mononuclear cells (PBMC) stimulated with Mtb antigens, and treated with anti-TNF antibody, demonstrated reduced T cell activation, IFNγ production [292] and proliferative responses [293]. Depletion of Mtb reactive CD8+ T cells by antibody binding to membrane TNF has also been described [294]. In humans receiving anti-TNF therapy, there is an increased risk of disseminated

mycobacterial disease [186, 187], which may be due to disrupted granuloma formation, contributing to reactivation of LTBI. An in vitro model of a human TB granuloma using human primary leucocytes and Mtb-infected macrophages, shows that addition anti-TNF antibody therapy leads to resuscitation of dormant bacilli [295]. Although the mycobacteria become more metabolically active following TNF blockade, it is not clear from this experimental model whether TNF has effect on the cellular structure of the microgranuloma.

TNF is also important in the production of ROS and RNS, which activate macrophages and contribute to killing of intracellular Mtb in animal models. Defects in production of ROS and / or RNS in animal models are associated with increased susceptibility to mycobacterial infection [226, 277, 296-298], and levels of ROS and RNS have been shown to be downregulated by etanercept in Mtb-infected rabbits [286].

Indeed excessive TNF production is also associated with higher susceptibility to TB. This has been shown in zebrafish and other animal models, as well as in TB meningitis patients, where a polymorphism at the LTA4H locus that causes increased TNF production, has been linked with more progressive disease symptoms [299, 300]. Elevated levels of TNF are associated with immunopathological responses in human pulmonary TB, with increased destruction of pulmonary tissue [301] and excessive inflammation associated with cell necrosis and cachexia [302, 303]. These data suggest that the role of TNF in Mtb is finely balanced as it is required for macrophage activation, apoptosis and cell recruitment, but if overproduced may cause excess injury to the host.

3.3.5 The effect of anti-TNF therapy on cell mediated immunity in the immune response to TB

Cell mediated immunity plays a vital role in controlling mycobacterial infection. Following in vitro addition of anti-TNF antibody therapy to whole blood stimulated with mycobacterial antigens, activation of CD4+ T cells is inhibited with reduced production of IFNγ and IL10 [292, 293]. In whole blood sampled shortly after infliximab infusion and stimulated with BCG, significant reduction is seen in chemokines CXCL8, CCL2, CCL3 and CCL4 [304]. Similarly macrophage production

of chemokines is reduced in response to Mtb infection by anti-TNF antibody added in vitro [279, 291], suggesting that TNF controls chemokine expression in response to mycobacterial antigens.

Infliximab also depletes a major subset of anti-mycobacterial CD8 effector memory cells, which may contribute to reactivation of LTBI [294], suggesting that anti-TNF antibody therapy has a major impact on T cell responses. Etanercept has not been shown to inhibit or deplete these cell types.

The restoration of Treg function with anti-TNF antibody therapy but not etanercept may also help to explain the differential risk of granulomatous disease, particularly reactivation of LTBI during and after anti-TNF therapy. Although both mechanisms of TNF blockade confer similar clinical efficacy in inflammatory arthritis, anti-TNF antibody therapy increases risk of active mycobacterial infection by 7-17 fold compared to etanercept [187].

3.4 WHAT ARE THE GAPS IN OUR KNOWLEDGE?

The biologics registries have established unequivocally that anti-TNF therapies increase the risk of active TB [186-189]. Because the incident rate of disease in the short term, typically within months of starting anti-TNF therapy, was perceived to exceed the likely rate of transmission of new infections, anti-TNF therapy is thought to increase reactivation of active TB [305]. Clinical practice has therefore focused on identifying LTBI before starting anti-TNF therapy [306]. The registries have not provided long-term data follow up on patients receiving anti-TNF therapy. Therefore, whether these drugs increase risk of active TB due to new infections remains unknown. This question has become increasingly important because a rising number of patients have now been established on long-term anti-TNF therapy. Subsequently the need for ongoing TB surveillance during anti-TNF therapy is not known.

The mechanism by which anti-TNF therapy leads to active TB also remains elusive. In part this is because the immune correlates of host protection and pathogenicity in active are also incompletely understood. In this context, the effects of anti-TNF therapies may offer novel insights into immune correlates of clinical outcome on TB infection. TNF activity is clearly evident in the host response to TB infection [307]. The available experimental data derived from animal models or in vitro cellular immunology, suggest that anti-TNF therapies may inhibit T cell reactivity [292-294], leucocyte recruitment [279, 291, 304] or TNF mediated pathways for intracellular Mtb restriction [286]. Remarkably, there are no in vivo human data to show that anti-TNF therapies block biological TNF activity within cell mediated immune responses and consequently no such in vivo data on the differential effects of anti-TNF antibodies and ETN, which exhibit differential risk of active TB. Moreover there is a dearth of experimental data that anti-TNF therapies affect Mtb growth in human macrophages, thought to represent the principal unit of infection.

These gaps in our knowledge are significant hurdles to being able to manage the risk of active TB in patients in anti-TNF therapies, to refine biological therapies in

order to minimise risk of infections and to obtain greater insights into the immune correlates of outcomes in TB.

4 HYPOTHESES

1. I hypothesised that anti-TNF therapy leads to reactivation of LTBI and also increases the risk of newly acquired TB infection. In order to test this hypothesis, I sought to identify all cases of active TB reported to the BSRBR and categorised cases according to the site of infection and time to infection, after starting anti-TNF therapy. By focusing on extrapulmonary infection, a time course of reactivation of LTBI can be established. If the incidence of pulmonary infections follow this trajectory, then I infer that all incident TB is due to reactivation, but if pulmonary disease continues to occur after extrapulmonary disease stops then I infer that acquisition of new infection also contributes to risk of TB.

2. Genome wide transcriptional processing has been used in RA previously, before and after anti-TNF treatment, to identify potential biomarkers that can stratify patients' response to treatment. Given that TNF-associated genes are downregulated following anti-TNF therapy, I hypothesised that TNF activity would be suppressed in the TST of RA patients receiving anti-TNF therapy, compared to healthy volunteers and RA patients only treated with methotrexate. I sought to test this hypothesis by assessing TNF-dependent transcriptional responses in vivo, responses at the site of the TST, and consequently evaluate the role of TNF in genome-wide assessments of cell-mediated immune responses.

3. The risk of active TB increases significantly to 7-17 times higher than the general population, when patients are treated with anti-TNF therapy. Although it has been shown that anti-TNF therapy may increase bacillary burden, and is likely to reactivate LTBI by resuscitating dormant intracellular mycobacteria, it has not been clearly demonstrated that anti-TNF therapy has a direct effect on intracellular mycobacterial growth in macrophages. Given these findings, I hypothesised that anti-TNF therapy is

also able to abrogate intracellular mycobacterial killing, thus leading to an increased total Mtb burden. In order to investigate this I treated Mtb-infected MDM with anti-TNF therapy and quantified mycobacterial growth inside and outside of MDM.

5   AIMS OF THE PROJECT

The aim of this thesis is to further understand the mechanism by which anti-TNF therapy increases the risk of active TB.

Specifically, this thesis will aim to understand:

1. Whether the risk of active TB during anti-TNF therapy is due to reactivation of latent TB infection, or also increases the risk of de novo TB infection

2. What impact anti-TNF therapy has at the site of the TST in vivo, and its effect on TNF inducible activity

3. What effect anti-TNF therapy has on growth of *Mycobacterium tuberculosis* in monocyte-derived macrophages in vitro

6   MATERIALS AND METHODS

6.1   BRITISH SOCIETY OF RHEUMATOLOGY BIOLOGICS REGISTRY DATA ANALYSIS

Patients who developed active tuberculosis (TB) who were registered in the British Society of Rheumatology Biologics Registry (BSRBR) database were identified using searched terms "tuberculosis" and "mycobacteria". The database search included patients registered from 2001 until December 2013.

64 cases were identified from the database and demographic, pharmacologic and disease data was collected on these patients.

6.2   STUDY SUBJECTS

This study was approved by the local research ethics committee (REC number 11/LO/1863); written informed consent was obtained from all participants.

Human blood and skin samples were obtained from patients with rheumatoid arthritis (RA) recruited from University College London Hospital (UCLH) and Northwick Park Hospital (NPH). Healthy subjects were recruited from University College London (UCL), NPH and UCLH.

6.3   INVESTIGATION OF PERIPHERAL BLOOD RESPONSES TO TB ANTIGENS - INTERFERON GAMMA RELEASE ASSAYS

6.3.1   IFNγ ELISpot
Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation of heparinised whole blood with Ficoll-Paque™ PLUS (GE Healthcare Biosciences) at 800 x g for 20 minutes according to the manufacturer's instructions. After repeated washing with PBS without calcium (GIBCO, Invitrogen). PBMC were cryopreserved in foetal calf serum (FCS) (Biosera) containing 10% DMSO (Sigma Aldrich) and stored at -80°C at a concentration of 2×10^7 cells/ml.

Sterile, clear 96-well filter plate with 0.45 μm pore size and a PVDF base membrane (Merck Millipore) were coated with 100μl 1μg/ml Anti-Human IFNγ

antibody (eBioscience) overnight at 4°C. Plates were washed with phosphate buffered saline (PBS) and blocked with Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine (GIBCO, Invitrogen) with 5% foetal calf serum (FCS) for 30 minutes. The plate was then washed and blotted dry. PBMC were thawed, washed in PBS and resuspended in X-VIVO15 media (Lonza) and plated at 2×10^5 cells/well. Antigens were added to each well in duplicate – PPD 10μg/ml (SSI, Denmark), TB antigen (ESAT6/CFP10 fusion protein – courtesy of Professor Tom Ottenhoff, University of Leiden) 10μg/ml, TT 10μg/ml (NIBSC), heat-killed *Candida albicans* 10^6/ml cells (Invivogen) and soluble anti-CD3 (HIT 3a) (eBioscience).

Plates were incubated for 48 hours at 37°C in the dark. The cell suspension was then removed and the plate was washed with PBS. 1μg/ml of biotinylated secondary detection antibody (eBioscience) was applied in 0.5% FCS/PBS and incubated for 60 minutes at room temperature (RT), flicked out and washed. 1μg/ml of streptavidin alkaline phosphatase (ALP) (Calbiochem) conjugate was applied in 0.5% FCS/PBS for 30 minutes at RT, flicked out and washed. The ALP substrate BCIP/NBT (Merck) was applied and reaction was allowed to develop in the dark for 8 minutes. The substrate was removed and the reaction stopped by addition of distilled water.

Plates were dried in the dark and analysed using AID ELISpot Software version 5.0 on the AID ELISpot Reader. Duplicate wells were averaged and the negative control was subtracted from the mean to normalise results.

6.4   TUBERCULIN SKIN TESTS

6.4.1   Tuberculin skin tests (TST) and biopsy collection

Research nurses recruited patients with RA from UCLH, under my supervision, and I recruited RA patients from NPH. I recruited healthy subjects from UCL, NPH and UCLH.

Participants were identified following a positive response to the PPD ELISpot (> 10 spots / 200 000 cells). An intradermal injection of 2TU tuberculin (Serum Statens Institute, Denmark) was performed into the left forearm and the injection site was

marked with a permanent marker. After 72 hours, the skin was infiltrated with 0.5ml 2% lignocaine and two 3mm punch skin biopsies were taken. Biopsies were collected into RNAlater (Qiagen) for RNA extraction, or snap frozen in OCT Compound (Tissue-Tek) for histological analysis. Samples were stored at -80°C. Details of clinical induration and erythema were noted at 72 hours.

I performed the TST and biopsies for 36 participants, a further 14 TST and biopsies were performed by the research nurses Jo Williams and Vicky Howard. I performed all of the RNA extraction and tissue processing.

6.4.2 Histological assessment of TST biopsy samples

Frozen sections were carefully thawed and fixed in 4% neutral buffered formalin, then embedded in paraffin wax (Sakura) by the UCL Pathology Core Facility. 3 µm sections were cut and stained using the Leica Bond III automated immunostaining platform, with the Leica Bond Polymer Refine detection kit (Leica DS9800) and a 3,3'-diaminobenzidine (DAB) chromogen. Immunostaining was performed by Dr Naomi Guppy, at UCL Advanced Diagnostics. Details of the antibodies used are seen in Table 6.1.

Whole slide images of the histology sections were acquired with an Axio-Scan microscope using Zen 2 core software at 20x magnification and are presented without any subsequent processing. Digital image analysis was performed using Definiens AG (Munich) Tissue Studio 4.3, by Dr Matthew Ellis, Institute of Neurology. Tissue detection automatically identified all the tissue within each image, then a machine learning method was used to separate the sample from background and non-tissue regions, and segment the sample into dermis and epidermis; manual correction was used to ensure valid separation of these regions of interest (ROI). A fixed threshold was then applied to each ROI to identify the chromogen positive areas (μm^2), which is represented as a percentage of the total tissue/ROI area. For MMP9 stained sections an alternative method was used to identify chromogen positive regions; due to variation in the stain properties between samples a dynamic threshold, based on the overall intensity of chromogen staining in each sample, was applied to calculate the coverage of chromogen positive tissue.

Table 6.1 Antibodies used for immunohistochemistry

Target	Clone	Catalogue number	Dilution
CD3	LN10	Leica NCL-L-CD3-565	1:100
CD14	7	Leica NCL-L-CD14-223	1:25
CD19	BT51E	Leica PA0843	1:1
CD163	10D6	Leica NCL-L-CD163	1:100
MMP9	56-2A4	Millipore MAB3309	1:2000
Neutrophil Elastase	NP57	Dako M0752	1:10

6.4.3   Genome wide transcriptional profiling of TST biopsy samples

Skin biopsy samples were thawed and transferred to 1ml Qiazol (Qiagen). Tissues were disrupted using a homogenisor (Omni International), and RNA was obtained following a chloroform extraction and RNEasy Mini kit (Qiagen) according to manufacturer's instructions. RNA samples were subjected to DNase treatment using the TURBO DNA-free kit (Ambion), as per the manufacturer's instructions to remove contaminating genomic DNA. RNA quality and integrity were measured electrophorectically (Agilent 2100 Bioanalyser).

RNA Samples were labelled in preparation for hybridisation and scanning of microarrays with the Agilent Low RNA Input Linear Amplification Kit according to manufacturer's instructions.

Total RNA was amplified, reverse transcribed into cDNA and then to cRNA and labelled with Cy5 or Cy3 using the Agilent Low RNA Input Linear Amplification Kit. Purification, labeling intensity and RNA concentration were verified used in a NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Scientific). Equal concentrations of Cy3 and Cy5 labelled samples were mixed and hybridised to Agilent 8x60k SurePrint G3 Human Gene Expression arrays as per manufacturer's instructions.

6.4.4   Whole genome transcriptional profiling analysis software

Array images were acquired with Agilent's dual-laser microarray scanner G2565BA and raw signal data were collected with Agilent Feature Extraction software (v10.7.1.1). The R package agilp was used to extract and $\log_2$-transform raw data from scanner files, remove probe duplicates, and apply Loess normalization [308]. All array data are deposited on ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession numbers E-MTAB-5095

(LPS MDM module), E-MTAB-5094 (whole blood stimulation), and E-MTAB-5093 (skin biopsies).

Differential gene expression was assessed in MultiExperiment Viewer v4.9.0 (http://www.tm4.org/mev.html), using a p value cut-off of 0.05 in t tests with Welch's approximation (paired where appropriate) to derive TNF modules from ex vivo and cell culture experiments, or in Wilcoxon tests to compare saline- and tuberculin-injected skin. The overlap of gene lists was visualized in Venn diagrams, using the web application Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/) (Oliveros 2007-2015). Upstream regulators were predicted with the upstream analysis tool within QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingeuity). Over-represented pathways were identified in InnateDB (http://www.innatedb.com), using default analysis parameters, and were visualised as network diagram in Gephi v0.8.2 beta (https://gephi.org/). Principal component analysis was performed using the prcomp function in R.

6.4.5 Derivation of cell type specific gene modules

Cell type specific modules were created by members of our group – specific thanks to Lucy Bell, Gabriele Pollara and Carolin Haas who have described the methods in a published paper [309].

In brief, a gene expression matrix of purified cells was derived from the processed dataset E-GEOD- 22886 available on ArrayExpress repository. The extracted data were not processed further aside from adding gene symbol annotations to probe names. Cell type specific modules were generated by identifying three to five gene probes, corresponding to validated markers that identify each cell type of interest. The markers were each used to identify co-correlated genes amongst all other gene probes in the expression data matrix. The top 1% of probes that were most co-correlated with the expression of each marker were identified using the Pearson correlation coefficient function in R, and cell type specific modules were then derived from the highly co-correlated probes that were common to all markers for each cell type. The specificity of these modules was validated by comparison of the geometric mean expression level for each module within

genome-wide data from each cell type in E-GEOD-22886 (from where the modules were derived) and also in E-GEOD-28490, an independent dataset of purified cell types. Furthermore, the sensitivity of cell type specific modules to detect changes for specific cell populations in tissue specimens was evaluated in published datasets, which described changes to cell composition confirmed by histological assessments.

In addition, a macrophage specific gene module was identified by overlapping module content in two data matrices comprising purified monocyte derived macrophages and other cell types. We have previously demonstrated that this approach results in modules more specific than those generated from single datasets [310]. For every gene, the expression difference between macrophages and all other cell types in the first data matrix was computed (ArrayExpress accession number: E-GEOD-22886). Genes that were two-fold or greater upregulated in macrophages compared to at least 90 % of the other cell types in that matrix were selected. This process was then repeated for the second data matrix (generated based on datasets E-TABM-759, E-TABM-998, E-MEXP-2032, E-TABM-1163 and E-TABM-1206), and genes identified in both analyses were selected to comprise the final macrophage module.

6.4.6 Integrated TST signature

Differential gene expression between TST and saline-injected skin samples was assessed with the Wilcoxon test in MultiExperiment Viewer v4.9.0 (http://www.tm4.org/mev.html). Only probes with a $\log_2$ signal intensity above the detection limit of six and a valid NM_RefSeq annotation were considered for this analysis. For each patient group, genes with a p value < 0.05 and a median expression $\geq$ two-fold higher in the TST compared to saline were defined as this group's TST signature. The group-specific TST signatures were then combined to form the integrated TST signature, which consequently consisted of 595 genes that were significantly up-regulated in the TST in at least one of the patient groups.

6.4.7 Derivation of TNF activity gene modules
TNF activity gene modules were developed by Lucy Bell (TNF (MDM)), Carolin Haas (Endogenous TNF and TNF (KC)) and myself (TNF – whole blood modules).

The TNF (MDM) module has previously been published and represents genes induced significantly and at least four-fold in human monocyte-derived macrophages after 24 hour stimulation with 10 ng/ml of recombinant human TNF [309]. Specificity of this module has been achieved through removal of genes that were also up-regulated at least two-fold in response to stimulation with other cytokines [309].

The TNF (KC) module was obtained from published transcriptomic data of primary human keratinocytes stimulated with different cytokines [311, 312]. Adapting the approach taken by Bell et al. to derive the TNF (MDM) module above, significant transcriptional responses to TNF (24 hours, 10 ng/ml) in comparison to unstimulated keratinocytes were identified by t test with Welch's approximation ($p < 0.05$) and a fold change of at least four-fold. Genes that were additionally up-regulated at least two-fold by IFN-γ, IL-17 or IL-22 were excluded, and the remaining genes formed the TNF (KC) module.

To generate the Endogenous TNF (MDM) module, human blood monocyte-derived macrophages were generated as previously described [313]. Differentiated MDM were pre-stimulated for 1 hr with 10 μg/ml etanercept (Pfizer) or medium control before ultra-pure LPS (100 ng/ml; Invivogen) was added for 24 hr (n=3 per group). Transcriptional profiling of stimulated MDM was then performed as previously reported [309]. LPS-inducible genes were defined through comparison with transcriptional data from unstimulated MDM (n=18), using a t test with Welch's approximation ($p < 0.05$) and a fold change cut-off of two-fold. Amongst these LPS response genes, those that were attenuated significantly ($p < 0.05$) and at least two-fold in the presence of etanercept were summarised as the Endogenous TNF (MDM) module.

The TNF (HV whole blood) module was generated using whole peripheral blood from four healthy volunteers incubated at 37 °C for three hours with or without 10ng/ml recombinant human TNF (Life Technologies). TNF-inducible genes were defined through comparison of the transcriptional data from TNF-stimulated and unstimulated whole blood, using a t test with Welch's approximation ($p < 0.05$)

and a fold change cut-off of two-fold. All genes which were up-regulated by at least two-fold were included in the TNF (HV WB) module.

In addition TNF modules were developed from patients with RA on specific therapies (Methotrexate or anti-TNF therapy) or amalgamated to make the "RA whole blood" module. These modules were developed in the same manner as the TNF (HV whole blood) module. The gene lists that make up each TNF module are summarised in Table 6.2.

Table 6.2 TNF modules - gene lists

Module	Constituent genes
TNF (MDM)	EBI3, SLC2A6, LAD1, LOC440896, IL8, PLEKHG3, SLC7A11, NFKBIA, BIRC3, MARCKS, LOC389634, ZNF205, KCTD19, SGPP2, MSC, NKG7
Endogenous TNF (MDM)	MYH11, IL2RA, EBI3, ORM1, TNFAIP6, NNMT, NKG7, ORM2, TMEM120A, ADAM19, IL32, SGPP2, SLC39A8, CFB, SIX5, CCL19, MAP1LC3A, CCM2L, ABTB2, SOCS3, PPA1, LIMK2, TMEM173, MCOLN2, ITGB8, BATF, CHI3L2, G0S2, RRAD, SIGLEC10, CAMK1G, IDO1, PNRC1, LAMB3, RHOH, ADORA2A, C1S, CCL20, CLEC4D, LAMP3, MAOA, CCL23, STXBP6, TNFRSF4, CCL14, LAD1, TNIP1, PDE4B, DUSP8, IL15RA, PKN3, JMJD7, STAP2, HAPLN3, ULK2, PSTPIP2, ZNF445, CD274, CRABP1, SAA2, NAMPT, APOL3, PFKFB3, CCL5, TSPAN33, NFKBIA, GRAMD1A, PTPN2, VEGFA, SOCS2, PTGIR, KRTAP10-10, JMJD7-PLA2G4B, C1orf122, PLEKHM3, CD82
TNF (KC)	CSF2, MMP9, IGFBP3, NRCAM, SEMA3C, INHBA, TMEFF1, SQSTM1, CD83, NFKB2
TNF (HV whole blood)	KCNJ2-AS1, ATP6AP1, NDUFV2, CCL16, ANP32A, LIMK2, PTAFR, PLEKHM3, PI4K2B, INSIG1, PTGES, PDLIM5, RASGEF1B, VAV1, ZHX2, MTF1, DMXL2, FAS, ANP32D, ECE1, CCL19, IL36G, IGSF6, ZC3H12C, TNFAIP3, WTAP, DNAJB9, BAZ1A, BCL2A1, PIM2, AK2, AZIN1, IL4I1, NFKB1, HIF1A, TTL, ALCAM, TRIP10, C3, KCNJ2, XBP1, NBN, SLC15A3, VILL, NFKB2, MIR155HG, RHOV, IVNS1ABP, PLK3, CFLAR, CD82, UPB1, SLC39A8, AQP9, SLC7A5, MCTP1, SERPINB9, TNIP1, CCL4L2, IRAK3, SAMSN1, BATF3, TCFL5, PLEKHF2, ADORA2A, SOD2, MAFF, CLEC4E, RILPL2, SH2D3A, IRAK2, GCH1, EMR1, ACSL1, SLC43A3, RAB21, CCM2L, RNF144B, PDE4DIP, CD83, MAP3K8, PIK3AP1, ANKRD33B, IRG1, IL1RN, TNFAIP6, NCR3LG1, CLEC4D, PI3, DRAM1, SLAMF7, TRAF1, FMNL3, CCL3L3, KRTAP5-11, FSCN1, CLIC4, MFSD2A, GPR84, SGPP2, EBI3, CCL20, ORM2, ORM1
TNF (RA whole blood)	AGAP3, SERPINB9, NCF1, MIR146A, SKIL, BCL3, SLC11A2, PTGIR, LIMK2, EYA3, HCAR3, CCL19, TTL, PTAFR, ARHGAP31, BID, SNN, CD58, CFLAR, IVNS1ABP, NCR3LG1, ADM, TNFAIP2, NCF1B, NFKBID, GADD45B, EMR1, NFE2L3, SLC15A3, DLEC1, WTAP, TLR2, CLEC4D, SIRPA, IGSF6, RELA, NR6A1, PLEKHF2, MAP1LC3A, HIVEP1, BMF, PIM2, IFIH1, PLEKHM3, TFEC, BCL2A1, MAP3K8, IER3, FAS, ATF5, ADORA2A, ZSWIM4, RIPK2, BTG3, C3, GBP1, LOC644090, FNDC3B, GPR35, DENND5A, EHD1, ZC3H12A, TICAM1, EDN1, RELB, ATG7, SLPI, IER5, SLC1A3, CCRL2, KYNU, IFNGR2, KMO, PIK3AP1, AQP9, NFKB1, LOC441268, RAB21, FFAR3, GRAMD1A, SCARF1, PSTPIP2, CD274, PLAGL2, MCTP1, SH2D3A, CDC42EP2, CXCL10, DDIT4, MTF1, SIX5, CD82, CLEC4E, MAFF, CCM2L, B4GALT5, LINC01268, PLEK, C1orf122, CXCL2, SOD2, FFAR2, CKAP4, PLAU, ACSL1, CCL4L2, RILPL2, LAMP3, NBN, FEZ1, TNIP1, KRTAP5-11, TNFAIP3, PLK3, NFKBIE, TNF, NFKB2, IRAK3, PDE4DIP, FMNL3, NFKBIA, GCH1, TCFL5, HILPDA, VILL, IL1B, CD83, AK4, LAMB3, UPB1, SAMSN1, KCNJ2, SLC39A8, TRAF1, SLC2A6, ICAM1, SGPP2, RNF144B, ANKRD33B, IL4I1, SLAMF7, CXCL8, CCL23, MARCKS, PI3, EBI3, DRAM1, CLIC4, MFSD2A, ORM2, IRAK2, CCL3L3, IL1RN, G0S2, ORM1, FSCN1, CCL3, TNFAIP6, GPR84, C15orf48
TNF (MTX whole blood)	ADA, ADORA2A. AK4, AMPD3, ANXA2, ANXA7, BIRC3, FAS, AQP9, ATF3, ATP6AP1, BCL2A1, BCL3, BID, C3, PTTG1IP, CD40, CD44, CD58, PLK3, MAP3K8, DMPK, EIF5A, EZH2, ACSL1, FCER1G, FKBP2, LRRC32, GBP3, GCH1, GPR35, FFAR3, HDAC2, HDGF, HIVEP2, HNRNPC, ICAM1, IL1B, IL1RN, IRAK2, CD82, KCNJ2, LAIR1, LIMK2, LIMS1, MARCKS, MLH1, MTF1, GADD45B, NDUFV2, NFKB1, NFKB2, NFKBIA, NFKBIE, ORM1, ORM2, P2RX4, PLAGL2, PLAU, PLEK, PSEN1, PSMA6, PSMD4, PTGER2, PTGER4, RELA, RELB, RGS16, RLF, SAT1, CCL3, CCL18, CCL20, CCL23, SDC4, SLC1A3, FSCN1, SOD2, SQLE, ADAM17, TLR2, TNF, TNFAIP3, TNFAIP6, TRAF1, TNFRSF4, UBE2H, UGP2, VDR, XBP1, PLA2G7, SNN, KMO, KCNK5, MARCO, RIPK2, TNFRSF18, CFLAR, HCAR3, IER3, KYNU, SOCS3, CCRL2, PSTPIP2, CD83, TRIP10, SEC22B, CCL4L2, WTAP, NFE2L3, FEZ1, PDE4DIP, DOCK4, DLEC1, OXSR1, HS3ST3B1, TANK, TOM1, SH2D3A, EBI3, LHFPL2, IGSF6, TNIP1, BTN2A2, ATG7, SSSCA1, DNPH1, PDLIM5, IVNS1ABP, TCFL5, EHD1, BTG3, PNRC1, CKAP4, CACFD1,

	BAZ1A, SLC2A6, IRAK3, MGLL, TFEC, ZHX2, RAB21, RGL1, DENND5A, NUP62, MAFF, TNFAIP8, CLIC4, CLEC4E, LAMP3, SLC43A3, CD274, REPIN1, DNTTIP2, VILL, IER5, SIRT6, UFM1, ACSL5, UPB1, GPR84, FTHL17, GSAP, GNG2, DRAM1, BATF3, MYDGF, SMG9, C15orf39, GPR108, GRAMD1A, SLAMF7, RAP2C, SAMSN1, SLC39A8, FNDC3B, MRPL14, MRPS24, RHBDF2, MCTP1, ZC3H12A, ITPKC, DNAJC5, VOPP1, ZMIZ2, C15orf48, NFKBID, MFSD2A, SPPL2A, PPP1R15B, ZC3H12C, FMNL3, DNAJA1P5, GTF3C6, TMEM106A, CARD16, AGAP3, PIK3AP1, DCUN1D3, C17orf49, C1orf122, SGPP2, TPRA1, NOL4L, CCM2L, DACT3, SIX5, TICAM1, MOB3C, MEI1, RASGEF1B, RILPL2, CRTC2, RNF144B, IL4I1, LINC01268, TMEM205, C11orf96, PLEKHM3, MIR146A, CCL3L3, PIM3, KRTAP5-11, CARD17, TIFAB, LOC644090, ANKRD33B, LINC0113, LOC730257, PFN1P2
TNF **(anti-TNF therapy whole blood)**	SQSTM1, IFIH1, PLAGL2, BID, BCL3, TNFAIP3, EHD1, CCRL2, HCAR3, GCH1, NFKB2, PLK3, PLEK, IER5, GCH1, IL1B, SNN, PLAGL2, VILL, B4GALT5, UPB1, SAMSN1, CLIC4, RELB, NFKBIA, NFKBIE, DRAM1, KCNJ2, ANKRD33B, CLIC4, ICAM1, CCL3L3, FFAR3, FSCN1, CCL4L2, MARCKS, IRAK2, CCL4L2, CCL3, C15orf48, TNFAIP6, GPR84

6.4.8 Integrated TST signature

Differential gene expression between TST and saline-injected skin samples was assessed with the Wilcoxon test in MultiExperiment Viewer v4.9.0 (http://www.tm4.org/mev.html). Only probes with a $\log_2$ signal intensity above the detection limit of six and a valid NM_RefSeq annotation were considered for this analysis. For each patient group, genes with a p value < 0.05 and a median expression ≥ two-fold higher in the TST compared to saline were defined as this group's TST signature. The group-specific TST signatures were then combined to form the integrated TST signature, which consequently consisted of 595 genes that were significantly up-regulated in the TST in at least one of the patient groups.

6.4.9 Quantification of module expression

To summarise the overall gene expression of each module with one single value, the geometric mean $\log_2$ expression of all contributing genes was computed in Microsoft Excel 2010, and referred to as module score. Relative enrichment of a particular module in the TST compared to saline was determined as the module score difference between individual TST samples and the respective saline pool, and referred to as module enrichment score.

6.5 WHOLE BLOOD TRANSCRIPTIONAL RESPONSE TO RECOBINANT HUMAN TNF STIMULATION

6.5.1 Genome wide transcriptional profiling of whole blood

Whole blood was taken from participants in each of the four study groups described above, and kept at 37°C for 3 hours, either unstimulated or stimulated ex vivo with TNF 10ng/ml (Life Technologies).

Samples were stored in RNALater and frozen (-80°C). RNA was isolated and DNA removed from the using RiboPure RNA Purification Kit Blood (Ambion, Life Technologies), according to manufacturer's instructions. Further purification of the RNA was performed using the RNeasy MinElute Cleanup Kit (Qiagen) and GLOBINclear kit (Ambion, Life Technologies), according to manufacturer's instructions. RNA quality and integrity were measured electrophorectically (Agilent 2100 Bioanalyser).

6.5.2 Labelling, hybridization, scanning and feature extraction

RNA Samples were labelled in preparation for hybridization and scanning of microarrays with the Agilent Low RNA Input Linear Amplification Kit according to manufacturer's instructions.

Total RNA was amplified, reverse transcribed into cDNA and then to cRNA and labelled with Cy5 or Cy3 using the Agilent Low RNA Input Linear Amplification Kit. Purification, labeling intensity and RNA concentration were verified used in a NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Scientific). Equal concentrations of Cy3 and Cy5 labelled samples were mixed and hybridised to Agilent 8x60k SurePrint G3 Human Gene Expression arrays as per manufacturer's instructions. Array images were acquired with Agilent's dual-laser microarray scanner G2565BA and raw signal data were collected with Agilent Feature Extraction software (v10.7.1.1).

Median Cy3 and Cy5 signal intensity was Log transformed and normalised using LOESS local linear regression against the mean signal of all the samples using the R package agilp (http://www.bioconductor.org/packages/release/bioc/html/agilp.html).

Principal component analysis (PCA) was performed using the prcomp function in R. Significant gene expression differences between data sets were identified using t-tests for non-parametric data (Mann Whitney tests) in MultiExperiment Viewer v4.9 (http://www.tm4.org/mev.html) and a filter for >two-fold difference in mean or median normalised expression values.

6.5.3 Statistical analyses

Statistical tests as specified in figure legends were performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). P values < 0.05 were considered significant.

6.6 PREPARATION OF HUMAN MONOCYTE DERIVED MACROPHAGES AND AUTOLOGOUS PERIPHERAL BLOOD LYMPHOCYTES

6.6.1 Monocyte Derived Macrophages (MDM)

PBMC were obtained by density gradient centrifugation of heparinised whole blood with Ficoll-Paque™ PLUS (GE Healthcare Biosciences) at 800 x g for 20 minutes according to the manufacturer's instructions. After repeated washing with PBS without calcium (GIBCO, Invitrogen), PBMC were resuspended at 1 x 10^7 cells/ml in RPMI 1640 with L-glutamine (GIBCO, Invitrogen) containing 5% heat inactivated (56°C for 45 minutes) pooled type AB human serum (ABS) (Sigma Aldrich) and seeded onto 96 well tissue culture plates (TPP) at a volume of 100µl per well.

After one hour at 37°C, non-adherent cells (peripheral blood lymphocytes [PBL]) were removed by sequential washes with PBS supplemented with calcium and magnesium. Adherent cells (predominantly monocytes) were incubated in RPMI 1640 with L-glutamine containing 10% autologous serum (AS) and 20ng/ml M-CSF (R&D Systems) for three days at 37°C. On day 3, any remaining non-adherent cells and the media was refreshed with RPMI 1640 with L-glutamine (GIBCO, Invitrogen) supplemented with 5% ABS (Sigma Aldrich).

MDM have been widely used as in vitro models to study host-pathogen interactions. I adopted this protocol using M-CSF differentiated MDM from other

members of our groups, which has been extensively characterised and validated [309, 314].

6.7 CULTURE OF MYCOBATERIUM TUBERCULOSIS

All experiments used a Mycobacterium tuberculosis (Mtb) H37Rv strain constitutively expressing a fluorescent reporter (mCherry) (courtesy of Tanya Parish, Queen Mary University of London, United Kingdom) cultured in Middlebrook 7H9 medium (BD Bioscience) supplemented with 10% ADC enrichment medium, 0.2% glycerol and 0.05% Tween 20 and agitated at 100 rpm at 37°C. For infection experiments, Mtb at mid log growth at an optical density (OD) of 0.6 was used, corresponding to 10^8 colony forming units per millilitre (CFU/ml) [315].

Growth curves of H37Rv mCherry were compared to wild type H37Rv, grown in log phase liquid culture and measured by optical density.

6.8 MYCOBACTERIAL INFECTION OF MDM

MDM were prepared as described above. Following six days of culture, cells were infected with H37Rv mCherry at a multiplicity of infection (MOI) 1 for 4 hours at 37°C. Supernatants were then removed and cells were washed vigorously with PBS supplemented with calcium and magnesium to remove extracellular mycobacteria.

For the four-hour time-point, cells were lysed with 200µl of distilled water and fixed with final concentration 4% paraformaldehyde (PFA) (Sigma) for flow cytometry. Plates for microscopy were fixed with 8% PFA.

For the later timepoints, cells were replenished with RPMI 1640 with L-Glutamine with 5% AB serum and incubated at 37°C for a further 24 or 120 hours.

At time points 24 or 120 hours, supernatants were removed and wells were washed with PBS again. MDM were lysed with 200µl of distilled water and fixed with final concentration 4% paraformaldehyde (PFA) (Sigma) for flow cytometry. Plates for microscopy were fixed with 8% PFA.

6.8.1 Flow cytometry analysis of H37Rv mCherry

Fluorescence of the mCherry reporter was measured by flow cytometry using the BD Fortessa. Results were recorded in BD FACSDiva Software v8.0.1 and analysed using FlowJo (version 7.6).

Mycobacteria were quantified by flow cytometry, by collecting 10 000 events, gated on standardised 10μm (nominal diameter) polystyrene fluorescent microsphere counting beads (Flow-Check Fluorospheres, Beckman Coulter). The counting beads were gated on the Fluorescein isothiocyanate (FITC) channel, thus could be excluded from further analysis on the mCherry channel, to ensure only fluorescent mycobacteria were quantified. Singlets beads were identified, and quantified for a more precise number of beads.

The normalised mycobacterial fluorescence was enumerated by multiplying fluorescence in the mCherry channel with the number of events, then dividing by fluorescence and number of events in the FITC channel, which represented the counting beads. Therefore normalised mycobacterial fluorescence represents number of mycobacteria per counting bead

$$\frac{\text{(mCherry fluorescence FSC x mCherry count)}}{\text{(Bead FSC x Bead count)/Singlet FSC}}$$

Sensitivity of flow cytometric quantitation was assessed by addition of the of the anti-mycobacterial drug, isoniazid used at concentrations of 0.4, 4 and 40ng/ml. Isoniazid was added to Mtb-infected cultures, four hours after inoculation, until 120 hours post infection.

6.8.2 Microscopy of H37Rv mCherry-infected MDM

Cells were processed as above and fixed with 8% Paraformaldehyde (PFA). 0.1% Triton X-100 (Sigma) was used to permeablilse cells, before blocking with 10% goat serum and staining cell nuclei with DAPI (Invitrogen) and macrophage cytoplasm with CD68-FITC (Biolegend).

Hermes WiScan microscope was used to image cells using 10x magnification (75% coverage, 35% density, 26 fields per well) and WiSoft automated software was used to count imaged cells per well.

6.9   ADDITION OF ANTI-TNF THERAPY TO H37RV INFECTED MDM

MDM were prepared as described above in 96 well plates. Adalimumab (Abbott), Etanercept (Pfizer) or natural human IgG1 protein (Abcam) were resuspended in RPMI 1640 with L-Glutamine with 5% ABS, at a concentration of 10μg/ml and added to MDM for one hour prior to Mtb infection.

Optical density of H37Rv mCherry measured, and prepared for inoculation of MDM in RPMI 1640 with L-Glutamine with 5% ABS with or without adalimumab, etanercept or natural human IgG1. MDM were then infected with H37Rv mCherry at a multiplicity of infection of 1, using the same additive (adalimumab, etanercept, IgG1 or no drug), as had been used prior to infection.

After 4 hours, adherent cells were washed vigorously. Wells for the "4 hours timepoint" were lysed in distilled water and collected in a final concentration of 4% PFA. Remaining wells were replenished with RPMI 1640 with L-Glutamine with 5% ABS with or without adalimumab, etanercept or natural human IgG1 for a further 24 or 120 hours. At later time points, supernatants were collected in a final concentration of 4% PFA, adherent cells were vigorously washed and lysed in distilled water for flow cytometry. Parallel plates were also washed and fixed in 8% PFA for microscopy at 4, 24 and 120 hour timepoints.

7 EVALUATING THE RISK OF NEW INFECTION VERSUS REACTIVATION OF LATENT TUBERCULOSIS IN PATIENTS ON ANTI-TNF THERAPY

7.1 INTRODUCTION

When anti-TNF therapy was approved for use in rheumatoid arthritis (RA) in the United Kingdom (UK) in 2001, a national registry was established as a large prospective cohort study. Clinical trials had proved the efficacy of these drugs for inflammatory arthritis [180-182], compared to standard disease modifying therapy, and were shown to be relatively safe with minimal observed side-effects. However following more general use of TNF inhibitors on a wider population, concerns arose regarding potentially serious infections including tuberculosis (TB) [183], reports of multiple sclerosis [184], and malignancy [185].

Given that patients with RA were already known to have an increased risk of infection [316] and malignancy [195], due to the underlying inflammatory disease and/or the immunosuppressing effect of DMARD therapy, it was not known whether the rate of adverse effects observed with the new anti-TNF drugs were above what would already be expected in patients with RA, or whether RA patients were now at an increased risk. Therefore a national registry, the British Society or Rheumatology Biologics Registry (BSRBR), was established with the main aim of safety monitoring and observing potential toxicities during treatment with biologic therapies, particularly with regard to infection (including active TB), malignancy and other severe co-morbidity. These rates were compared to the observed rates in a simultaneous cohort of RA patients treated with traditional, non-biologic DMARDs. The aim was to recruit all patients starting on anti-TNF therapy in the UK, thereby encompassing a broader range of patients both by age and co-morbidity than those recruited in clinical trials, thus more reflective of the UK RA population. Eligibility criteria included a diagnosis of RA (rather than any other inflammatory disease) and patients over the age of 16 years. Data collected included diagnosis (including the presence or absence of those features in the American College Rheumatology diagnostic criteria for RA), age, gender, and year

of disease onset, previous drug history of disease modifying agents, including duration of therapy, significant co-morbidity, all current drug therapy, including more details concerning biologic treatment, disease activity score (DAS28), anthropometric measurements (height and weight), blood pressure, whether they had a chest X-ray prior to starting therapy and questionnaires regarding disability and patient reported health status (HAQ and SF-36) [317].

The registry's initial goal was to recruit 12000 patients on anti-TNF therapy, equally divided between infliximab (IFX), adalimumab (ADA) or etanercept (ETN) and a comparison group of 3900 active RA subjects, treated with conventional non-biological therapies. Patients were recruited to the IFX and ETN cohorts from 2001 onwards, while recruitment to the ADA cohort started in 2003. Recruitment targets were met for the ETN cohort in 2005, IFX in 2007 and ADA in 2008. It was estimated that before recruitment targets were met, > 80% anti-TNF treated patients with RA in the UK, were registered with the BSRBR. After the initial success of the cohort and renewed funding, recruitment restarted at the end of 2011 alongside other newly opened comparison cohorts for further biologic drugs and inflammatory diseases.

This registry aimed to recruit patients from a broad mix of secondary and tertiary hospitals, and a wide mix of socio-economic and geographic diversity, however as numbers of patients on anti-TNF therapy grew, many hospitals were unable to keep up with the paperwork relating to data registration and follow-up, thus lessening the diversity of those recruited. There was also a large gap in recruitment between the first round of recruitment (ending in 2005-8) and second round of recruitment at the end of 2011, by which time fewer patients were registered. The registry is reliant on healthcare professionals and patients reporting outcomes and adverse events, therefore patients may have been lost to follow up, or adverse events not fully recorded.

Other national registries have also been established for a similar purpose. These include Sweden's ARTIS, Germany's RABBIT, Spain's BIODASER and France's RATIO.

Early reports by the BSRBR, other registries and clinical trials, indicated that the majority of cases of active TB occurred soon after initiation of treatment with anti-TNF therapy [183, 186, 187, 318]. It is thought that this is most likely reflecting reactivation of latent TB infection (LTBI), as very few patients had reported recent exposure to TB, were living in countries with a low incidence of TB, and were not recent migrants from countries of high TB burden. Most initial reports of TB during anti-TNF therapy occurred in more developed countries with low TB burden, most likely because it is an expensive drug and was far less available in poorer countries, where TB is more prevalent.

Screening for LTBI prior to starting anti-TNF therapy was therefore introduced, with a program to treat patients found to have LTBI (or at high risk of TB) with TB chemoprophylaxis [306]. Since the introduction of LTBI screening and treatment prior to starting anti-TNF therapy, rates of active TB have significantly reduced [319].

The majority of publications assessed the risk of anti-TNF therapy with respect to TB up to circa 5 years after starting anti-TNF treatment. A recent analysis from the Swedish ARTIS registry compared the number of cases of active TB before and after 2007, and reported a reduced relative risk of TB with longer follow-up (< 5 years HR 7.8 vs > 5 years HR 1.2) [191], which suggested that this supports the notion that anti-TNF therapy reactivates LTBI rather than increases of acquisition of new TB infection. However currently there are no clinical investigations which are able to delineate reactivation of LTBI from new TB infection, therefore it is difficult to ascertain the cause of active TB during anti-TNF therapy. Being able to differentiate between reactivation and acquisition of new TB infection in immunosuppressed individuals is important for risk stratification and monitoring during anti-TNF therapy. If there were evidence that new TB infection is a risk during anti-TNF therapy, more emphasis should be placed on routine screening for TB during treatment.

From clinical trials, national registries and case series, it is evident that anti-TNF antibody therapies – IFX and ADA confer a higher risk of active TB (7-17 times higher) than ETN [186, 187]; it is also reported that anti-TNF therapy-associated

TB leads to a high proportion of extrapulmonary and disseminated mycobacterial infection, compared to pulmonary infection [186-188, 190, 320]. As *Mycobacterium tuberculosis* is transmitted via aerosol droplets and inhaled as a respiratory pathogen, it is thought that pulmonary and miliary TB infection are more likely to arise from primary infection or reactivation of LTBI, but we presume that extrapulmonary TB arises exclusively from reactivation of LTBI. Given that rates of new infection are relatively low in developed countries, from which our existing data are derived, assessment of the effect of anti-TNF therapies on acquisition of new infection may only become evident during longer follow up. There are currently no laboratory strategies for distinguishing new infection from reactivation, hence this analysis would give us a new approach to screening for TB and managing patients during treatment with anti-TNF therapy.

We hypothesised that anti-TNF therapy leads to reactivation of LTBI and also increases the risk of newly acquired TB infection. In order to test this hypothesis, we sought to identify all cases of active TB reported to the BSRBR and categorised cases according to the site of infection and time to infection, after starting anti-TNF therapy. By focusing on extrapulmonary infection (and the presumption that all of these cases arise from LTBI), a time course of reactivation of LTBI can be established. If the incidence of pulmonary infections follow this trajectory, then we infer that all incident TB is due to reactivation, but if pulmonary disease continues to occur after extrapulmonary disease stops then we infer that acquisition of new infection also contributes to risk of TB.

7.2 OBJECTIVES

To test whether cases of pulmonary TB continue to occur after cases of extrapulmonary disease during anti-TNF therapy.

7.3   RESULTS

As the UK has a low transmission rate of new cases of TB, assessment of the effect of TNF inhibition on acquisition of new TB infection may only become evident during longer follow-up. Therefore I analysed the BSRBR database to evaluate active TB disease in all RA patients receiving anti-TNF therapy. Up until 2013, long-term registry data had not extended beyond five years [186-188, 321], although more recently the Swedish registry, ARTIS reported on patients receiving anti-TNF therapy for up to 9 years [191]. The BSRBR database to end of 2013, included 64 cases of active TB in patients receiving anti-TNF therapy for a duration of almost 11 years, extending the follow-up data available in previous reports which have reviewed TB cases occurring between 3-9 years after starting anti-TNF therapy.

7.3.1   Anti-TNF therapy incurs a long-term risk of active TB

The demographic data of the cohort are summarised for each anti-TNF agent in the table below (see Table 7.1).

Recruitment of each anti-TNF agent was staggered due to licencing within the UK. Patients may have started anti-TNF therapy up to 12 months before registration with the BSRBR. IFX was the first anti-TNF agent to be licenced in the UK, and therefore was used more readily initially. However, this drug has to be given intravenously as opposed to ETN and ADA, which are given as subcutaneous injections. Intravenous injections are generally less convenient for patients, and incur a higher cost due to additional nursing requirements and infusion facility provision. Therefore once ETN and ADA were licenced, patients tended to be prescribed these drugs, rather than IFX. This is reflected in the number of patients starting each agent, who were registered to the BSRBR (see Figure 7.1 A), that there was an initial surge in IFX usage, which then steadily declined as ETN and ADA prescriptions increased. Following the end of initial recruitment to each group, there was a pause in recruiting new patients to the registry. Patients already recruited continued to be followed up, but no new patients were registered until new ethics was obtained, and recruitment restarted at the end of

2011. As the ETN group was the first to complete recruitment in 2005, there was a much longer break in first and second round recruitment (starting in 2011) compared to the anti-TNF antibody therapies.

Despite the differences in pattern of recruitment, similar numbers of patients were in each group by the end of 2013, when this analysis was performed. Numbers of patients recruited were: IFX – 4231, ADA – 4822, ETN – 4970 and 3774 in the standard treatment (DMARD) comparator group.

No cases of TB occurred in the standard treatment (DMARD) comparator group. However 64 cases (0.46% of the cohort) of active TB were reported across all of the anti-TNF therapy groups. Comparing the cases of active TB that occur in each treatment group, more patients developed active TB whilst taking anti-TNF antibody therapy – IFX (19 cases, 0.45%) and ADA (30 cases, 0.62%), compared to the soluble receptor, ETN (15 cases, 0.30%). The majority of cases (49 of 64 cases, 76.6%) occurred within the first 24 months of anti-TNF therapy (Figure 7.1 B), although active TB continued to present up to 129 months after starting anti-TNF treatment. Cases of active TB occurred in all three treatment groups after 24 months (Figure 7.1 C), although no ADA-related cases occurred after four years of treatment. No significant difference was seen in time to presentation of active TB between each treatment group.

Table 7.1 Demographics of patients with active TB from the BSRBR

	Infliximab	Adalimumab	Etanercept
Recruitment to cohort, n	4231	4822	4970
Cases of active TB, n (%)	19 (0.45)	30 (0.62)	15 (0.30)
Female, n (%)	16 (84.21)	26 (86.67)	12 (80.00)
Age, mean (standard deviation)	62.6 (10.7)	60.5 (13.0)	60.3 (12.2)
Place of birth, n (%)			
UK	9 (47.2)	17 (56.7)	10 (66.7)
Non UK	6 (31.5)	8 (26.7)	4 (26.7)
Unknown	4 (21.1)	5 (16.7)	1 (6.7)
Ethnicity, n (%)			
White	14 (73.7)	21 (70.0)	10 (66.7)
Non white	3 (15.8)	5 (16.7)	5 (33.3)
Unknown	2 (10.5)	4 (13.3)	0 (0)
Site of TB disease			
Pulmonary, n (%)	5 (26.3)	13 (48.3)	5 (33.3)
Extrapulmonary, n (%)	10 (52.6)	9 (30.0)	8 (53.3)
Miliary, n (%)	4 (21.1)	8 (26.7)	2 (13.3)
Length of anti-TNF treatment until active TB (months)			
Range	1-107	1-48	2-129
Mean +/- SD	22.4 +/- 34.1	14.8 +/- 11.5	25.6 +/- 33.3
Treatment with another biologic agent prior to current anti-TNF therapy, n (%)			
Anti-TNF therapy	0	5 (16.6)	4 (26.7)
Which therapies:	0	3 IFX, 2 ETN	3 IFX, 1 ADA
Other biologic	0	0	0
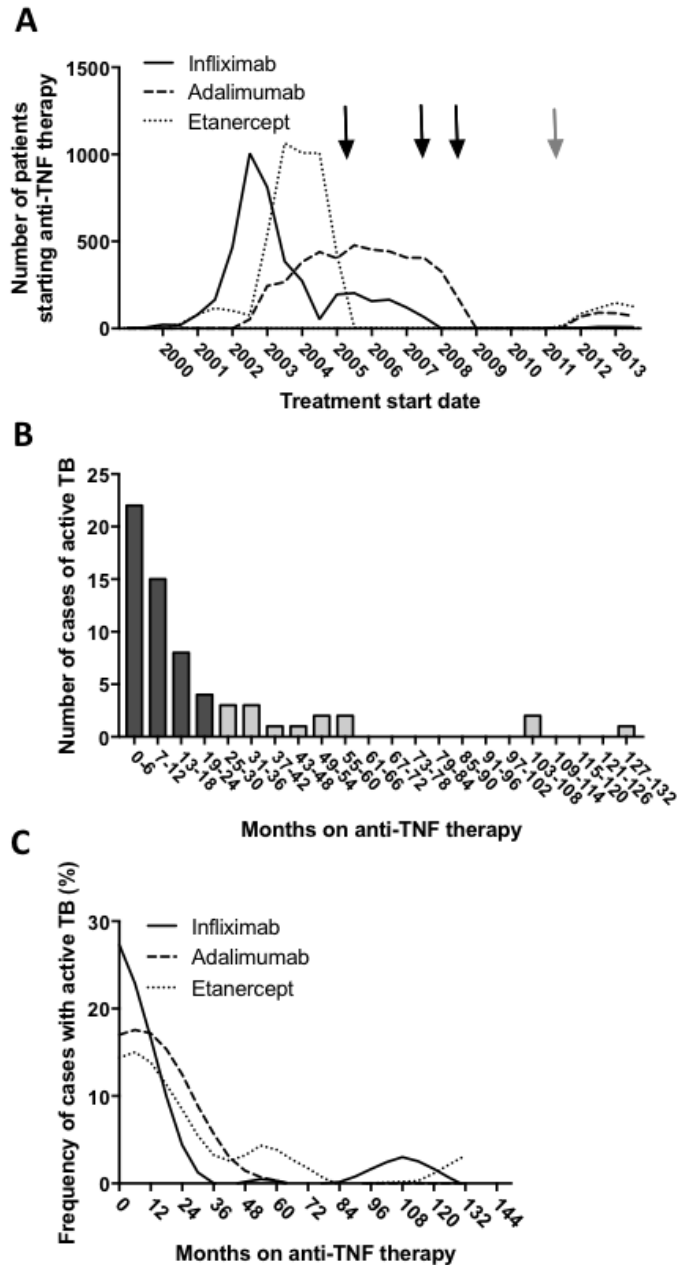
Figure 7.1 Cases of active TB occurring after starting anti-TNF therapy

Patients were recruited to the registry up to 12 months after starting anti-TNF therapy. Date of starting anti-TNF therapy was recorded for each drug **(A)**. Black arrows show end of initial recruitment for each drug and grey arrow shows start of second round recruitment. Cases of active TB were identified from the BSRBR by search terms for "tuberculosis" and "mycobacteria". Time to active TB disease was calculated after starting any anti-TNF agent **(B)**. The dark bars represent number of cases presenting before 24 months of therapy and light bars, after 25 months of therapy. **(C)** The frequency distribution of cases was categorised by the drug, which the patient was receiving at the time of active TB. No significant differences are seen between the time of presentation of active TB for each drug, by a Kruskal-Wallis test with Dunns post test analysis.

7.3.2 Reactivation of latent TB infection is the sole cause of active TB following anti-therapy

On the presumption that pulmonary or miliary TB may arise from primary infection or reactivation of latent infection, but extrapulmonary disease is most likely to be due to reactivation of latent infection [322, 323], I aimed to define the period of risk of reactivation by identifying the longest duration of anti-TNF therapy associated with extrapulmonary disease. By inference, any pulmonary or miliary disease that presents after this time is most likely due to new acquisition of TB infection.

On this basis, I tested the hypothesis that all extrapulmonary TB presents within 24 months of the start of anti-TNF therapy and that any TB cases arising after 24 months were exclusively pulmonary or miliary TB. However, the incident case frequency distribution of pulmonary, miliary and extrapulmonary disease within the registry were almost identical, approximating to zero after 72 months follow up, with albeit very infrequent cases of both patterns of disease between 72-129 months follow-up (Figure 7.2 A). No cases of pulmonary TB occurred after the last cases of extrapulmonary or miliary TB occurred, suggesting all of these cases were due to reactivation of TB.

The median values for pulmonary TB in all three drugs was within 24 months, however those on infliximab with extrapulmonary disease and those taking infliximab or etanercept with miliary TB had longer median times to active TB infection (Figure 7.2 B). These values were not significantly different, but this is likely to be due to the low numbers of cases occurring after 24 months in each group. In the IFX and ETN groups, the majority of cases presented as extrapulmonary TB, but this was not the case for ADA (Table 7.1). Recruitment to the ADA group was started two years later than that of IFX and ETN, by which time physicians had started to screen for LTBI. This may confound the data and influence time to reactivation of LTBI and site of infection, unfortunately information was not collected on TB screening prior to anti-TNF therapy.
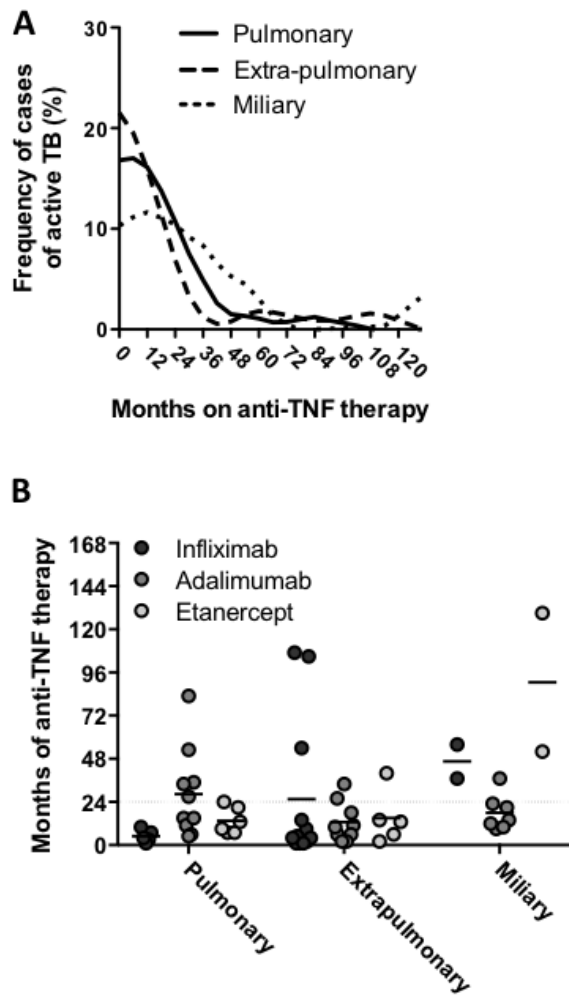
Figure 7.2 Time to active TB from start of anti-TNF therapy, determined by site of infection

(A) A smoothed histogram shows the frequency distribution of cases of active TB, determined by the site of infection – pulmonary, extrapulmonary and miliary. The cases were further categorised by the drug which the patient was receiving at the time of active TB infection **(B)**. No significant differences are seen between the time of presentation of TB for each anatomical site by a Kruskal-Wallis test with Dunns post test analysis, or between drug therapies at each site. In (B) each dot represents an individual case with lines indicating median value. The dotted line on the *y* axis identifies 24 months after starting anti-TNF therapy.

7.3.3 The majority of cases of active TB during anti-TNF therapy occurred in UK-born residents

The majority of active TB in the UK occurs in non-UK born individuals [324], who are largely migrants from high TB incidence countries in areas such as South-East Asia, Africa and Eastern Europe. The high occurrence of TB in this group is principally thought to be caused by reactivation of LTBI [324]. Of those born in the UK, non-White ethnic groups have higher rates of active TB which is likely to reflect the increased risks associated with travel or exposure to people from high TB burden countries. Therefore I wanted to test the hypothesis that extrapulmonary TB (reflecting reactivation of LTBI) occurs exclusively in non-UK born individuals or UK-born, non-White ethnic groups.

Overall, the majority of cases, and in each treatment group, were from the White ethnic group (Figure 7.3 A). A significant proportion of cases of extrapulmonary TB did not have a record of ethnicity or country of birth, however most cases were from the White ethnic group. The pattern of country of birth was very similar between extrapulmonary and pulmonary disease, with the majority of patients being born in the UK, although there were a few individuals born in high TB burden countries (Figure 7.3 B). Unfortunately there was no record of the ethnicity of a large proportion of the infliximab and adalimumab-treated cases with extrapulmonary TB.
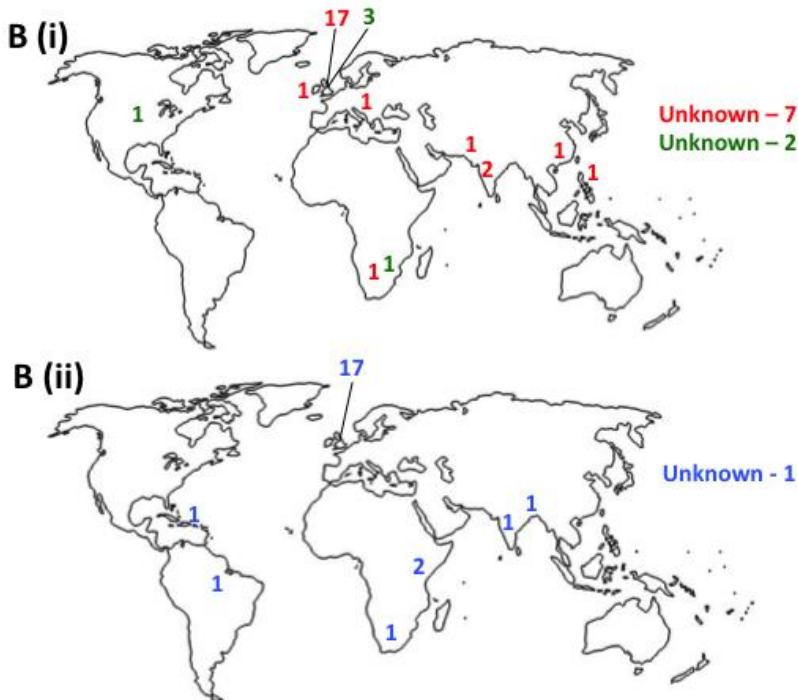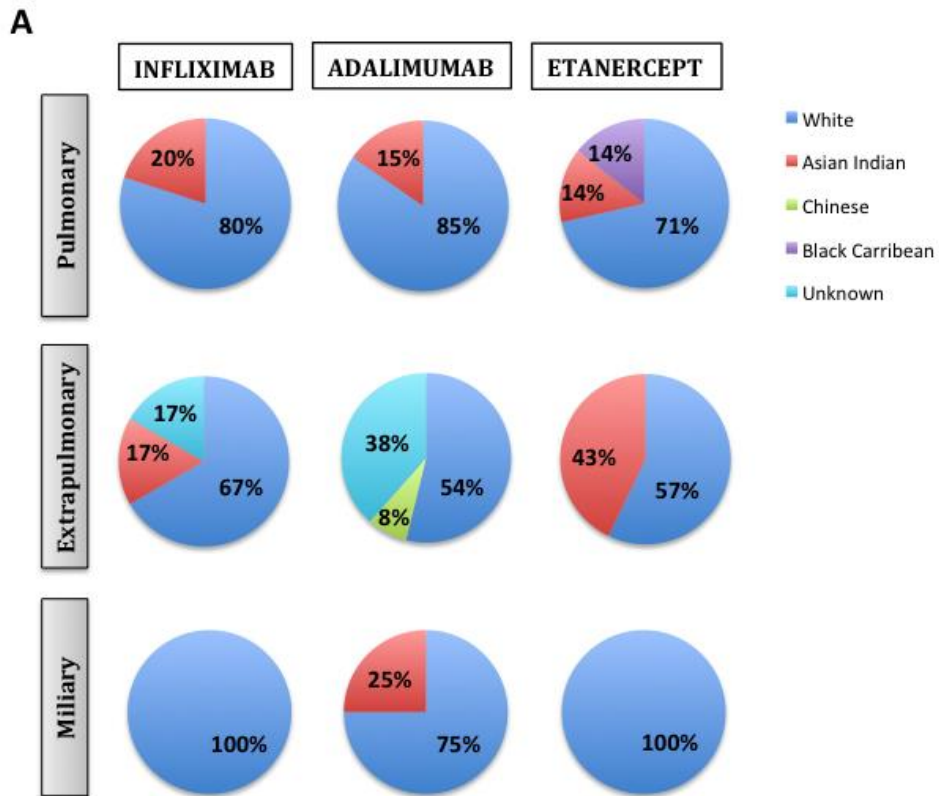
Figure 7.3 Ethnicities and Country of Birth of active TB patients from the BSRBR

(A) Ethnicities are shown for each category of drug and site of TB infection. (B) Shows the distribution of country of birth in the world (i) displays those with extrapulmonary TB (red) and miliary TB (green) and (ii) pulmonary TB (blue).

7.4 DISCUSSION

With the increasing use of anti-TNF therapies, the risk of active TB during treatment persists, although screening for LTBI reduces this risk [191], cases of active TB may still occur [325, 326].

Although it has been extensively reported that anti-TNF therapy increases the risk of reactivation of LTBI, it is unclear how long this risk persists for [183, 186, 187, 305, 318, 327, 328]. Registry data from across Europe has followed patients up to 9 years after starting anti-TNF therapy, but it is still not clear whether anti-TNF therapy also increases the risk of new TB infection [329]. The BSRBR now has more than ten years worth of follow-up data, which affords the opportunity to make continual assessment of rates of active TB compared to the general population. By studying this dataset, it is evident that cases of active TB are still occurring even after a decade of anti-TNF therapy. 64 cases were identified in patients with RA who were taking anti-TNF therapy, none were reported in the DMARD cohort. The patients with active TB largely reflected the typical RA population in the UK, with a higher proportion being female, of White ethnic origin and in the fifth to sixth decade of life.

However there were some issues with the dataset which may reduce confidence in the results. Although I was able to quantify numbers of patients in each of the treatment cohorts, there was some cross-over between these groups, as patients switched between agents after initial registration. Therefore patients were re-registered to the new treatment group. Patients with active TB who had switched therapy, were allocated to the treatment group for the drug they were prescribed when the TB occurred.

I would have also liked to calculate the frequency of cases of active TB per person-years of treatment for each group. However the data suggested many patients started and stopped medication, as well as switching. Start and stop dates were either inaccurate or not complete for many patients, which is a result of relying on patient and physicians to report changes in treatment to the registry.

The majority of cases do occur within the first 24 months suggesting that reactivation of LTBI may be the main determinant of active TB in this cohort, with

most extrapulmonary cases occurring within 72 months follow up, and albeit very infrequent cases of both patterns of pulmonary and extrapulmonary disease between 72-129 months follow-up. However no new cases of pulmonary TB occurred after extrapulmonary or miliary cases suggesting that there is no evidence for new infection of TB and these are all due to reactivation of LTBI.

One limitation of this analysis is the low prevalence of new TB infections occurring in the UK. Cases of active TB continued to occur after more than five years of therapy in both the IFX and ETN groups. There were no cases of TB in the ADA group more than four years after therapy; This may be confounded by the fact that recruitment to the ADA group started later than the other groups, therefore follow-up times were shorter.

One limitation of the BSRBR is the limited amount of data that was collected in relation to risk of TB infection. Data was not collected on details of LTBI screening or TB prophylaxis treatment, or TB risk factors (other than ethnicity, country of birth and whether they had a pre-treatment chest x-ray). Treatment for LTBI reduces the risk of reactivation occurring during treatment with TNF inhibition, but it is not clear what the risk is for patients who continue to be exposed to TB, by visiting countries with a high TB burden or close TB contact after initial screening. Now that a robust LTBI screening programme is in place, it would also be interesting to calculate rates of active TB during anti-TNF therapy and compare these to an age, sex and ethnicity-matched comparator group from the general population, to see whether these rates are now equivalent.

Consistent with the published literature of extrapulmonary and disseminated disease being the mainstay of clinical presentation of reactivation of LTBI in immunocompromised individuals [322, 330-334], this cohort of RA patients on immunomodifying therapy has a higher proportion of particularly miliary TB, compared to the general population in the UK – where rates of pulmonary TB are 52.9%, miliary 2.8% and extrapulmonary 44.3% [335].

Given their analogous antibody structure it is surprising that IFX and ADA have different proportions of pulmonary and extrapulmonary TB, with ADA having a higher preponderance for pulmonary TB. This may be confounded by the fact that

IFX was the first anti-TNF therapy to be licenced in the UK, with ADA arriving several years later, when the risk of TB was more evident and therefore patients receiving ADA were more likely to have undergone LTBI screening. It is unclear what extent LTBI screening had on the earlier patients recruited to the registry, as some may have been screened and some not. Now new patients starting on anti-TNF therapy are unlikely to be prescribed IFX, as it is given intravenously (rather than via subcutaneous injection), thus less convenient for patients, and a higher cost to deliver.

Interestingly in this cohort of active TB patients the most common ethnicity was White and the majority of patients were born in the UK. These are usually regarded as lower risk groups for acquisition of LTBI (compared to the general population where high risk groups include non-UK born individuals from South-East Asia, Africa and Eastern Europe [324], although we do not have information regarding additional social risk factors for TB (current or history of homelessness, imprisonment, drug misuse and alcohol misuse). This may also be a reflection that RA is more common in people of Northern European origin [35].

Those who were born abroad did tend to be from high TB burden areas such as South Africa, South-East Asia and Eastern Europe. Since the introduction of TB screening in 2002, with formal guidance from the British Thoracic Society in 2005 [306], bias may have been introduced to the cohort, as these high risk patients may have been treated with alternative biologic therapies. The registry did not collect information on LTBI screening or TB chemoprophylaxis which may have given us useful insight into the benefit of the screening programme. Screening has been shown to be effective [191], although cases of TB still occur despite an active screening programme [325, 326]. As there is a discrepancy in the patients recruited as to whether they were screened and/or treated for LTBI; those recruited early to the registry before the screening programme was introduced may confound results, as those recruited later should have been screened and treated for LTBI. Those who were deemed as high risk may have had a selection bias towards ETN, with a reported lower TB risk, or a different biologic therapy altogether (with a lower TB risk) – such as B cell depletion (Rituximab).

A further concern is the under-reporting of cases to this national registry, thereby underestimating the true TB rate. Initially the registry was set-up to capture 4000 patients per anti-TNF therapy, however those targets were reached between 2005-7. As the number of patients on anti-TNF treatment has increased in each hospital significantly, reporting to the BSRBR has taken a lower priority than clinical management of patients and is often not completed.

7.5 CONCLUSIONS

Therefore we conclude that active TB is caused by reactivation of latent TB infection (based on the presumption that all extrapulmonary TB is caused by reactivation of LTBI), and there is no evidence for acquisition of new TB infection occurring in patients on anti-TNF therapy. Hence our analysis of the BSRBR data does not support a change in screening strategy in patients taking these drugs.

8 IN VIVO ASSESSMENT OF THE IMMUNE RESPONSE TO THE TUBERCULIN SKIN TEST IN RHEUMATOID ARTHRITIS

8.1 INTRODUCTION

Excessive TNF activity contributes to the complex pathogenesis of rheumatoid arthritis (RA) [95], which is associated with a pro-inflammatory cascade that includes the production of IL1, IL6, IL8, activation of macrophages, neutrophils and eosinophils and ultimately drives tissue destruction through activation of synovial fibroblasts and osteoclasts [73, 96, 105, 106]. High concentrations of TNF and upregulated TNFR expression have been found in the synovial fluid and on the synovial membrane of joints affected by RA [95, 97-101]. Furthermore mice genetically engineered to over-express human TNF, developed a spontaneous inflammatory arthritis with synovial hyperplasia and destruction of cartilage and bone [108], giving evidence for TNF as a key driver in the pathogenesis of inflammatory arthritis. In humans, increased expression of TNF is found in many inflammatory diseases and inhibiting TNF in these diseases has substantially improved the outcome and clinical course of the disease [109].

The five licensed TNF inhibitors (see The development of anti-TNF therapy and its clinical applications) were developed to competitively inhibit the binding of TNF to its cognate receptors and consequently block its biological activity. However, a comprehensive characterisation of their in vivo inhibitory effect on TNF activity has yet to emerge. Variable effects of TNF inhibition on the level of TNF itself in serum or synovium of RA patients have been described [158-161], which do not necessarily correlate with the clinical response to anti-TNF therapy. In general the level of pro-inflammatory mediators in serum and synovium, and pro-inflammatory cytokine production by peripheral blood mononuclear cells is reduced following anti-TNF therapy [162, 163, 336].

Infliximab has been shown to induce apoptosis of monocytes and activated T cells in vivo and in vitro, which is not seen with etanercept or certolizumab [141-143]. However both etanercept and infliximab induce apoptosis in monocytes and

macrophages in synovial fluid and of monocytes in peripheral blood in vivo [144]. Moreover anti-TNF antibody therapy also reduces the number of infiltrating synovial granulocytes and macrophages in the joint as well as reduced expression of chemokines, IL8 and CCL2 [145]. In vitro, infliximab readily binds tmTNF leading to cell lysis via complement-dependent cytolysis and antibody dependent cell-meditated cytotoxicity [147], whereas etanercept forms less stable complexes with tmTNF. This may be associated with a transient leucopaenia, as four months after starting adalimumab, peripheral blood from RA patients showed a significant reduction in CD4^{+} and CD8^{+} producing IFNγ cells [148], however in another study attenuated Th1 responses are restored after 12 weeks of anti-TNF therapy [149]. Infliximab is also able to restore CD28 expression on activated CD4^{+} T cells, which may lead to dampened sensitivity of co-stimulatory signals in T cell responses [150].

While these data suggest that anti-TNF therapies ameliorate the immunopathogenesis of RA, they provide limited insight as to how they regulate TNF activity. Genome-wide transcriptional profiling has identified a set of genes involved in NFκB and cAMP signalling after 72 hours (including NFKB1A, IL1B and TNFAIP3), in those responding to etanercept [174] and changes to the peripheral blood transcriptome four weeks after treatment with infliximab, have shown a downregulation of genes involved in inflammation, angiogenesis, T- and B-cell activation, which is thought to represent a dampening of TNF-associated genes, in all patients irrespective of clinical response to treatment [172]

Thus it is not known whether these agents antagonise TNF activity in selected physiological compartments, and whether they equally inhibit steady state TNF activity associated with chronic inflammatory diseases and inducible TNF activity at foci of acute immune responses. Moreover, our group has described at least one indirect mechanism of action, in which anti-TNF antibodies unexpectedly promoted an interaction between membrane bound TNF on monocytes and TNFR2 on regulatory T cells leading to enhanced Treg activity that may contribute to disease control [153].

A well-recognised complication of anti-TNF therapy is increased susceptibility to granulomatous infections, especially with Mtb [183, 328], in which cell mediated immune responses are thought to represent the principal mechanism of host defence [337]. The role of TNF in immune protection against TB was primarily derived from observations in TNF receptor deficient mice, which do not assemble well-formed granuloma [284, 285]. This observation was replicated by administration of anti-TNF agents in wild type mice [277, 338].

Consequently, increased risk of TB associated with anti-TNF therapy is also widely interpreted to be due to deficient TNF activity in cell mediated immune protection, leading to reactivation of LTBI, and indeed in in vitro models of Mtb-infected granuloma anti-TNF therapy can resuscitate dormant mycobacteria, which may lead to LTBI reactivation [295]. Interestingly, anti-TNF antibodies such as IFX and ADA invoke significantly greater risk of active TB in man, than the soluble TNF receptor, ETN [186, 187, 327]. One possible mechanism for the differential risk is reported to be apoptosis of monocytes and activated T cells [141, 142, 144], or depletion of Mtb reactive CD8$^+$ T cells by antibody binding to membrane TNF [294].

Our group has previously described transcriptional profiling at the site of the TST to make molecular and systems level assessments of in vivo human immune responses at the site of a standardised experimental challenge [309, 339]. Clinical inflammation in the TST has been widely used as a surrogate for T cell memory for mycobacterial antigens [340], but transcriptional profiling of biopsies from the injection site reflects all the components of integrated innate and adaptive immune responses, each of which can be quantified with independently derived transcriptional modules [309]. Importantly, this approach also revealed immune responses in the absence of clinically evident inflammatory induration, allowing unprecedented sensitivity to measure immune responses that were previously described as anergic [309, 339].

Although genome wide transcriptional data can provide valuable insights into mechanisms of disease and has the potential for biomarker discoveries, the extraction of relevant information from the large amounts of data produced can be

difficult. As such, modules, or gene lists associated with the cell, pathway or cytokine of interest have been used and verified to help process information to further understand pathogens and for biomarker discovery [341].

Genome wide transcriptional processing has been used in RA previously, before and after anti-TNF treatment, to identify potential biomarkers that can stratify patients' response to treatment. Given that TNF-associated genes are downregulated following anti-TNF therapy [172, 174], I hypothesised that TNF activity would be suppressed in the TST of RA patients receiving anti-TNF therapy, compared to healthy volunteers and RA patients only treated with methotrexate. I sought to test this hypothesis by assessing TNF-dependent transcriptional responses in vivo, responses at the site of the TST, and consequently evaluate the role of TNF in genome-wide assessments of cell mediated immune responses.

8.2 OBJECTIVES

1. To establish the effect of anti-TNF therapy on TNF inducible activity in peripheral blood, both in steady state and in response to a surge in soluble TNF

2. To determine whether anti-TNF therapy attenuates inducible TNF activity in vivo at the site of the TST

3. To identify effects of anti-TNF therapy on cell-mediated immunity, in vivo at the site of the TST

8.3 RESULTS

8.3.1 Derivation of TNF-inducible transcriptional modules

The effects of anti-TNF therapies are not directly reflected by measurement of TNF levels, but require measurement of induced TNF function. TNF exerts its biological functions via stimulation of the TNFRs and intracellular signalling cascades that converge on regulation of transcription factors and consequently gene expression[342]. My first aim was to derive and validate gene expression modules, which could be used to detect and quantify functional TNF activity. In monocyte-derived macrophages (MDM), our group have previously reported one such module that discriminated cellular responses to TNF from a selection of other cytokines including IFNγ, IL4 and IL13, or IL10 – TNF (MDM) [309]. In the present study I reasoned that the transcriptional response to TNF stimulation may be affected by its context and that any individual gene expression module may be inadequate to detect all TNF activity. Therefore I sought to derive novel TNF-inducible gene expression modules by a range of alternative approaches.

On the basis that TNF activity does not occur in isolation, I first sought to identify gene expression attributable to TNF in the presence of other bioactive cytokines. I used innate immune stimulation of MDM with bacterial lipopolysaccharide (LPS) as a prototypic model of a stimulus to invoke secretion of a wide range of immunologically active factors, including TNF, which may contribute to LPS-inducible gene expression by their downstream autocrine and paracrine activity. In order to identify gene expression attributable to LPS-induced TNF, I compared the transcriptome of MDM 24 hours after LPS stimulation in the presence and absence of the soluble TNF receptor fusion protein, ETN. I found significantly lower expression of 76 genes in the presence of ETN, the expression of which I infer to be TNF-dependent – Endogenous TNF (MDM) (Figure 8.1 A).

The ultimate aim was to evaluate TNF activity in response to an in vivo immunological challenge using the TST [339]. Differential transcriptional responses to the same stimulus in different cell types are widely recognised [343]. Therefore, I took advantage of previously published transcriptomic data from keratinocytes stimulated with a selection of cytokines including TNF [311, 312] to

derive a third TNF-specific module in keratinocytes – TNF (KC) (Figure 8.1 B). We were not able to identify specific TNF-inducible genes using 2-fold changes in gene expression, but 4-fold changes did achieve the desired level of specificity. In addition I aimed to investigate the effect of anti-TNF therapies on functional TNF activity in blood. Therefore I derived a fourth TNF gene expression module – TNF (HV WB), by identifying significantly upregulated genes following ex vivo whole blood stimulation with TNF from healthy volunteers (Figure 8.1 C).

Direct comparison of the genes included in each of the four modules associated with TNF activity showed only modest overlap (Figure 8.1 D). The gene lists can be found in Table 8.1. Cross-validation of each gene list in the different experiments which were used to derive them also highlighted the context specificity of functional gene expression modules, such that modules derived in MDM were not enriched in TNF stimulated keratinocytes and modules derived in keratinocytes were not enriched in TNF or LPS stimulated MDM (Figure 8.2 A). Importantly however, independent bioinformatic analysis of predicted upstream regulators of each of these gene lists showed statistically robust associations with TNF (Figure 8.2 B).
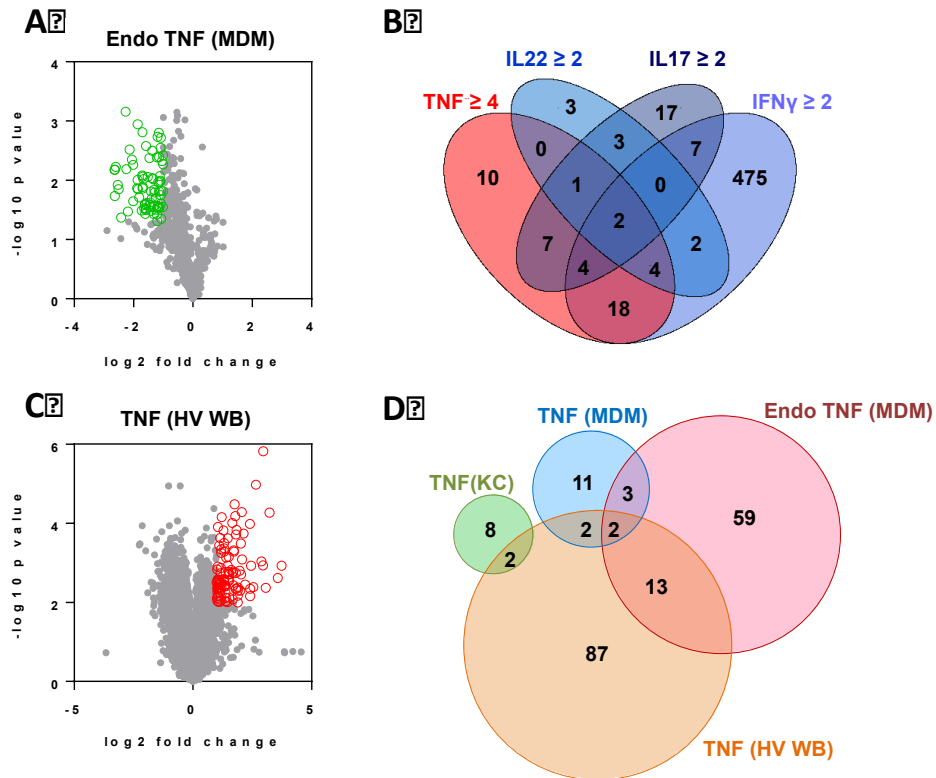
Figure 8.1 Derivation of transcriptional modules to measure TNF activity

(A) Volcano plot of the effect of etanercept (ETN) on LPS-induced genes in monocyte derived macrophages (MDM) after 24 hours. Open black circles represent 76 genes that were significantly ($p < 0.05$ in t test) and at least two-fold attenuated in the presence of ETN and which were combined in the 'Endogenous TNF (MDM)' module. **(B)** 4-way Venn diagram showing the number and overlap of keratinocyte genes upregulated significantly ($p < 0.05$ in t test) and at least two-fold (IFNγ, IL22, IL17) or four-fold (TNF) by different cytokines after 24 hours. The 10 TNF-specific genes yielded the 'TNF (KC)' module. **(C)** Transcriptional response to TNF (10 ng/ml for 24 hours) in ex vivo whole blood (WB) stimulation experiments. Open black circles represent 104 genes that were significantly ($p < 0.05$ in t test) and at least two-fold upregulated in response to TNF and which were combined in the 'TNF (HV WB)' module. **(D)** Venn diagram of the number and overlap of genes making up each TNF transcriptional module, derived as described in (A-C) and by Bell et al. ('TNF (MDM)').
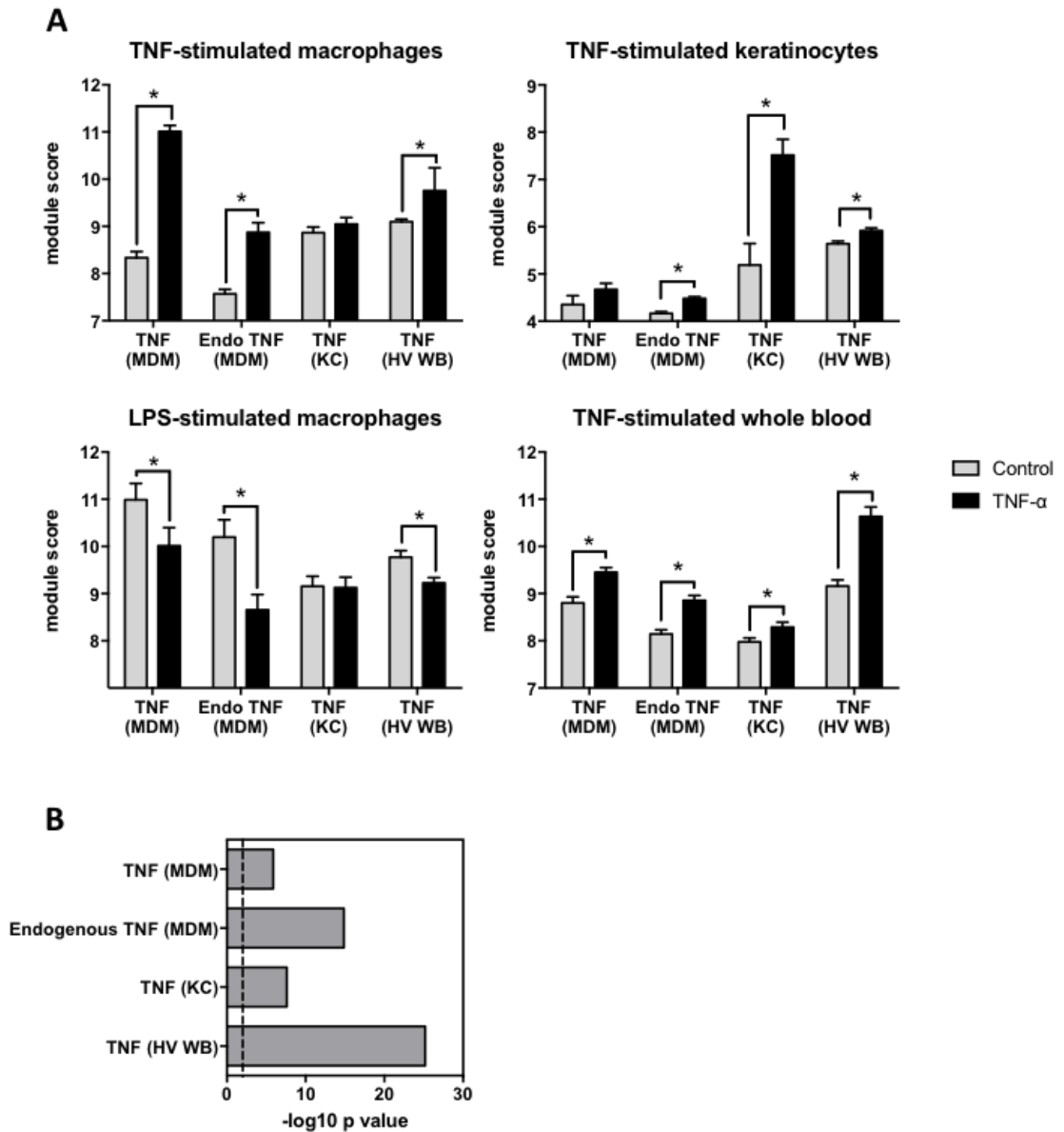
Figure 8.2 Validation of transcriptional TNF-inducible activity modules

Cross-validation of TNF modules in the different experiments which were used to derive them. Module scores were calculated as geometric mean expression of the genes contributing to each module, and are shown as mean + SD (n = 3-5). * p < 0.05 in t tests. **(B)** Statistical prediction of TNF as upstream regulator of each TNF gene module. The dashed line denotes an overlap p value of 0.01.

Table 8.1 Gene lists of TNF-inducible activity modules

Module	Constituent genes
TNF (MDM)	EBI3, SLC2A6, LAD1, LOC440896, IL8, PLEKHG3, SLC7A11, NFKBIA, BIRC3, MARCKS, LOC389634, ZNF205, KCTD19, SGPP2, MSC, NKG7
Endogenous TNF (MDM)	MYH11, IL2RA, EBI3, ORM1, TNFAIP6, NNMT, NKG7, ORM2, TMEM120A, ADAM19, IL32, SGPP2, SLC39A8, CFB, SIX5, CCL19, MAP1LC3A, CCM2L, ABTB2, SOCS3, PPA1, LIMK2, TMEM173, MCOLN2, ITGB8, BATF, CHI3L2, G0S2, RRAD, SIGLEC10, CAMK1G, IDO1, PNRC1, LAMB3, RHOH, ADORA2A, C1S, CCL20, CLEC4D, LAMP3, MAOA, CCL23, STXBP6, TNFRSF4, CCL14, LAD1, TNIP1, PDE4B, DUSP8, IL15RA, PKN3, JMJD7, STAP2, HAPLN3, ULK2, PSTPIP2, ZNF445, CD274, CRABP1, SAA2, NAMPT, APOL3, PFKFB3, CCL5, TSPAN33, NFKBIA, GRAMD1A, PTPN2, VEGFA, SOCS2, PTGIR, KRTAP10-10, JMJD7-PLA2G4B, C1orf122, PLEKHM3, CD82
TNF (KC)	CSF2, MMP9, IGFBP3, NRCAM, SEMA3C, INHBA, TMEFF1, SQSTM1, CD83, NFKB2
TNF (HV WB)	KCNJ2-AS1, ATP6AP1, NDUFV2, CCL16, ANP32A, LIMK2, PTAFR, PLEKHM3, PI4K2B, INSIG1, PTGES, PDLIM5, RASGEF1B, VAV1, ZHX2, MTF1, DMXL2, FAS, ANP32D, ECE1, CCL19, IL36G, IGSF6, ZC3H12C, TNFAIP3, WTAP, DNAJB9, BAZ1A, BCL2A1, PIM2, AK2, AZIN1, IL4I1, NFKB1, HIF1A, TTL, ALCAM, TRIP10, C3, KCNJ2, XBP1, NBN, SLC15A3, VILL, NFKB2, MIR155HG, RHOV, IVNS1ABP, PLK3, CFLAR, CD82, UPB1, SLC39A8, AQP9, SLC7A5, MCTP1, SERPINB9, TNIP1, CCL4L2, IRAK3, SAMSN1, BATF3, TCFL5, PLEKHF2, ADORA2A, SOD2, MAFF, CLEC4E, RILPL2, SH2D3A, IRAK2, GCH1, EMR1, ACSL1, SLC43A3, RAB21, CCM2L, RNF144B, PDE4DIP, CD83, MAP3K8, PIK3AP1, ANKRD33B, IRG1, IL1RN, TNFAIP6, NCR3LG1, CLEC4D, PI3, DRAM1, SLAMF7, TRAF1, FMNL3, CCL3L3, KRTAP5-11, FSCN1, CLIC4, MFSD2A, GPR84, SGPP2, EBI3, CCL20, ORM2, ORM1

8.3.2 Anti-TNF therapy attenuates TNF-inducible gene expression in blood

Numerous reports have interpreted the reduction in circulating pro-inflammatory mediators following initiation of anti-TNF therapies in RA as evidence for inhibition of TNF activity [158, 162, 163, 336]. Therefore I reasoned that anti-TNF therapy would be associated with steady state reduction in levels of TNF-dependent gene expression in blood and attenuate upregulation of these gene expression modules following ex vivo stimulation with TNF. In order to test this hypothesis I quantified expression of each of the four TNF-dependent transcriptional modules following ex vivo TNF stimulation of blood from RA patients treated with anti-TNF therapies and compared these to blood samples from RA patients on methotrexate only. To avoid the confounding of differential disease activity, I restricted recruitment to patients on a stable treatment regimen for at least three months and who showed low levels of background inflammation, using C-reactive Protein (CRP) and the rheumatoid arthritis disease activity score of 28 joints (DAS28) (see Table 8.2).

Baseline levels of TNF-dependent activity were not significantly different in blood from RA patients on anti-TNF therapies compared to that of patients on MTX (Figure 8.3 A). Ex vivo whole blood stimulation with TNF upregulated the expression of each of the TNF-dependent modules in all groups of patients, but this was significantly attenuated in patients on anti-TNF therapies (Figure 8.3 A, B). This observation was more clearly evident in the MDM and blood derived TNF-dependent transcriptional modules, than in the keratinocyte-derived module consistent with context specific associations of each of the modules described above (Figure 8.3 B).

Using Ingenuity Pathway Analysis I searched for regulators predicted to act upstream of the genes significantly attenuated by anti-TNF therapy in TNF-stimulated blood. As expected, TNF was amongst the top predicted upstream regulators of these genes, thus validating the modular analysis (Figure 8.3 C). Therefore, I concluded that anti-TNF therapies undoubtedly attenuate the response to circulating TNF, which I modelled by ex vivo stimulation of peripheral blood, consistent with the expected neutralising activity of the anti-TNF agents.

Table 8.2 Summary of demographics, characteristics and laboratory data of the TST study population

Participant characteristics	TST				Saline Controls			
	HV	MTX	ADA	ETN	HV	MTX	ADA	ETN
n	10	10	10	8	3	3	3	3
Age (median and range)	32.5 (28-42)	61 (53-69)	59.5 (38-75)	62.5 (30-67)	31 (29-38)	76 (57-76)	68 (66-74)	36 (31-61)
Gender								
Male (%)	40	20	40	37.5	0	0	33.3	0
Female (%)	60	80	60	62.5	100	100	66.7	100
Ethnicity								
White British (%)	70	100	70	87.5	100	66.7	100	100
White other (%)	20	0	10	0	0	0	0	0
Asian Indian (%)	10	0	0	0	0	33.3	0	0
Other (%)	0	0	20	12.5	0	0	0	0
Rheumatoid factor status								
Positive (%)	n/a	60	40	37.5	n/a	66.7	66.7	33.3
Negative (%)	n/a	40	40	25	n/a	33.3	33.3	33.3
Unknown (%)	n/a	0	20	37.5	n/a	0	0	33.3
CRP mg/dl (median and range)	n/a	2.3 (<0.6-11)	1.0 (<0.6-2.4)	3.2 (<0.6-11)	n/a	2.4 (2.1-2.5)	2.15 (<0.6-3.1)	0.85 (<0.6-0.9)
DAS28 score (median and range)	n/a	2.0 (0.6-2.8)	2.6 (0.8-3.4)	2.3 (1.5-4.3)	n/a	2.9 (2.9-3.0)	3.6 (1.9-4.2)	2.4 (2.3-2.6)
PPD IFNγ ELISpot, spots/200 000 cells (median and range)	52.25 (11-300)	48.25 (10-225)	130.35 (15-300)	35.5 (11-138)	41 (15-98)	13.5 (13-50)	43.5 (38-60)	100 (20-122)
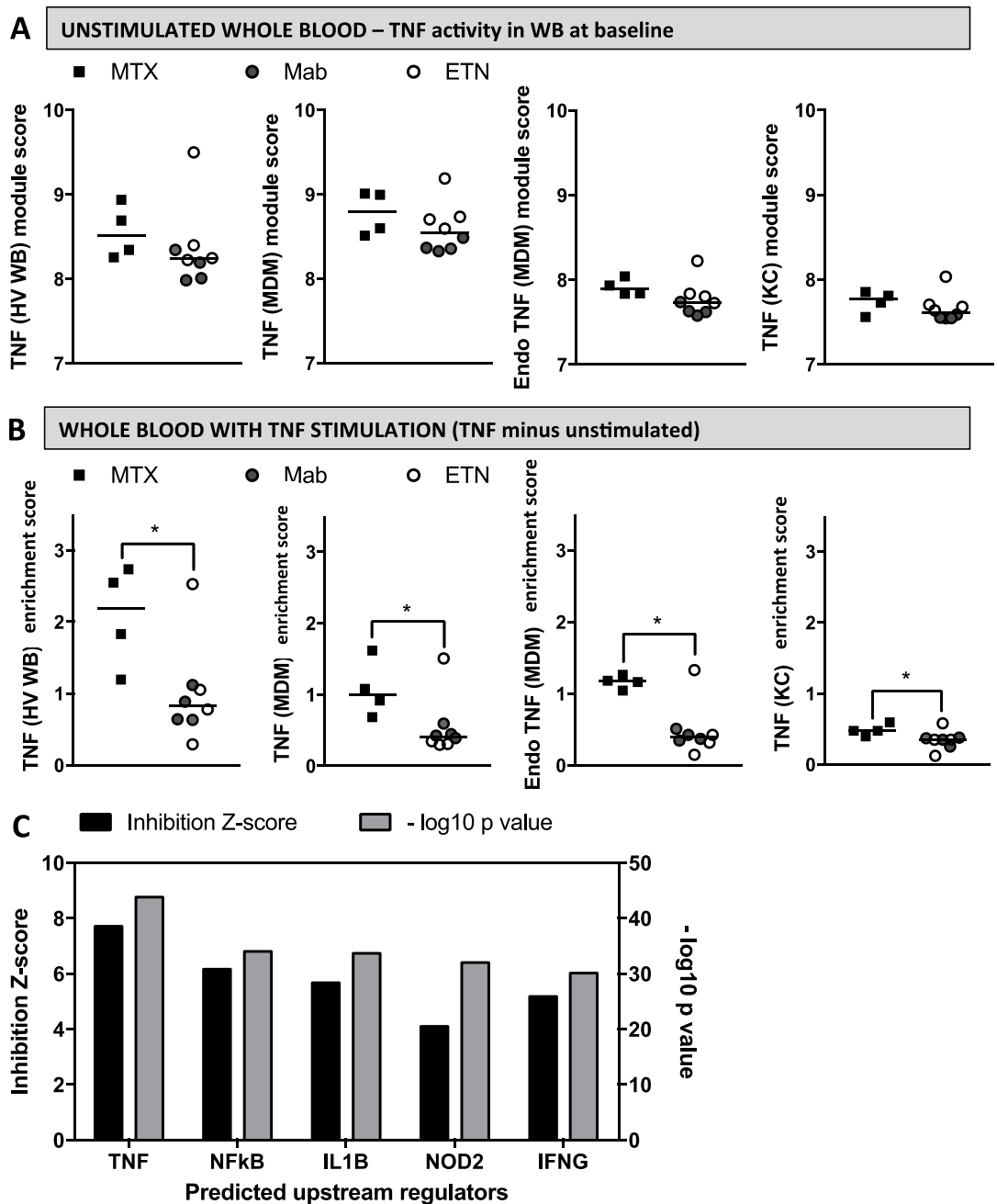
Figure 8.3 Anti-TNF therapy attenuated TNF inducible gene expression in blood

Transcriptional TNF activity, represented by each of four TNF gene expression module scores, in peripheral blood of rheumatoid arthritis patients treated with methotrexate (MTX), monoclonal anti-TNF antibodies (Mab) or etanercept (ETN) at baseline **(A)** and the change in each module score (enrichment score) associated with TNF stimulation (10 ng/mL for 3 hours) **(B)**. Data points were derived from individual experiments. Line represents the median. * $p < 0.05$ in Mann-Whitney test. **(C)** Inhibition Z-scores and overlap p values for the top five upstream regulators of genes significantly attenuated in TNF-stimulated blood during anti-TNF therapy (Mab and ETN) compared to MTX.

The modules described above (see Derivation of TNF-inducible transcriptional modules) were derived from healthy volunteer data (TNF (MDM), Endogenous TNF (MDM), TNF (HV WB)) and either primary human keratinocytes, TNF (KC).

However in RA, monocyte derived cells are highly activated with increased adhesion [66, 71] and augmented production of proinflammatory cytokines including TNF, IL6 and IL1β [60]. In addition they massively infiltrate inflammatory sites – such as the synovial membrane [61, 62, 344]. Furthermore TNF and IL6 genes are highly expressed in active RA, together with upregulation of chemokines and acute phase proteins [345].

Therefore I sought to establish whether modules derived from RA whole blood samples were consistent with those derived from healthy volunteers (HV).

In addition to the previously derived module TNF (HV WB), two further modules of TNF inducible activity were developed from peripheral blood of RA patients treated with either MTX or anti-TNF therapy by identifying significantly upregulated genes following ex vivo whole blood stimulation with TNF. Direct comparison of the genes included in each of the three modules associated with TNF activity showed significant overlap of the two modules derived from RA patients, but only modest overlap of RA modules with that derived from healthy volunteers suggesting there is a distinct set of genes which are upregulated in RA (Figure 8.4 A). However despite these differences, a similar pattern of TNF activity was seen in the unstimulated and TNF-stimulated blood (Figure 8.4 B - C), suggesting that the disease from which the modules are derived from is unimportant in this context.
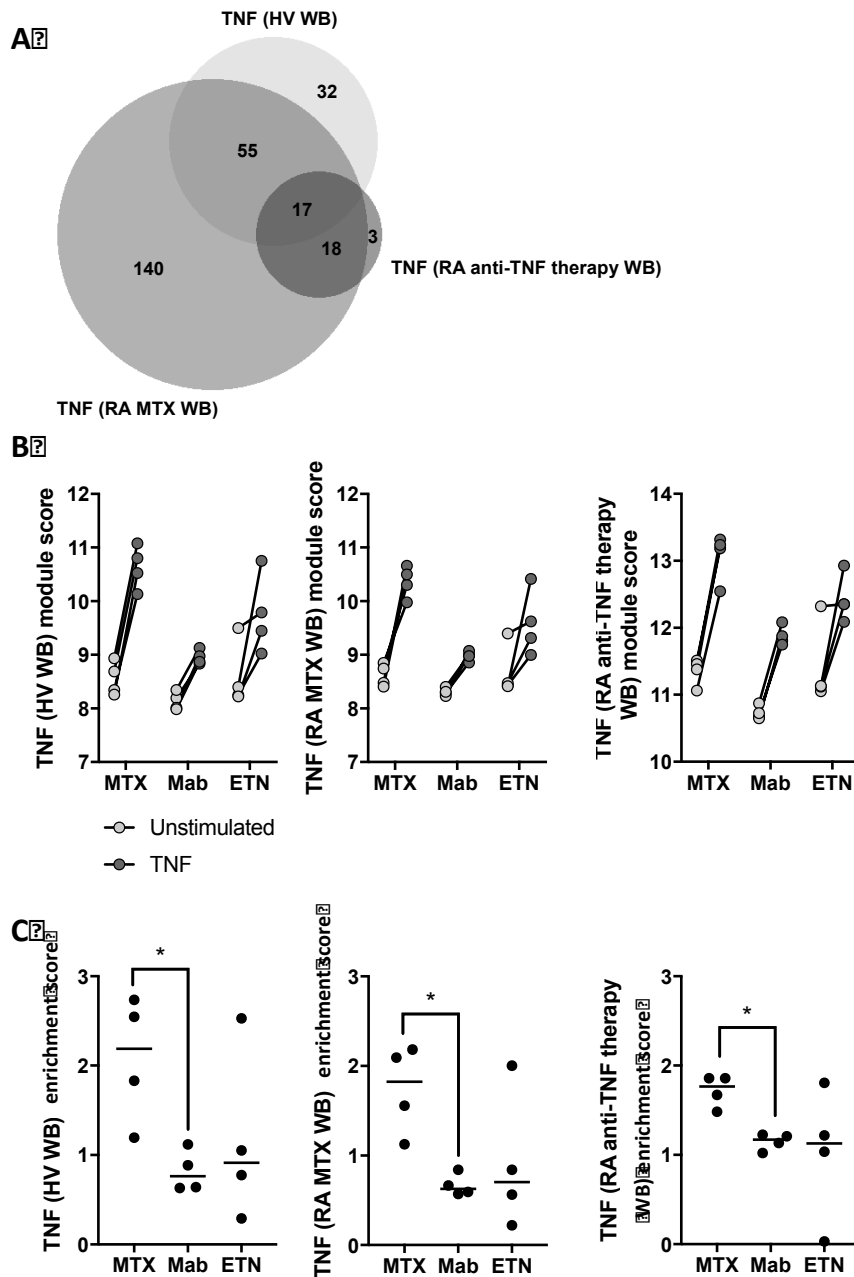
Figure 8.4 Transcriptional TNF activity derived from healthy or RA subjects is equivalent

TNF inducible activity modules developed from blood of RA patients treated with MTX or anti-TNF therapy. **(A)** 3-way Venn diagram shows the number and overlap of genes significantly upregulated at least two-fold ($p < 0.05$ in t test) in three different TNF modules. **(B)** Transcriptional TNF-inducible activity was measured in peripheral blood before and after 3 hours of ex vivo stimulation with TNF. Enrichment scores were calculated as fold change of TNF activity after TNF stimulation, and are shown with separate data points for each patient (n = 4 per treatment group). * $p < 0.05$ in Mann-Whitney test. **(C)** Enrichment of TNF activity in TNF-stimulated compared to unstimulated blood. Enrichment scores were calculated as difference between the module scores displayed in (B), and are shown as separate data points for each patient (n = 4 per treatment group) with median indicated. * $p < 0.05$ in Mann-Whitney test.

8.3.3 Quantitation of TNF activity in the tuberculin skin test (TST) in healthy participants

The TST invokes a complex multivariate immune response, which includes innate immune responses, immune cell recruitment and cytokine responses [309, 339]. In the TST of patients with active tuberculosis, our group has previously reported enrichment of the specific TNF dependent gene expression module derived from TNF stimulation of MDM [309], consistent with the hypothesis that the TST invokes TNF responses. I confirmed the presence of a largely Th1 response and infiltration of myeloid derived cells, so I next sought to consolidate and extend our previous observations in a new cohort of healthy volunteers using all four of the TNF dependent gene expression modules described above.

In line with the application of the TST as a prototypic model of Th1 dependent cell mediated immune responses, I recruited healthy individuals with detectable peripheral blood IFNγ T cell responses to ex vivo stimulation with purified protein derivative (PPD) of Mtb (see Table 8.2). Participants then received an intradermal injection of either saline or PPD and the injection sites were biopsied after 72 hours. Comparison of the genome-wide transcriptome at the site of PPD compared to saline injection revealed processes of significant enrichment of transcripts associated with multiple immune pathways that are known to participate in cell mediated immunity and included enrichment of T cell associated and IFNγ inducible genes as previously described (see Figure 8.5). Importantly, these also included robust enrichment of TNF gene expression (Figure 8.6 A) and all four of the TNF dependent gene expression modules derived from MDM, keratinocytes and blood cells representing a broad range of TNF responsive cells in the context of the TST (Figure 8.6 B).

In order to extend the assessment of TNF activity beyond transcriptional responses to the protein level, I also quantified expression of the matrix metalloproteinase (MMP)9 by immunostaining tissue sections from the site of PPD and saline injections. MMP9 expression has been associated with TNF activity in previous studies [346] and was also a component of the TNF dependent gene expression module derived from keratinocytes, identifying it as specific protein

target in the skin to discriminate between TNF and IFNγ activity. Six of the ten participants undergoing TST injection showed increased MMP9 expression compared to the range seen in participants receiving control saline injections (Figure 8.6 C). In order to consolidate the evidence for the functional relationship between TNF expression and the independently derived TNF dependent transcriptional modules or MMP9 immunostaining, I showed that the variability in TNF modules and MMP9 staining each demonstrated a statistically significant correlation with TNF transcript levels in different individuals (Figure 8.6 D-E).
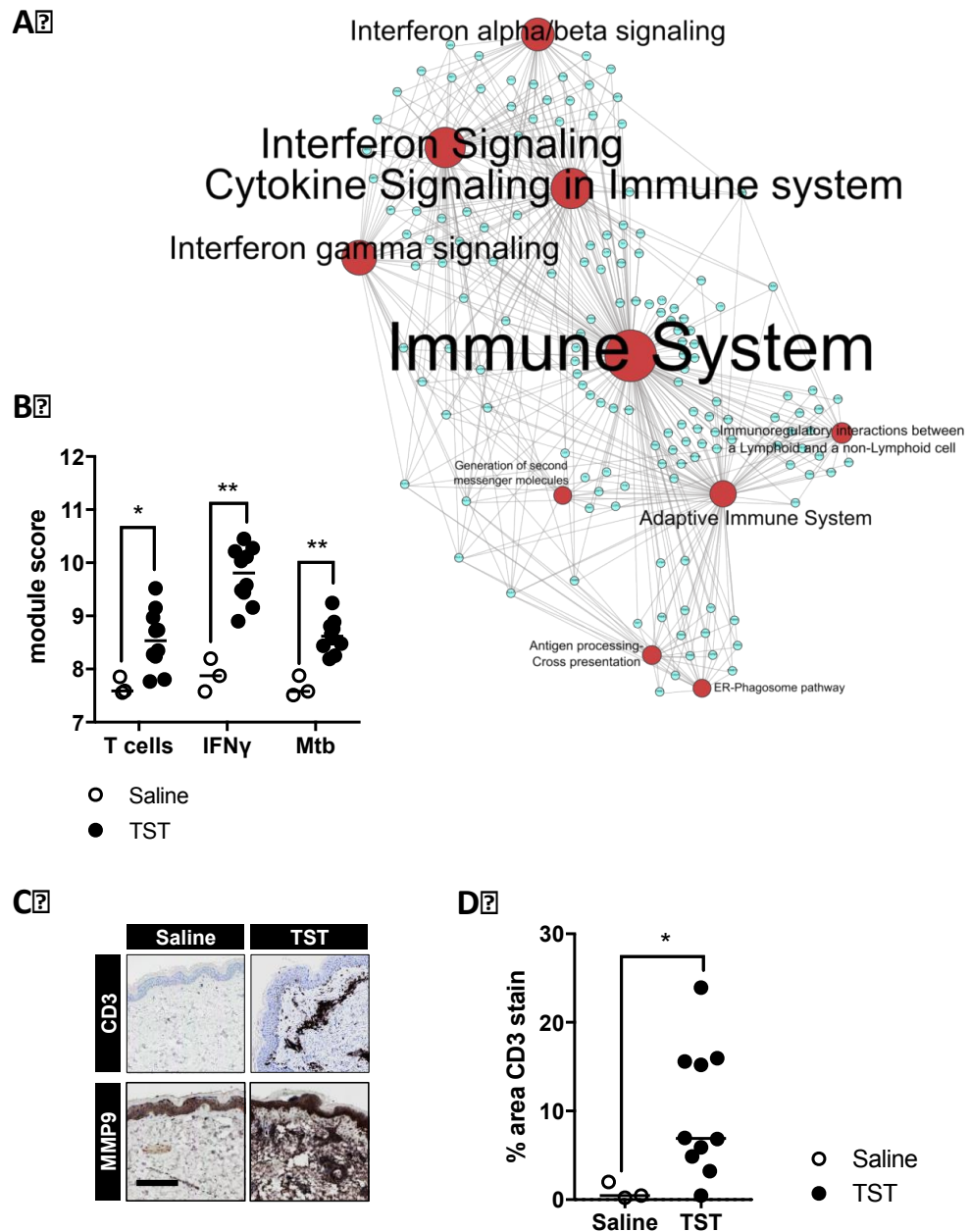
Figure 8.5 The transcriptional TST response of healthy volunteers

(A) Network graph of the top 10 Reactome pathways enriched amongst the genes upregulated in TST (N=10) compared to saline (N=3) samples from healthy volunteers. Red nodes represent pathways and blue nodes represent genes. Node and label font size denote the significance of the respective pathway and are proportional to its $-\log_{10}$ p value. **(B)** Transcriptional module scores representing T cell recruitment, IFNγ activity, and responses to *Mycobacterium tuberculosis* (Mtb) in each biopsy. **(C)** Representative immunostaining (scale bar= 500μM) of CD3 and MMP9 in skin biopsy samples from the site of TST and saline injections. **(D)** Quantitation of CD3 immunostaining in each biopsy. Data points represent individual participants and group medians are indicated by red lines. * p < 0.05, ** p < 0.01 in Mann Whitney tests.
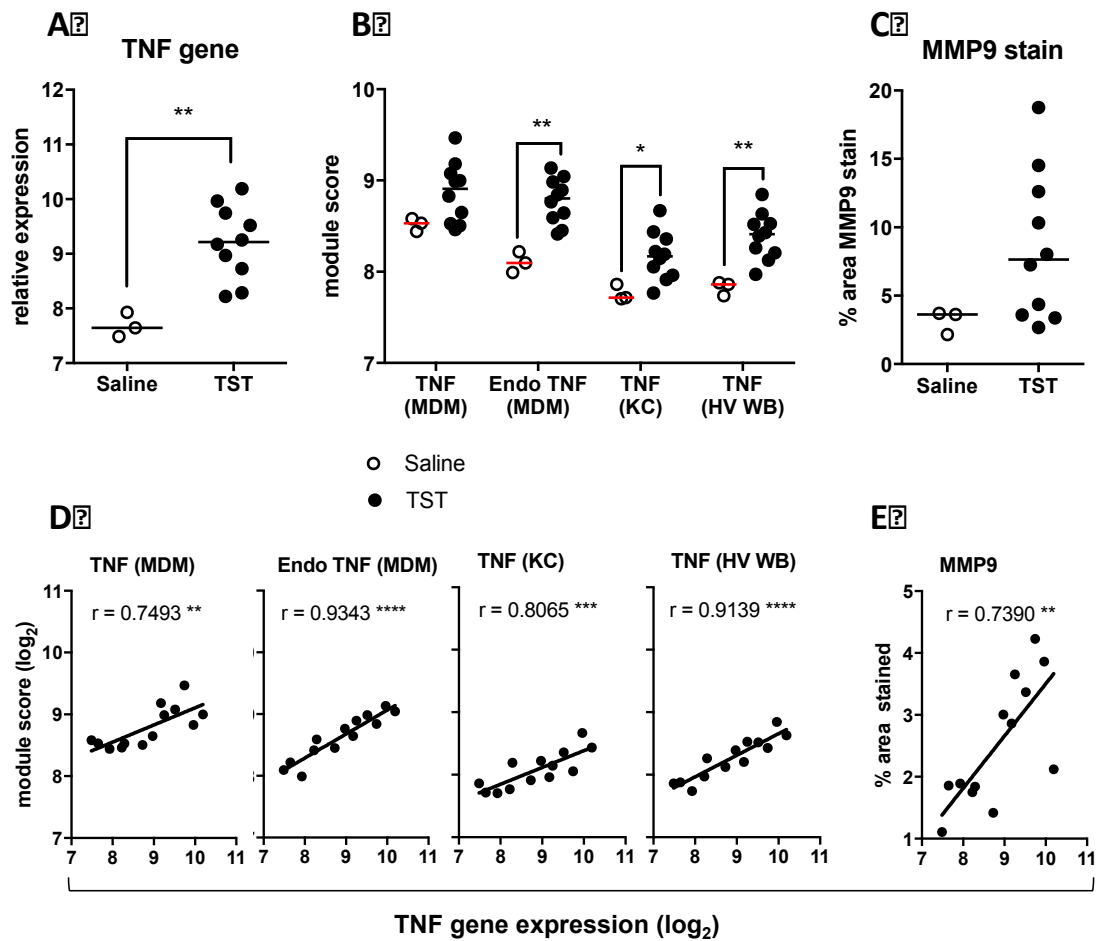
Figure 8.6 Quantitation of TNF activity in the tuberculin skin test (TST) of healthy volunteers

Skin biopsies from healthy volunteers 72 hours after injection of saline or tuberculin (TST) were analysed for **(A)** $\log_2$ TNF gene expression by gene microarrays, **(B)** module scores of four separate TNF modules derived from genome-wide microarrays, and **(C)** positive immunostaining for MMP9 in skin biopsies from the site of TST (n = 10) or control saline injections (n = 3) in healthy volunteers. Data points represent individual experiments. * p < 0.05, ** p < 0.01, Mann-Whitney test. **(D-E)** Correlation between TNF expression and each of the TNF module scores or positive MMP9 immunostaining. Correlation coefficients (r) and p values were derived from Pearson correlation analyses. **p < 0.01, ***p < 0.001, ****p < 0.0001.

8.3.4 Functional TNF activity is preserved at the site of an immunological challenge in vivo despite anti-TNF therapy.

My analyses showed that TNF constitutes a component part of the immune response in the TST and that I was able to quantify variability in the TNF response and its downstream biological activity within human tissue at the site of a standardised experimental immune challenge. Therefore, I applied this approach to test the primary hypothesis that anti-TNF therapies will attenuate TNF activity at the site of cell-mediated immune responses in vivo. To this end I compared TSTs in RA patients treated with anti-TNF therapies with those on methotrexate only. Patients in each treatment group were of similar age and ethnic background, all with low background levels of inflammation and low disease activity at the time of the study (see Table 8.2). The participants received an intradermal injection of either saline or PPD, and the injection sites were biopsied after 72 hours for transcriptional profiling and immunohistochemistry.

Firstly, I assessed whether the global TST response was affected by anti-TNF therapy. Through transcriptional profiling of the skin biopsies, I defined an integrated TST signature consisting of 595 genes that were significantly enriched in the TST compared to saline injection sites in at least one of the study groups (Figure 8.7 A). Consistent with the TST response of healthy volunteers, the integrated TST signature in RA patients was enriched for immunological pathways (Figure 8.7 B).

The enrichment of the integrated TST signature showed substantial discordance to the PPD response in blood (see Figure 8.8 A) but showed a statistically significant positive correlation with the extent of clinical induration reflecting inflammation at the site of the TST (see Figure 8.8 B). Of note, several TST biopsies showed enrichment for the integrated TST signature compared to individuals receiving saline injections despite a lack of clinically detectable inflammatory induration (see Figure 8.8 B). This was consistent with our previous observations where we were able to detect transcriptional changes in biopsies from so-called clinically anergic TSTs in healthy volunteers and HIV infected patients [309, 339], and

extends the inference that transcriptional profiling afforded greater sensitivity than clinical assessments in RA patients.
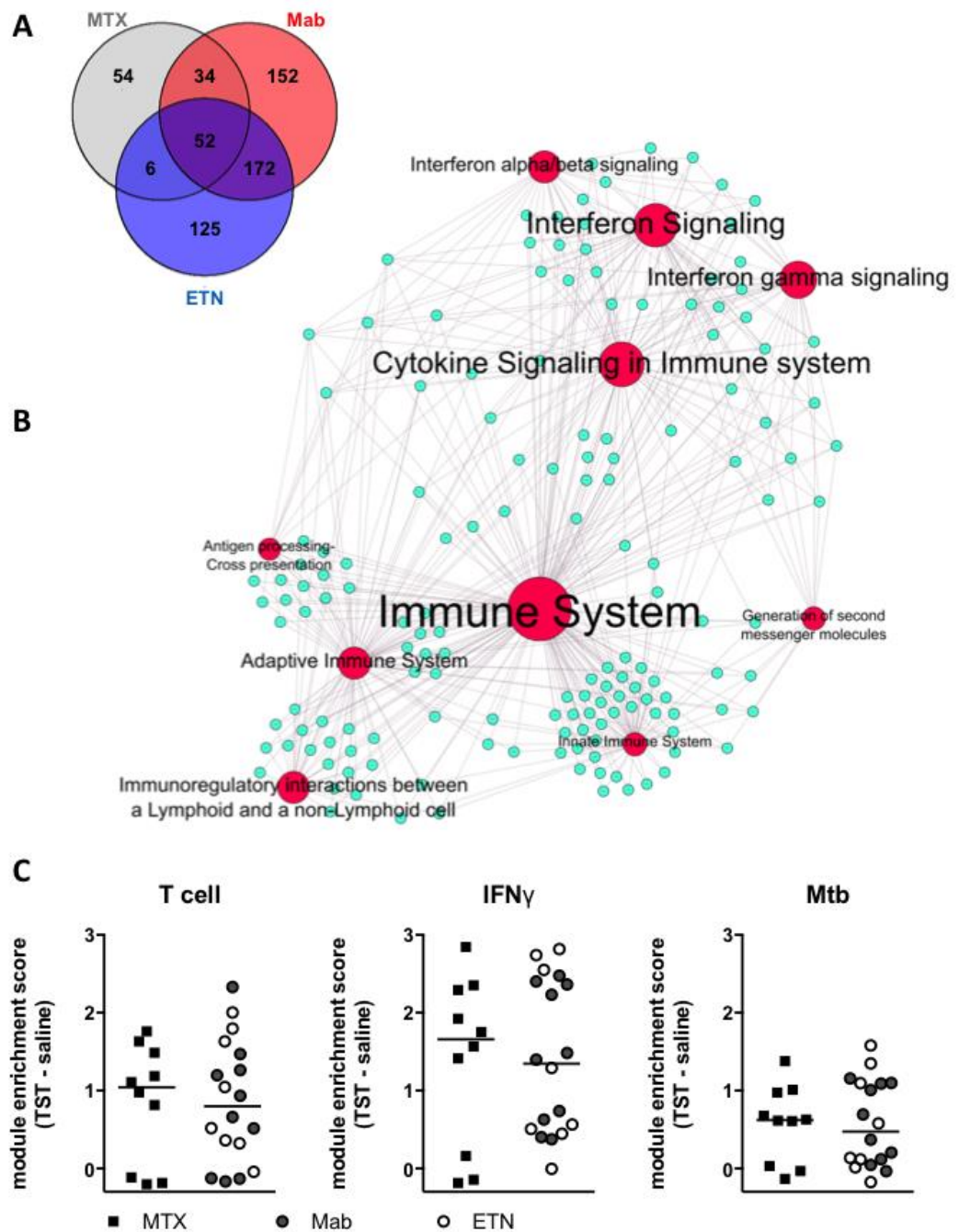
Figure 8.7 The transcriptional TST response of rheumatoid arthritis patients

(A) Number and overlap of genes upregulated in TST compared to saline samples in patients treated with MTX, monoclonal anti-TNF antibodies (Mab) or etanercept (ETN). The total number of genes displayed in this Venn diagram was combined in the integrated TST module. **(B)** Network graph of the top 10 Reactome pathways enriched in the integrated TST signature genes. Red nodes represent pathways and blue nodes represent genes. Node and label font size denote the significance of the respective pathway and are proportional to its $-\log_{10}$ p value. **(C)** Transcriptional modules representing enrichment of T cells, IFNγ activity and innate immune responses to Mtb in TST compared to saline baseline as shown by $\log_2$ module enrichment scores. Individual patients are represented with separate data points and group medians are indicated by black lines.
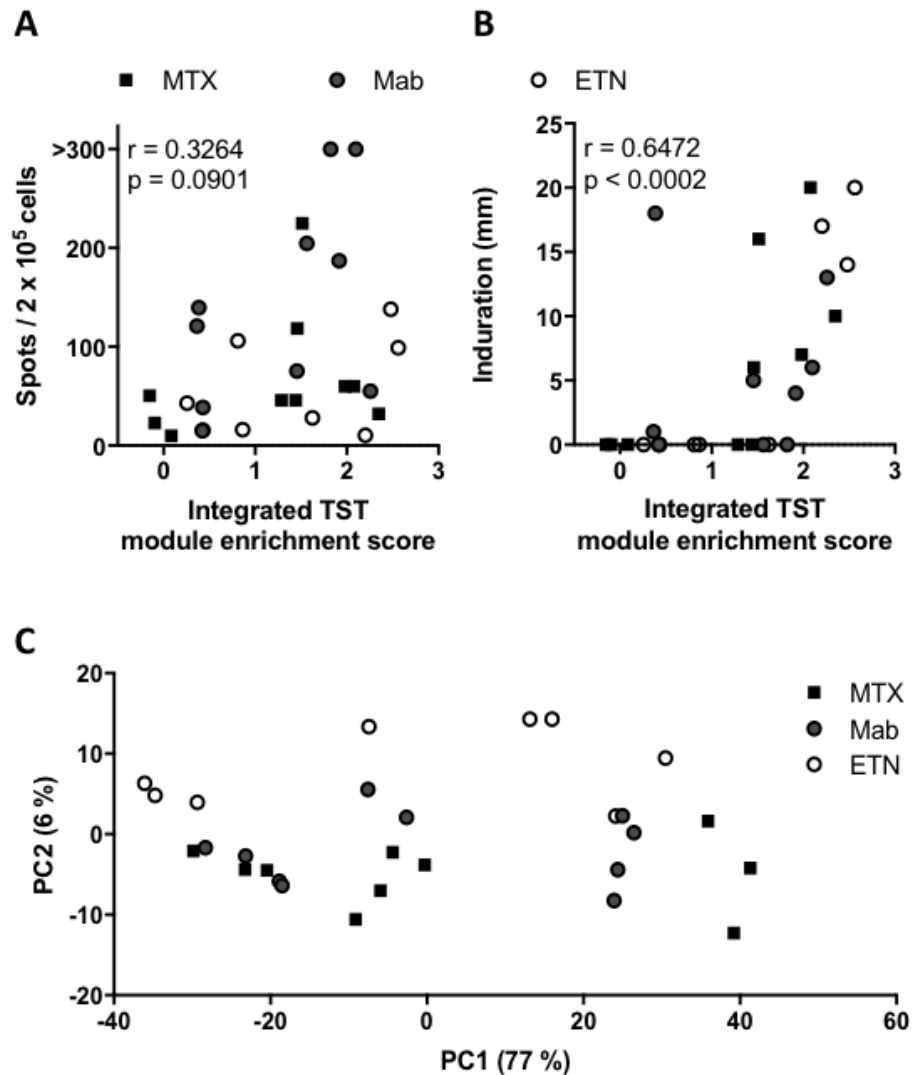
Figure 8.8 The transcriptional TST response in patients with rheumatoid arthritis

The transcriptional TST response after 72 hours in rheumatoid arthritis patients treated with methotrexate (MTX), monoclonal anti-TNF antibodies (Mab) or the soluble TNF receptor etanercept (ETN) was integrated by summarising genes upregulated in the TST compared to saline injections in at least one of the patient groups (see also Figure S2). Correlation plots of the integrated TST module enrichment score with **(A)** IFNγ responses measured by PBMC ELISpot in response to PPD stimulation, and **(B)** skin induration at the site of injection 72 hours after TST. Correlation coefficients (r) and p values were derived from non-parametric Spearman correlation analyses with all data points (n = 28). **(C)** Principal component (PC) analysis of integrated TST signature genes showed no treatment-specific clustering in PC 1 and 2, which together explained 83% of variation in this data set. Data points represent individual experiments.

Principal component analysis (PCA) of the integrated TST signature gene expression levels and their fold increases in TSTs compared to saline injections revealed no treatment-specific clustering of the data from different groups of RA patients (see Figure 8.8 C). Therefore, I concluded that the broad range of transcriptional responses in the TST of RA patients was not affected by anti-TNF therapy. Consistent with this analysis, I found comparable enrichment of a T cell associated transcriptional signature, and comparable enrichment of IFNγ dependent gene expression modules in all RA patients (see Figure 8.7 C). Inter-individual variation exceeded any effect of anti-TNF therapies on cellular recruitment and Th1/IFNγ responses in the TST. The TST response in RA patients included enrichment of TNF gene expression (see Figure 8.9). The extent of TNF induction was similar in all patient groups and therefore not affected by anti-TNF therapy (see Figure 8.10 A). Surprisingly however, comparable levels of the TNF dependent gene expression modules were also observed in all the study groups (see Figure 8.9C and Figure 8.10 B). In accordance with these transcriptional data, the expression of MMP9 was increased to comparable degree in the tuberculin-injected skin of all RA patients, independent of anti-TNF therapy (see Figure 8.9 B - C and Figure 8.10 C - D). I conclude that TNF-inducible activity in the TST was not attenuated by anti-TNF therapy in RA patients, and by extension I infer that anti-TNF therapies in humans do not attenuate inducible TNF activity within tissue foci of immunological responses to an in vivo challenge.
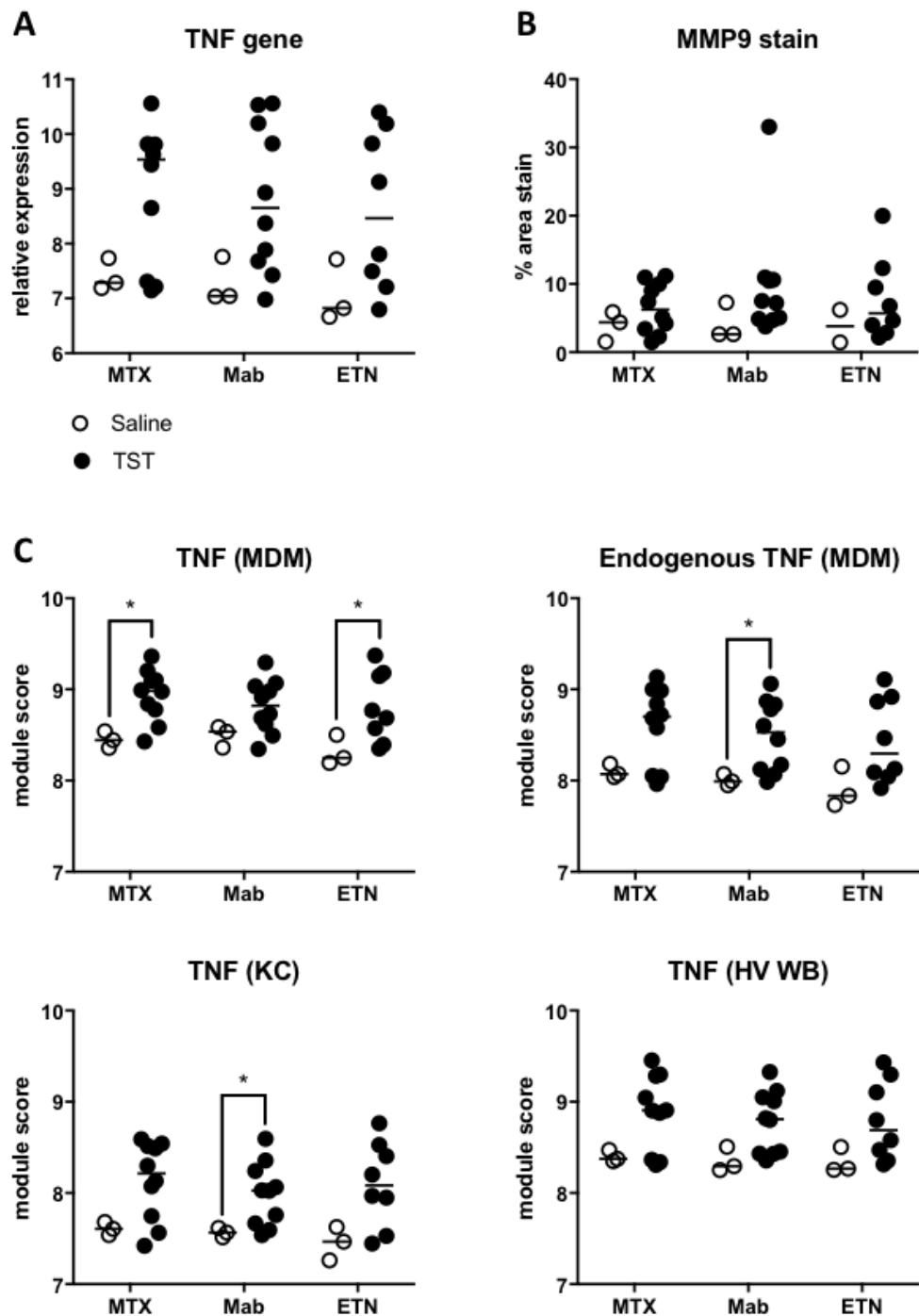
Figure 8.9 TNF gene expression and TNF module expression in skin biopsy transcriptomes from the site of TST or saline injections in rheumatoid arthritis patients

(A) TNF gene expression, and TNF activity quantified with **(B)** positive MMP9 immunostaining or **(C)** TNF module gene expression scores in TST and saline samples from rheumatoid arthritis patients on methotrexate (MTX), anti-TNF antibodies (Mab) or etanercept (ETN). Individual patients are represented with separate data points and group medians are indicated by black lines. Data points represent individual experiments. *$p < 0.05$, in Mann-Whitney test.
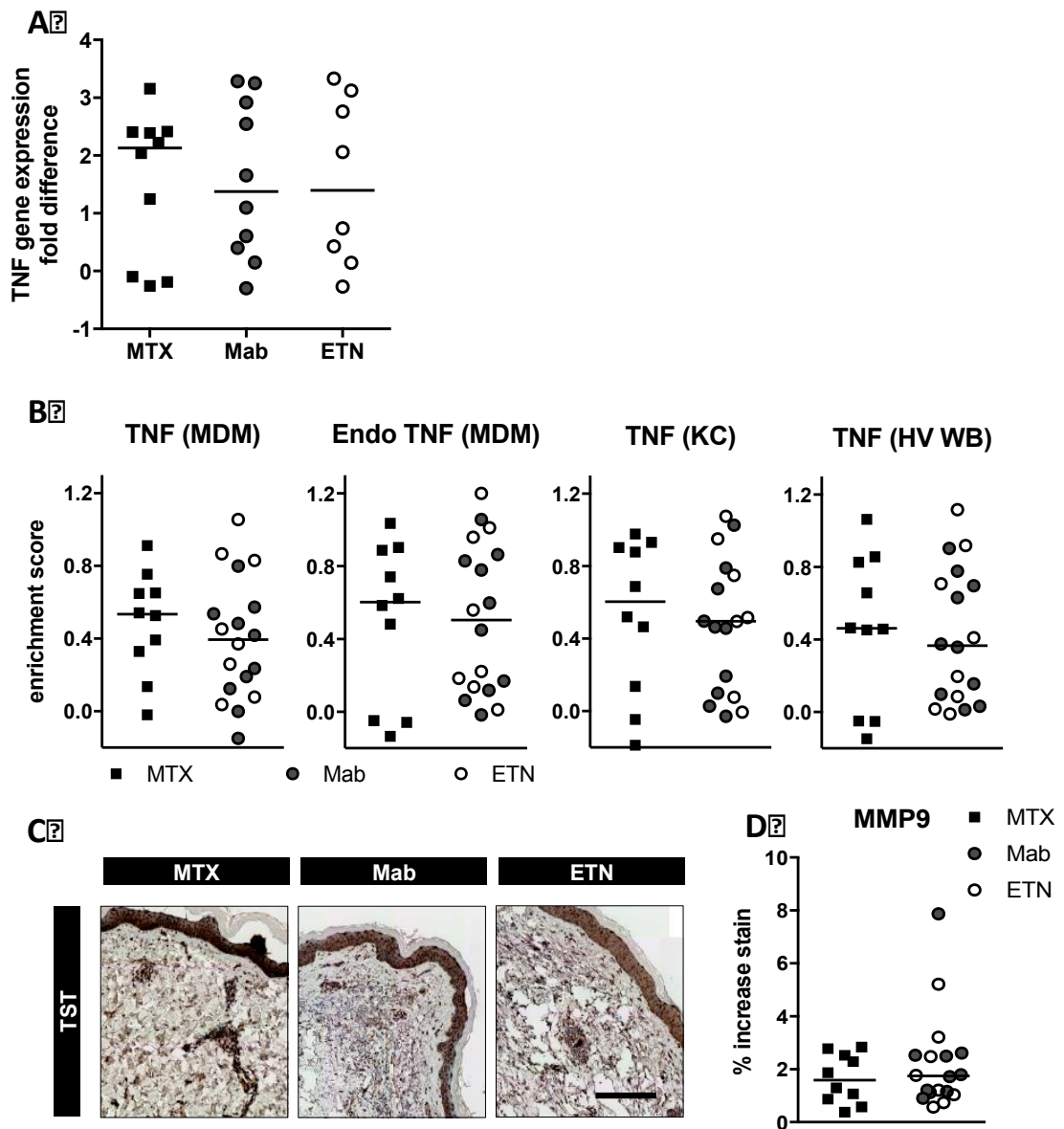
Figure 8.10 Inducible TNF activity is preserved in the TST of rheumatoid arthritis patients despite anti-TNF therapy

The change in **(A)** TNF gene expression and **(B)** module scores of four separate TNF-dependent transcriptional modules derived from genome-wide microarrays, in skin biopsies from the site of TST of rheumatoid arthritis patients treated with methotrexate (MTX, n = 10), monoclonal anti-TNF antibodies (Mab, n = 10) or etanercept (ETN, n = 8), compared to patients receiving saline injections (n = 3 per group). **(C)** Representative images of MMP9 immunohistochemical staining (scale bar represents 500μm) and **(D)** positive MMP9 staining in skin biopsies from the same patients.

Data points are derived from individual experiments. There were no significant differences between anti-TNF therapies (Mab & ETN) and MTX treatment in Mann Whitney tests (p>0.05).

117

8.4 DISCUSSION

The rationale for the development of anti-TNF therapies in RA was based on local expression of TNF at the site of disease, the understanding of its functional role in proinflammatory immune cell recruitment to tissues and data from animal models in which over expression of human TNF caused a chronic inflammatory arthritis which could be ameliorated by anti-TNF therapy [108]. In this context, the unequivocal therapeutic effects of anti-TNF antibodies and ETN in RA led to two conclusions. Firstly, that anti-TNF therapies mediate their beneficial effect by inhibiting TNF activity at the site of disease and consequently, that TNF function makes an essential contribution to the immunopathogenesis of disease. Secondly, the same rationale applied to the detrimental effect of TNF inhibition, as increased risk of certain infections amongst patients on anti-TNF therapies [186, 321], suggested that TNF activity is an essential component of protective immune responses.

Several lines of evidence suggest that despite the wealth of literature supporting these conclusions the effects of anti-TNF agents are not wholly understood. For example, it has become evident that anti-TNF antibodies and ETN have differential biological activity. Although, they mediate similar therapeutic effects in RA, anti-TNF antibodies but not ETN are effective in other chronic inflammatory disease such as Crohn's colitis [347]. Likewise, the use of anti-TNF antibodies incurs significantly greater risk of active tuberculosis than that of ETN [186, 187, 327]. In addition, our group has previously shown that anti-TNF antibodies, but not ETN, induce increased emergence of functionally active regulatory T cells [151]. Counterintuitively, this last example may be mediated by augmentation of TNF function through enhancing the interaction of membrane bound TNF and TNFR2 [153]. Therefore, the biological effects of anti-TNF therapies may extend beyond the canonical view that they block TNF activity at the site of immune responses in tissue.

In order to test this question directly, I adopted a human experimental challenge approach using the TST as a standardised stimulus with which to invoke a focus of cell mediated immune responses [339]. Transcriptional profiling from skin punch

biopsies from the site of TST afforded us the opportunity to make comprehensive genome-wide assessments of immune responses at the molecular level with unprecedented sensitivity. I complemented this approach with the derivation of independent experimentally derived TNF-dependent gene expression modules representing the functional bioactivity of TNF. The evaluation of TNF activity at the transcriptional level revealed striking differences between TNF-dependent transcriptional responses in macrophages, blood cells and keratinocytes. The mechanisms underlying the context specificity in the functional activity of TNF likely reflect distinct epigenetic landscapes in different cell types [348]. Despite the limited overlap between each of the four TNF-dependent gene expression modules I derived, in silico bioinformatics analysis that yielded TNF as the top predicted upstream transactivator of each list of genes. These independently derived transcriptional modules associated with different cell types present at the site of the TST, namely keratinocytes, macrophages and recruited blood leukocytes enhanced the sensitivity of my analysis and provided cross-validation.

Disease or treatment-specific modules of TNF activity developed from whole blood of patients with RA demonstrated a distinct set of genes which are upregulated in RA, however despite these differences, a similar pattern of TNF activity was seen in the unstimulated and TNF-stimulated blood suggesting that the disease from which the modules are derived from is unimportant in this context.

In healthy subjects, I showed that TNF expression was enriched in the TST and that the variability in its enrichment correlated with concordant enrichment of each of the four TNF dependent transcriptional modules. In addition, I extended this analysis to the protein level, by showing correlation with MMP9 as a TNF-inducible protein [349]. Taken together these data showed unequivocally that the TST invokes a functional TNF response and that I can quantify variability in TNF bioactivity in situ within this response.

In order to the test the effects of anti-TNF agents I sought to compare RA patients with quiescent disease on methotrexate to those receiving anti-TNF agents. In blood, baseline TNF bioactivity represented by the expression of TNF modules was not significantly different between RA patients on MTX and those on anti-TNF

therapies. Therefore, the data suggest that the widely reported diminution of pro-inflammatory mediators in RA patients with quiescent disease following anti-TNF therapy may not be related to a targeted reduction in steady state expression of TNF-dependent gene expression, but could simply represent successful control of active disease in patients on treatment with methotrexate or anti-TNF therapies albeit through possibly divergent mechanisms. Nonetheless, I confirmed that enrichment of TNF-dependent gene expression following ex vivo TNF stimulation of whole blood was significantly attenuated in blood from patients on anti-TNF agents. However, in the same groups of patients, receipt of anti-TNF agents did not attenuate the enrichment of the TNF-dependent transcriptional modules or MMP9 immunostaining within the TST. Hence, I concluded that although anti-TNF agents can inhibit TNF bioactivity in the circulation, but they have no discernable effect on in vivo TNF activity at the site of an acute immune challenge.

The most likely explanation for the observations in skin is that anti-TNF agents do not reach sufficient concentration within the tissue microenvironment to neutralise the level of TNF produced in an acute cell mediated immune response, modelled by the TST. Therefore, I speculate that their therapeutic effects may be achieved by neutralising lower levels of TNF in foci of chronic inflammation or by neutralising the endocrine functions of circulating TNF. The latter would suggest a previously unrecognised model by which TNF may contribute to the pathogenesis of RA or other inflammatory diseases for which anti-TNF agents have been deployed successfully. Although anti-TNF therapies are associated with increased risk of some infectious diseases, the fact that most recipients of anti-TNF therapy do not suffer from recurrent infections, has challenged the view that TNF is a non-redundant component of host-protective immune responses to infection. The data reveal this inference to be false because patients receiving anti-TNF therapies can exhibit normal TNF activity at the site of an acute inflammatory challenge. My findings are consistent with a model in which the contribution of TNF to chronic inflammatory processes is more sensitive to neutralisation by anti-TNF agents than TNF's actions in acute inflammation. The differential sensitivity of acute and chronic inflammation most likely reflects different levels of TNF and generates the

therapeutic window in which anti-TNF agents have provided such effective treatments for chronic inflammatory conditions. In such a model, current anti-TNF therapies may be ineffective in acute inflammatory pathology, and strategies that effectively inhibit TNF activity in acute inflammation may incur much greater risk of acute infections.

8.5   CONCLUSION

In conclusion, using modular analysis of TNF inducible bioactivity, I was able to use transcriptional processing of blood and skin to assess the response to an acute inflammatory challenge (either TNF in whole blood, or the TST) in RA patients treated with anti-TNF therapy.

Using the TST as an in vivo challenge model to test the immune response to Mtb, I have established by genome wide transcriptional processing and immunohistochemistry, that cellular infiltration or TNF inducible activity are not attenuated by either RA or anti-TNF therapy, compared to healthy participants. Hence anti-TNF therapy is unable to block a surge in TNF activity following an acute challenge in the skin.

9 THE EFFECT OF ANTI-TNF THERAPY ON MYCOBACTERIAL GROWTH IN MONOCYTE-DERIVED MACROPHAGES

9.1 INTRODUCTION

Immunological protection against *Mycobacterium tuberculosis* (Mtb) is dependent on cell mediated immunity, largely though interactions between Mtb-infected macrophages and T cells. Importantly immunological control of mycobacterial growth is thought to be the main determinant in the outcome of Mtb infection [350].

Following ingestion of Mtb, alveolar macrophages become activated and secrete pro-inflammatory cytokines, including TNF, IL6 and IL12 [222]. TNF is a critical cytokine in the host response to Mtb, as it stimulates neutrophils [351] and macrophages [352] to act in an autocrine and paracrine fashion to stimulate apoptosis and release ROS and RNS to augment killing of intracellular mycobacteria in animal models. Upon exposure to Mtb, mouse macrophages are stimulated to produce TNF in a TLR2-dependent manner, which is abolished in TLR2 knockout cells [353]. TNF has also been shown to be important in controlling both early and latent infection in non-human primates (NHP) [282], and is thought to be essential for the formation and maintenance of granulomas, by regulating the tight apposition of macrophages and lymphocytes [284]. High concentrations of TNF have been found in the broncho-alveolar lavage fluid of smear-positive TB patients (with high bacterial burden) [354]. Neutralisation of TNF by anti-TNF antibody therapy in mice and humans, or in TNF-knockout mice increases the susceptibility to granulomatous infections [183, 277, 285].

However the role of TNF in the host response to TB is not entirely clear, as it appears to have varying effects in different animal species. In mouse models, administration of anti-TNF antibodies resulted in a loss of granuloma cellularity and protective function [287, 288] and displayed more disorganised granuloma [277, 289, 291], and rabbits treated with etanercept (ETN) displayed increased bacilliary load and larger granuloma with more extensive necrosis and cavity formation [286]. Moreover latently-infected NHP and zebrafish maintained a

normal granuloma structure following TNF neutralisation, with an increased bacterial burden [282, 290], suggesting early granuloma formation is independent of TNF signalling, at least in NHP and zebrafish.

Much of the role of TNF in limiting growth of intracellular Mtb infection can be attributed to the induction of apoptosis in infected cells [355-357]. Indeed, addition of TNF to *Mycobacterium avium*-infected macrophages in vitro reduced *M. avium* growth over seven days, but also destroyed approximately 40% of the cells in culture [358]. TNF itself was innocuous to mycobacteria growing in tissue culture medium or liquid culture (7H9) [358], suggesting that TNF activates the macrophages to kill the mycobacteria, and subsequently the macrophages may undergo apoptosis. However it is not clear whether the apparent killing of mycobacterium is an artefact of macrophage cell death from toxic doses of TNF.

Further to this, Mtb itself has the ability to downregulate host cell TNF production in order to evade the host immune mechanisms. It has been demonstrated that specific Mtb genes encode mycobacterial components that can downregulate macrophage production of TNF [359-361]. Certain mutant Mtb strains deficient in such downregulating elements, have attenuated mycobacterial virulence [359-362].

In vitro addition of anti-TNF antibody therapy, but not ETN, inhibits phagosome maturation of infected macrophages despite pre-treatment with IFNγ [363], implying only anti-TNF antibody therapy is able to inhibit intracellular mycobacterial killing, albeit not directly tested.

Patients with RA are at increased risk of infection [316] and in particular TB risk is 2-4 times higher than the general population [189, 192-194]. The mechanisms for this have not been studied and although RA patients are known to have more highly activated monocytes and macrophages [94, 364], it is not clear whether these monocytes and macrophages are more permissive to infection than those from healthy subjects. Therefore I hypothesised that MDM from RA patients would be more permissive to Mtb infection, and have less control of mycobacterial growth compared to MDM from healthy subjects.

The risk of active TB increases significantly to 7-17 times higher than the general population, when patients are treated with anti-TNF therapy [186, 187, 189, 327]. Although it has been shown that anti-TNF therapy may increase bacillary burden, and is likely to reactivate LTBI by resuscitating dormant intracellular mycobacteria [295], it has not been clearly demonstrated that anti-TNF therapy has a direct effect on intracellular mycobacterial growth in macrophages. Given these findings, I hypothesised that anti-TNF therapy is also able to abrogate intracellular mycobacterial killing, thus leading to an increased total Mtb burden. In order to investigate this I treated Mtb-infected MDM with anti-TNF therapy and quantified mycobacterial growth inside and outside of MDM.

9.2   OBJECTIVES

1. To determine whether monocyte-derived macrophages (MDM) from rheumatoid arthritis (RA) patients are more permissive to Mtb infection than those from healthy subjects.

2. To assess the impact of anti-TNF therapy on intra- and extracellular mycobacterial growth in MDMs and to determine whether the effect differs in MDMs from RA patients and healthy subjects.

9.3 RESULTS

9.3.1 Development of methodology to use fluorescent mycobacteria to quantitate intracellular and extracellular mycobacteria

Traditional methods of quantifying mycobacterial load in cell cultures or infected tissues depends largely on seeding bacteria onto culture media (Middlebrook 7H10 or Lowestein Jensen) and counting colony-forming units after weeks of culture. Due to a slow growth rate, methods such as colony counting are potentially biohazardous, laborious, time-consuming and often inaccurate due to mycobacterial clumping.

Together with Dr Elspeth Potton (PhD student in our group), we developed a high throughput flow cytometric assay for quantitation of intracellular mycobacterial growth. Using a virulent strain of Mtb, H37Rv, which constitutively expresses a fluorescent reporter protein mCherry fluorophore (H37Rv mCherry) (a gift from Tanya Parish, QMUL), which is stable and detectable under hypoxic conditions in vitro, non-replicating cells or in low cell density conditions [365].
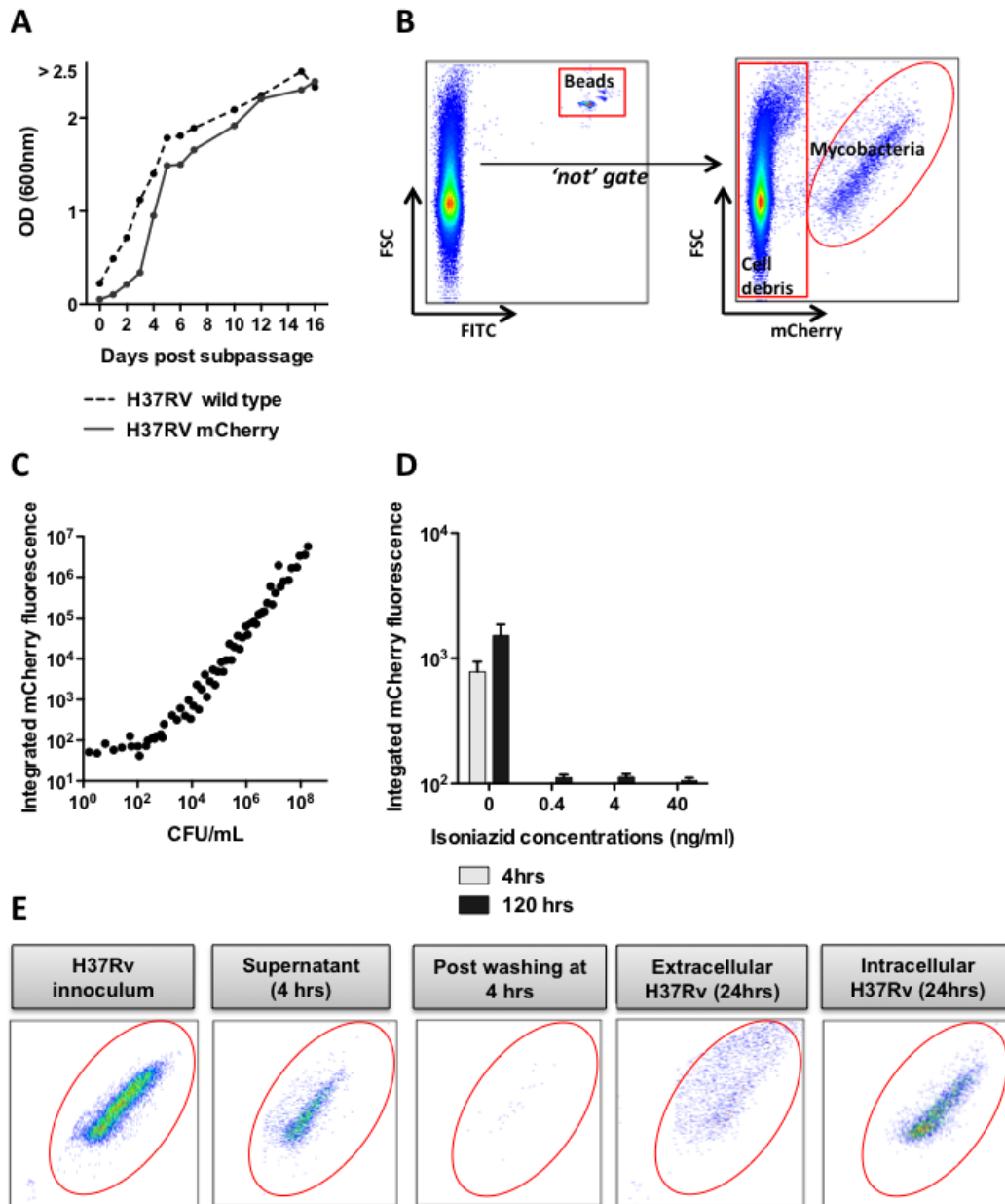
Figure 9.1 Growing and quantifying Mtb - H37Rv mCherry

(A) H37Rv wild type and H37Rv constitutively expressing an mCherry fluorophore (H37Rv mCherry) were grown simultaneously and measured by optical density (OD) at varying time points after passage to assess log-phase growth. Fluorescence of H37Rv mCherry was quantified by flow cytometry. **(B)** The gating strategy for measuring fluorescence of mycobacteria involves enumerating fluorescent beads and mycobacteria, excluding debris and calculating mycobacteria per bead. **(C)** mCherry fluorescence measured by flow cyotmetry is proportional to a standard method of quantification – colony forming units (CFU) and with a sensitivity of 100 mycobacteria. **(D)** Growth of intracellular mycobacteria from 4-120hours +/- varying concentrations of isoniazid. Bars represent mean +/- SEM, (n = 4). **(E)** Representative flow cytometry plots to demonstrate inoculum of H37Rv mCherry, supernatant at 4 hours post infection, supernatant after effective washing at 4 hours and extracellular H37Rv mCherry collected from supernatant of infected MDMs at 24 hours, and following lysis of MDM, intracellular mycobacteria, measured at 24 hours.

In order to show that the constitutive expression of mCherry by H37Rv did not affect bacterial fitness, I compared growth curves with wild type H37Rv, measured by optical density (see Figure 9.1 A). I was able to confirm a linear correlation between flow cytometric quantitation of H37Rv mCherry and conventional colony counting for bacteria grown on agar medium (see Figure 9.1 C). This relationship plateaued at ~100 bacteria, representing the lower limit for sensitivity of flow cytometric quantitation. I confirmed that intracellular mycobacterial growth can be quantitated in this assay by showing reduction of intracellular H37Rv mCherry in infected MDM incubated with increasing concentrations of the anti-mycobacterial drug, isoniazid (see Figure 9.1 D). A limitation of this approach remains that we do not know the rate at which fluorescence from dead bacteria decays and were not able to easily evaluate bacterial killing as distinct from lack of bacterial growth.

To confirm that it is possible to discriminate between intra- and extracellular bacteria in Mtb-infected MDM cultures, adherent MDM cultures were infected with H37Rv mCherry at a multiplicity of infection of one (MOI 1) for four hours to allow H37Rv uptake into adherent macrophages. After four hours, extracellular H37Rv mCherry in the supernatant was removed from the culture, and adherent cells were washed thoroughly, demonstrating little evidence of H37Rv mCherry in supernatants after washing (see Figure 9.1 E). Culture medium was replaced and at further time-points (24 or 120 hours), the supernatant of the cell culture was again analysed by flow cytometry, representing extracellular bacteria (see Figure 9.1 E). Adherent MDM were then washed thoroughly again to ensure removal of any remaining extracellular bacteria, and subsequently cells were lysed with distilled water to release intracellular bacilli, which were readily detectable (see Figure 9.1 E).

Plates of infected and uninfected MDMs were also set up in parallel to quantify cell number in each experiment and at each timepoint using microscopy (see Figure 9.2), to assess whether cell number varies over time and subsequently to normalise intracellular mycobacteria to cell number.

4 hours post infection	24 hours post infection	120 hours post infection
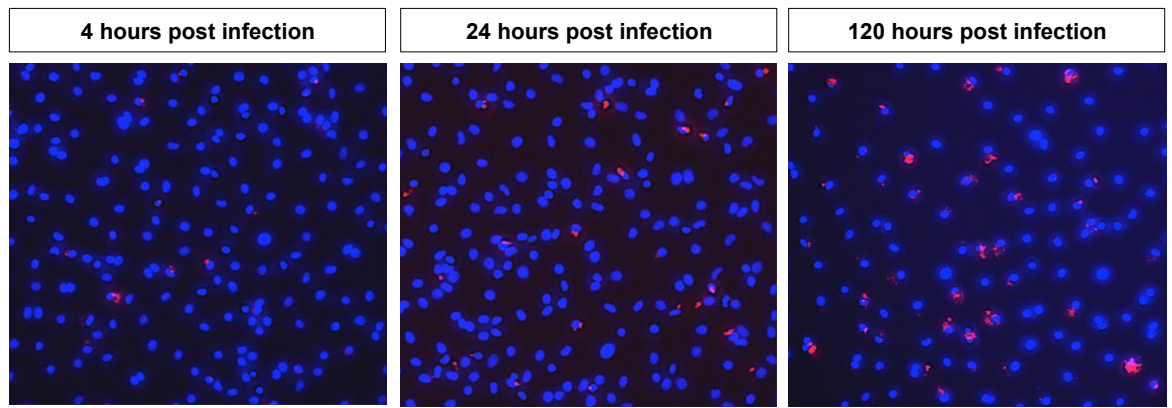
Figure 9.2 Imaging of H37Rv mCherry infected monocyte-derived macrophages

H37Rv mCherry infected macrophages were fixed with 8% paraformaldehyde (PFA) before permeabilisation with 0.1% Triton X and nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI). These are representative plots from healthy MDM at 4, 24 and 120 hours post infection with H37Rv mCherry. DAPI staining is shown as blue, mCherry as red.

9.3.2 Monocyte-derived macrophages from RA patients are not more permissive to Mtb infection compared to healthy controls

It is not fully understood why patients with RA (on standard disease modifying treatment) have a 2-4 times increased risk of TB infection compared to the healthy population [189, 192-194], therefore I tested whether this increased TB infection risk is due to increased permissivity by RA macrophages. I infected MDM from HV and RA patients treated with MTX with H37Rv mCherry and quantified intra- and extracellular TB for an estimate of total TB burden.

Firstly I established that cell number was not significantly affected by either H37Rv mCherry infection, or RA disease (see Figure 9.3 A), by comparison to uninfected MDM cultures from healthy volunteers. However cell numbers in the uninfected, infected, healthy and RA groups were all significantly reduced at 120 hours post infection. As this occurred across all culture conditions, this is likely to be due to technical handling of the cultures (such as the vigorous washing steps to remove extracellular bacteria), rather than a biologically relevant phenomenon.

Intra-, extracellular and total H37Rv mCherry burden was assessed at 4, 24 and 120 hours post infection (see Figure 9.3 C). When H37Rv mCherry is normalised to the inoculum only, RA MDM are more permissive to Mtb infection in the first 24 hours, however this phenomenon does not persist up to 120 hours. In addition, after normalising H37Rv mCherry to cell number in each experiment (see Figure 9.3 B), RA MDM are more permissive to intracellular Mtb within the first 24 hours, compared to healthy volunteers this may be due increased loss of cells at 24 hours in the RA group in individual experiments, (although a significant overall difference between cell number between HV and RA MDM was not seen). However Mtb is a slow-growing pathogen and thus the majority of growth was seen 120 hours post infection, where there was no overall increased permissivity of RA macrophages to Mtb.

Extracellular data could not be measured at 4 hours, as the remaining inoculum that had not been phagocytosed would have confounded this. Significantly more extracellular H37Rv mCherry was seen at 120 hours in both groups, which reflected reduced cell number, probably due to live Mtb being released from dead

or dying cells. However, there was no significant difference in extracellular Mtb between groups, and this overall Mtb burden was not significantly different between RA and HV MDM (see Figure 9.3 C). Therefore RA MDM overall are not more permissive to TB infection than healthy MDM.
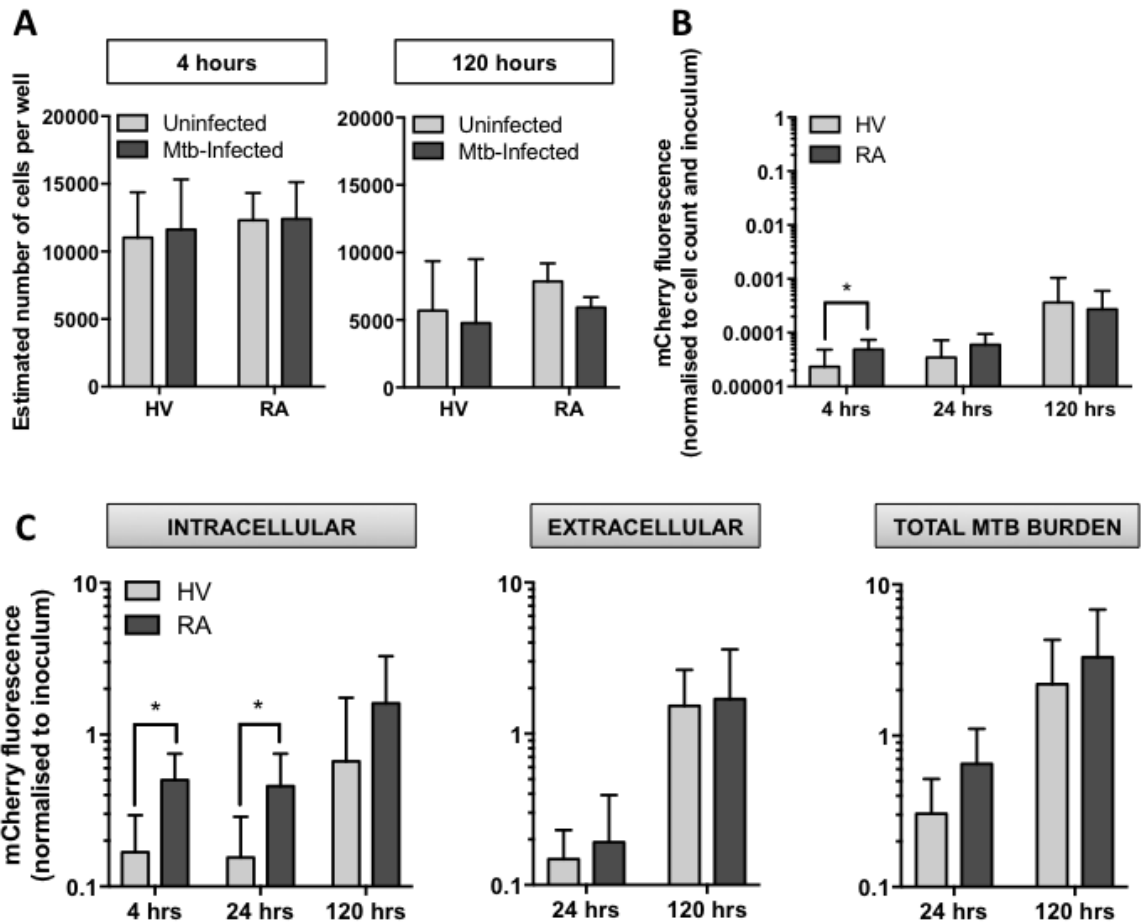
Figure 9.3 Monocyte-derived macrophages from RA patients may show an early deficit , but not sustained permissivity to Mtb infection compared to HV

Monocyte-derived macrophages (MDM) from healthy volunteers (HV) and rheumatoid arthritis (RA) patients with stable disease, treated with methotrexate, were infected with H37Rv mCherry at a multiplicity of infection of 1. **(A)** Adhesive MDM were stained with DAPI and imaged using the Hermes microscope. WiSoft software was used to quantify number of cells per well in Mtb-infected and uninfected samples, 4 hours and 120 hours post infection (24 hours post-infection data also collected, but not shown) in healthy volunteers (n = 6) and RA patients (n = 6) **(B)** 4 hours post-infection, wells were washed vigorously and lysed with distilled water or replenished with media for 24 or 120 hours, at which point they were lysed to quantitate intracellular bacteria. Intracellular mycobacteria were quantitated using flow cytometry and normalised to infecting inoculum and cell number for each experiment (HV n = 6, RA n = 6). **(C)** Intracellular bacteria were collected at 4, 24 and 120 hours post infection, and extracellular bacteria were collected and quantified from supernatants of Mtb-infected MDM at 24 and 120 hours post-infection. This was quantified by flow cytometry and normalised to the infecting inoculum (HV n = 6, RA n = 6). Total burden of mycobacteria at 24 and 120 hours post-infection represents the sum of intracellular and extracellular mycobacteria at each time point (normalised to inoculum only). (HV n = 6, RA n = 6). Bars represent mean, and error bars represent standard deviation. P values were derived from a Mann-Whitney test, * p < 0.05.

9.3.3 Anti-TNF therapy reduces number of in vitro Mtb-infected monocyte-derived macrophages

Anti-TNF antibody therapy has been previously associated with monocyte and T cell apoptosis in vitro in Crohn's disease [141-143], and both IFX and ETN have been shown to increase apoptosis of the monocyte/macrophage population in synovial fluid, but less so in peripheral blood from RA patients [144]. The mechanism of this apoptosis is not clear, but may be mediated by reverse signalling via tmTNF, which is activated by anti-TNF antibody therapy [146].

Therefore in order to assess whether adding in vitro anti-TNF therapy to cell cultures affected cell number, I quantified the number of Mtb-infected and uninfected MDMs in the presence of anti-TNF therapy (ADA or ETN), an isotype control to IgG1 (isotype control for ADA) or no drug in HV and RA (treated with MTX) MDMs.

As seen previously (see Figure 9.3 A), cell number reduced over time in both the healthy and RA groups in both Mtb-infected and uninfected MDM (see Figure 9.4 A – B). In the healthy subjects, neither anti-TNF therapy nor the isotype control alone had any impact on MDM number (see Figure 9.4 A). However, after 120 hours the number of RA MDM that were Mtb-infected and treated with either ADA or ETN, reduced by 54 or 57% respectively, which was significantly reduced compared to the number of uninfected cells treated with anti-TNF therapy, which only reduced by 26 or 25% respectively (see Figure 9.4 B). Although a similar trend was seen in the untreated uninfected and Mtb-infected RA MDM, but this did not reach statistical significance. This suggests that anti-TNF therapy may increase apoptosis of Mtb-infected MDM. Further assays of cell death or apoptosis would need to be studied to confirm this.
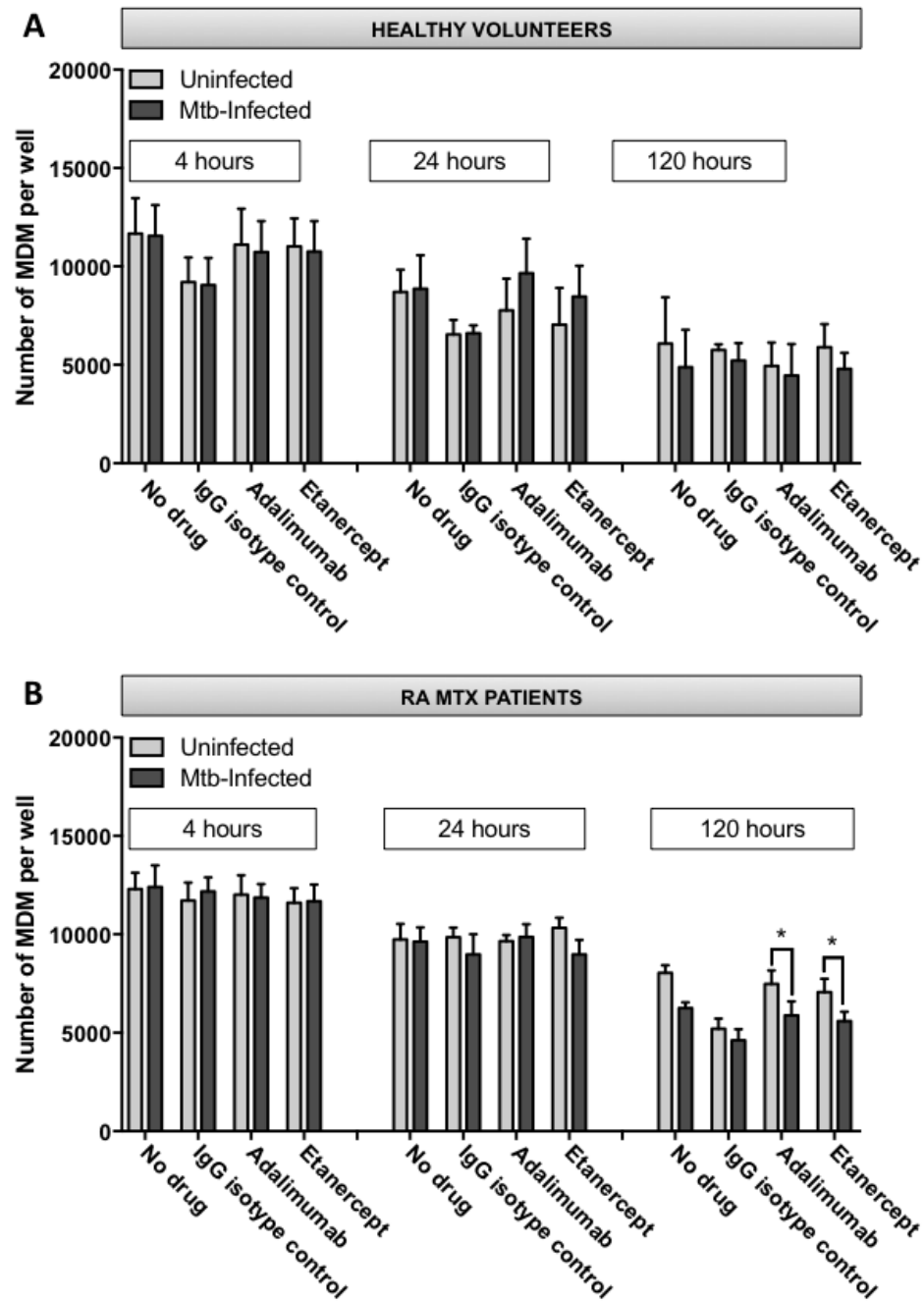
Figure 9.4 Anti-TNF therapy and Mtb infection reduce number of adherent MDM in RA, but not HV at 120 hours

Monocyte-derived macrophages (MDM) were pretreated with Etanercept (ETN), Adalimumab (ADA), an IgG1 isotype control or media alone, one hour before infecting with H37Rv mCherry (or no infection), at a multiplicity of infection of one. Cells were washed at 4 hours, and either fixed with 8% paraformaldehyde (PFA) or replenished with media (containing ADA, ETN, IgG1 isotype control or nil) for a further 24 or 120 hours at which point cells were fixed with 8% PFA. Adhesive MDM were stained with DAPI and imaged using the Hermes microscope. WiSoft software was used to quantify number of cells per well in Mtb-infected and uninfected samples, in **(A)** healthy subjects (n = 6) and **(B)** RA patients (n = 6). P values were derived from a Wilcoxon matched-pairs signed rank test * p < 0.05. Bars represent mean, and error bars represent standard error of the mean.

9.3.4 In vitro anti-TNF therapy enables greater Mtb growth in RA monocyte-derived macrophages compared to healthy subjects

In vitro studies have shown that TNF increases the ability of macrophages to phagocytose and kill mycobacteria, which largely thought to be via activation of macrophages and subsequent apoptosis [355-358]. It has been shown that anti-TNF antibody therapy, but not ETN blocks phagosome maturation in macrophages (PMA-differentiated THP-1 cell lines), but did not report whether this impacted on TB growth [363]. Anti-TNF antibody therapy has also been shown to diminish IFNγ effects and stimulate apoptosis of key immune cells, including monocytes [142], CD4^{+} T helper cells [366], and Mtb-reactive CD8^{+} T cells [294]. Therefore it is not clear what effect TNF or TNF blockade has on intracellular mycobacterial growth, and subsequently the total burden of mycobacterial growth if cells do undergo apoptosis.

I have already shown that ETN effectively blocks TNF dependent responses in MDM stimulated with LPS (see Derivation of TNF-inducible transcriptional modules). Therefore to establish whether MDM-derived TNF has a protective role in the immune response to Mtb, I sought to assess Mtb growth in the presence of anti-TNF therapy. I infected MDMs from healthy subjects and RA patients treated with MTX, with H37Rv mCherry, in the presence of anti-TNF therapy (ADA or ETN) or media alone. I sought to assess intracellular, extracellular and total Mtb burden in healthy and RA MDM treated with or without anti-TNF therapy. An isotype control for IgG1 (as a control for ADA) was also used.

Adherent MDM from HV or RA patients treated with MTX were primed with ADA, ETN, an IgG isotype control or no drug for one hour prior to infection with H37Rv mCherry. Cells were then infected with H37Rv mCherry at an MOI 1 for four hours, at which point they were washed and either lysed to collect intracellular Mtb or replenished with media, containing the same drug as previously (ADA, ETN, an IgG isotype control) or no drug for a further 24 or 120 hours.

A

B
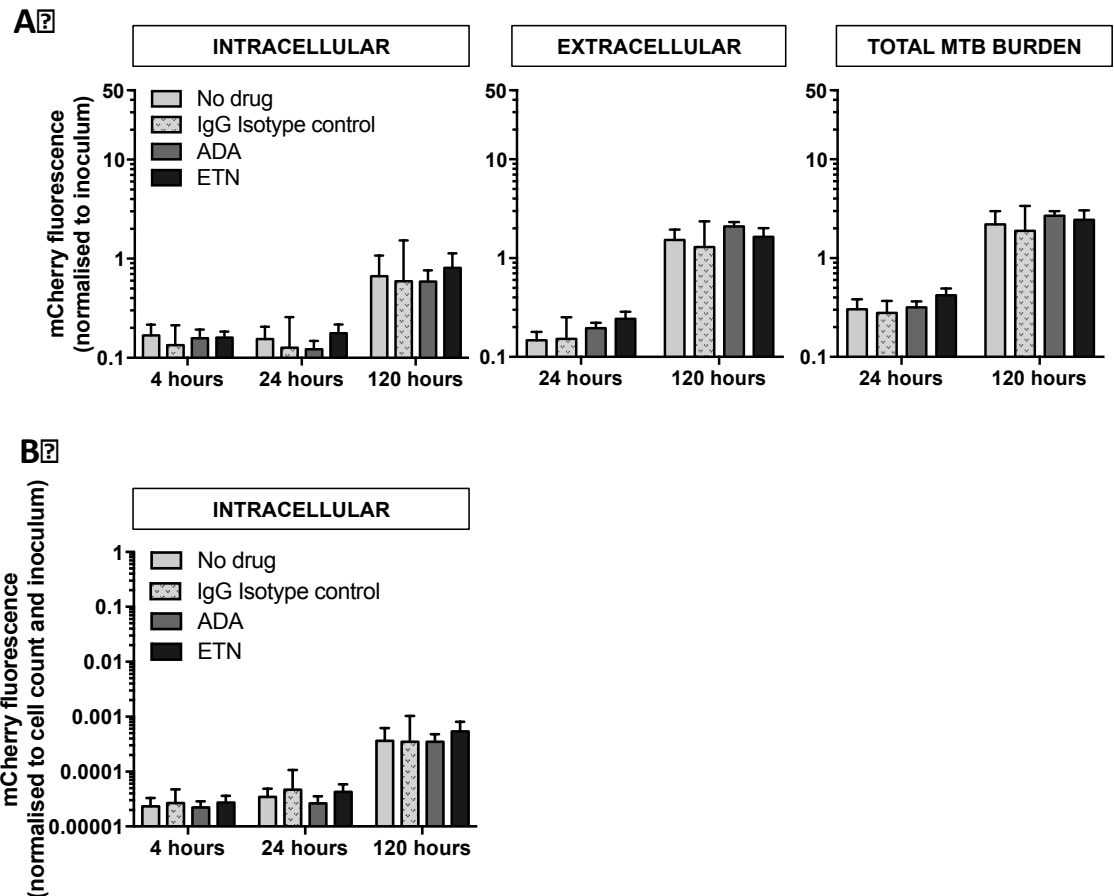
Figure 9.5 In vitro addition of anti-TNF therapy does not affect mycobacterial growth in MDM from healthy subjects

Monocyte-derived macrophages (MDM) from healthy volunteers were primed with no drug, Adalimumab (ADA), Etanercept (ETN) or an IgG isotype control, one hour prior to infection with H37Rv mCherry for 4 hours (multiplicity of infection of 1). Adherent cells were then washed and appropriate culture medium was replaced for a further 24 or 120 hours. **(A)** Intracellular mycobacterial growth was assessed by flow cytometry from washed and lysed cells at 4, 24 and 120 hours, and extracellular mycobacteria were collected and quantified from supernatants of Mtb-infected MDM at 24 and 120 hours post-infection. Total burden of mycobacteria represents the sum of intracellular and extracellular mycobacteria at each time point, this was quantified by flow cytometry and normalised to the infecting inoculum. n = 6 for each experiment. **(B)** Intracellular mycobacterial growth represented, normalised to inoculum and MDM cell count.

No significant p values were determined with a Wilcoxon matched-pairs signed rank test. Bars represent mean, and error bars represent standard error of the mean.
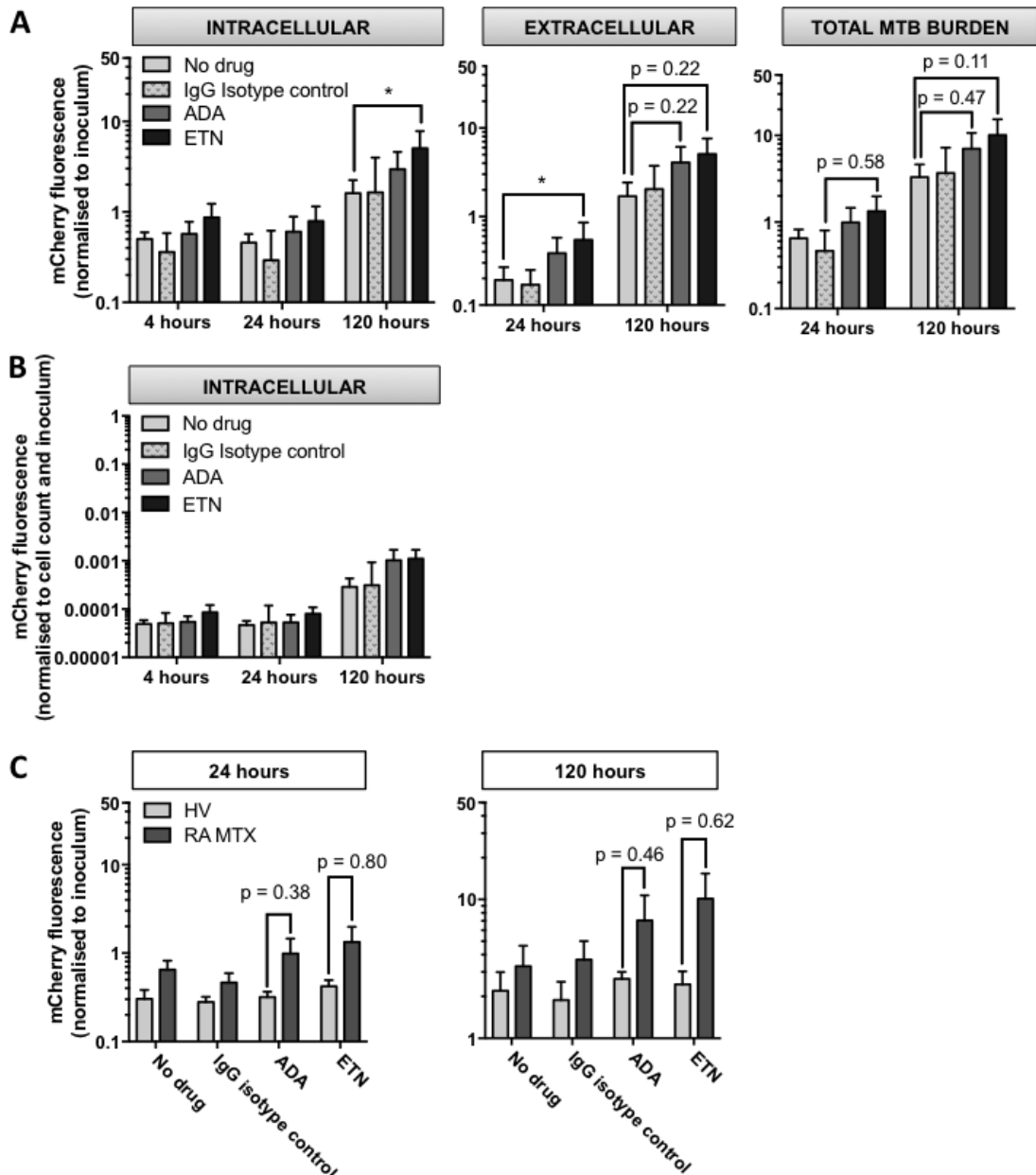
Figure 9.6 In vitro addition of anti-TNF therapy does not consistently increase mycobacterial growth in MDM from RA patients

MDM from RA patients treated with MTX were primed with no drug, ADA, ETN or an IgG isotype control, one hour prior to infection with H37Rv mCherry for 4 hours (MOI 1). Adherent cells were then washed and appropriate culture medium was replaced for a further 24 or 120 hours. **(A)** Intracellular mycobacterial growth was collected from washed and lysed cells at 4, 24 and 120 hours, and extracellular mycobacteria were collected and quantified from supernatants of Mtb-infected MDM at 24 and 120 hours post-infection by flow cytometry. Total Mtb burden represents the sum of intracellular and extracellular mycobacteria at each time point (n = 6), all normalised to inoculum only. p values were determined by Wilcoxon matched-pairs signed rank test, *p<0.05. Bars represent mean, and error bars represent standard error of the mean. **(B)** Intracellular mycobacterial growth represented, normalised to inoculum and MDM cell count. **(C)** Total Mtb burden at 24 and 120 hours comparing MDM from HV and RA on MTX. No significant differences were found by Mann-Whitney test.

Maximal intracellular H37Rv mCherry growth occurred after 120 hours in MDM from both HV and RA MTX patients. (see Figure 9.5 A – B and Figure 9.6 A – B). Neither of the anti-TNF therapies significantly increased intracellular mycobacterial growth or had an impact on extracellular mycobacterial growth in MDM from HV (see Figure 9.5). However, ETN did significantly increase intracellular H37Rv mCherry growth in RA MDM, but this was not significant once adjusting for number of MDMs per well (see Figure 9.6 A - B). Extracellular and total Mtb growth appeared to be greater following addition of anti-TNF therapy in RA MDMs, but this was not significant (see Figure 9.6).

Total growth of Mtb appeared to be greater in all RA conditions, compared to HV MDM at both 24 and 120 hours, but this was not significantly different (see Figure 9.6 C). Interestingly following addition of the IgG isotype control to Mtb cultures, results were more in keeping with 'no drug' than ADA, suggesting this possible increase in Mtb growth is due to blockade of TNF activity rather than non-specific antibody binding.

9.4   DISCUSSION

Mtb has evolved to subvert intracellular killing by macrophages, by preventing acidification of the phagosome and fusion with lysosomes [227]. However, macrophages still play an important role in at least partly containing mycobacterial growth [301, 367, 368].

As traditional methodology for quantifying intracellular mycobacterial growth, such as colony counting, is assumed to be slow and inaccurate, I have established a high throughput flow cytometric assay for quantitating mycobacterial growth, which can be normalised to both infecting inoculum and MDM cell number to provide greater accuracy. This methodology has also enabled me to quantify cell number over time by microscopy, and growth of extracellular mycobacteria by flow cytometry, thereby assessing total mycobacterial burden, which is not usually measured by traditional methods.

The advantages of the flow cytometric assay to quantify fluorescent reporter proteins in H37Rv is that the fluorophore mCherry, grows steadily over time and follows a similar growth curve to wild type H37Rv. H37Rv mCherry is quantifiable by flow cytometry with good sensitivity and this methodology allows the ability to quantify mycobacteria in large numbers of MDM cells, allowing for a high throughput of results. This method also allows for quantifying both intra- and extracellular Mtb, so that total Mtb burden can be assessed.

Once fluorescent mycobacteria die, they no longer fluoresce (as evidenced by Mtb-infected MDM treated with isoniazid), however it is not clear at what point dying mycobacteria lose fluorescence. This is important to establish, to see whether extracellular mycobacteria are in a dying stage. We did initially establish that extracellular H37Rv mCherry did contain live mycobacteria (by colony counting on agar plates – data not shown), but again colony counting does not account for mycobacteria that may be dying. Similarly other methods have been harnessed to quantify mycobacteria; qPCR is unable to discriminate live or dead bacteria, has a poor sensitivity for low numbers of mycobacteria and the thick lipid-rich cell wall of Mtb makes DNA extraction more difficult [369], imaging of fluorescent reporter proteins such as Green Fluorescent Protein (GFP) can estimate viable intracellular

pathogens [370] but is better suited to quantifying smaller numbers of infected MDM and colorimetric assays using 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) can quantitate intracellular mycobacteria [371], but MDM are usually lysed in the process, therefore it is not possible to quantify extracellular bacteria.

Using this method I have seen that over 120 hours, RA MDM are not more permissive to Mtb infection than HV MDM, despite the increased risk of TB in anti-TNF therapy-naïve RA patients. There is initially an increase in Mtb infection in RA MDM within the first 24 hours, which may give rise to an early permissivity, but this is not sustained and after 120 hours there is no discernable difference in intracellular, extracellular or total Mtb growth between RA and healthy MDM. Therefore the increased susceptibility to Mtb in RA seen in national registry data [189, 194] and from large databases of patients with RA from California [193] and Ontario [192], is not due to macrophage handling of the mycobacterium, but may be due to other immune mechanisms, such as defects in adaptive immunity.

It would have been interesting to characterise the phenotype of the HV and RA MDMs in this model before and after infection, as RA macrophages are thought to be more highly activated [94, 364], with a higher propensity to the CD14^{low}CD16^{high} phenotype and express more tmTNF compared with their healthy counterparts [72]. In this series of experiments, all MDMs were differentiated in macrophage colony stimulating factor (M-CSF) for consistency. Extensive functional heterogeneity in macrophages is widely recognised, although it is thought that monocytes differentiated in vitro with M-CSF are thought to have a more anti-inflammatory phenotype, whereas those differentiated in granulocyte macrophage colony stimulating factor (GM-CSF) may be more pro-inflammatory and with a similar phenotype to alveolar macrophages [372-374]. Therefore further work might include extension of the current experiments using alternative macrophage models, such as differentiation of MDMs in GM-CSF, to more closely resemble alveolar macrophages. However macrophages are highly plastic cells, and will adapt their phenotype according to a variety of environmental cues, including Mtb infection and therefore the initial differentiation phenotype may be irrelevant.

TNF is an important cytokine in macrophage activation, is released by macrophages once activated and is also thought to be important in granuloma formation [222, 277, 284, 285]. Mycobacterial infection of macrophages induces production of TNF [278, 375, 376], which improves the ability to phagocytotose and kill mycobacteria [358, 377]. Therefore I wanted to establish what effect TNF blockade had on mycobacterial growth, by both adding anti-TNF therapy in vitro and also using MDMs from patients receiving anti-TNF therapy. I had confidence in this methodology, given I have previously shown that ETN is able to block TNF-induced activity in MDM stimulated with LPS (see Derivation of TNF-inducible transcriptional modules). However it would also be interesting to repeat these experiments in the presence of exogenous TNF and also to model T cell contributions, by adding activated T cells to Mtb-infected MDM.

It has also been reported that anti-TNF therapy and ETN can induce apoptosis of monocytes and macrophages in vitro [141-144], hence I measured in vitro cell numbers over time in both Mtb-infected and uninfected MDM. Between the early and late time points there was up to 50% cell loss, but this was independent of infection or drug and seen in both HV and RA MDM. This cell loss is likely to be due to cell death and apoptosis, and also loss of adherence to the plate over time, as dynamic cytokine concentrations in the environment (supernatant) after Mtb infection will have contributed to altered phenotype of the MDMs. However there was a statistically significant decrease in cell number in Mtb-infected RA MDM compared to uninfected when anti-TNF was added in vitro. Mtb-infected MDMs in HV, and those without drug or treated with the IgG isotype control were also lower in number, but did not reach statistical significance, compared to uninfected MDM. Furthermore this cell loss over time highlighted the importance of normalising quantitation of intracellular Mtb to cell number. Further experiments should be conducted to explore the mechanism of cell loss, and whether anti-TNF therapy is contributing to apoptosis of Mtb-infected MDM.

Interestingly anti-TNF therapy had no effect on intra- or extracellular mycobacterial growth in healthy volunteers. However in RA patients, it appeared that anti-TNF therapy did contribute to total mycobacterial burden, but these results did not reach statistical significance. Only six individuals were used per

group, and there was large inter-individual variability between donors that may have contributed to the lack of statistical significance. It would be prudent to repeat these experiments with increased numbers of donors to see whether statistical significance can be reached.

It would also be interesting to examine whether MDM from RA patients treated with anti-TNF therapy behave in the same manner as those where TNF blockade has been added in vitro. This would address whether anti-TNF therapy modulates circulating monocytes (such as increasing tmTNF) and whether these changes are also applicable to monocyte-derived macrophages. I would also like to assess the monocytes and differentiated macrophages for tmTNF levels, in HV and RA patients treated with and without anti-TNF therapy to see whether the increased levels of tmTNF that has previously been reported in monocytes is still seen in macrophages.

9.5 CONCLUSIONS

In conclusion I have established an effective and accurate method of quantifying intracellular and extracellular mycobacteria in vitro, using flow cytometry and microscopy of a virulent strain of Mtb, H37Rv, which constitutively expresses a fluorescent reporter protein mCherry fluorophore (H37Rv mCherry).

I have demonstrated this is a robust method for quantifying intra- and extracellular mycobacteria and for assessing total mycobacterial burden in Mtb-infected MDM. However cell loss occurs with increasing time post-infection. This is increased by anti-TNF therapy in Mtb-infected MDM, although at this point it is not clear whether this is due to technical handling of the cultures or a biological phenomenon.

Furthermore, in vitro anti-TNF therapy does not increase the total burden of Mtb growth in MDM from either HV or RA patients treated with MTX.

10 GENERAL DISCUSSION

10.1 EPIDEMIOLOGY AND ACQUISITION OF TB IN RA PATIENTS ON ANTI-TNF THERAPY

Anti-TNF therapies have transformed the management of RA, since being licenced nearly 20 years ago. They are regarded as relatively safe drugs, given that TNF is thought to play a non-redundant role in the healthy immune system, but there is an increased risk of active tuberculosis (TB) during, and after treatment [183, 186-189, 328]. It appears that this is most likely to be due to reactivation of latent TB infection (LTBI), rather than acquisition of new infection due to the higher risk of extrapulmonary and disseminated disease, and majority of onset being within the first 24 months of therapy [183, 186-189, 328]. Indeed screening for and treating LTBI has significantly reduced the cases of active TB [191]. From the BSRBR dataset that I analysed, I did not find conclusive evidence that *de novo* TB infection was occurring, as cases of pulmonary infection (representing primary infection), did not become an increasingly bigger proportion of active cases with time on anti-TNF therapy.

Of interest the patients from the BSRBR registry who did develop active TB were mainly white females, aged in their sixties and born in the UK. This is not generally regarded as a high risk LTBI group – as it is more commonly seen in immigrants who have been in the UK less than 5 years, who were born in South-East Asia and Africa [335]. However these patients from the registry with LTBI reflect the demographics for RA patients who have a higher tendency to be female and usually present in the 4th-5th decade of life [35].

I would have also liked to compare the active TB cohort from the BSRBR with Public Health England's (PHE) national TB database, to establish whether the number of cases and case mix are in keeping with TB in the general population. It would have also been interesting to look at age and sex-matched controls and compare outcomes of treatment, and mortality rates. I also wanted to compare what effect guidelines for LTBI screening may have had on incidence of TB in patients on anti-TNF therapy. However, the BSRBR did not collect data on LTBI

screening in recruited patients. In addition, once the BSRBR reached their initial recruitment targets, recruitment was stopped for several years, and subsequently recruitment has been poor. This makes true comparisons between BSRBR and PHE very difficult to perform and may not have given an accurate reflection of events, therefore these analyses were not pursued.

Genotyping of Mtb samples has been used in outbreak investigations, long-term surveillance and the detection of laboratory contamination. I wanted to see whether we could identify the genotype from Mtb samples of RA patients by the mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR) strain types, and compare these with cases occurring in the same area during a similar time-period, to assess whether these cases were reactivation of LTBI and/or matched other cases of primary TB infection. However this was not possible, as many of our cases pre-dated MIRU-VNTR strain-typing, and this has not proven useful at identifying transmission chains previously. In addition the risk of new TB infection in most UK populations is low, other than hard to reach groups and some ethnic minority populations – mostly being due to reactivation of LTBI. Therefore this may not have provided us with enough information regarding local clusters of new TB infection to link the RA cases. It may have been that one or two cases had the same or similar MIRU-VNTR, but this could have been due to a common origin, rather than an epidemiological link or transmission between subjects. Therefore identifying MIRU-VNTR strains was not pursued.

10.2 ASSESSING A GENOME WIDE APPROACH TO INDUCIBLE TNF ACTIVITY

Transcriptomic analysis of tissue samples is used widely for an in vivo systems level assessment of multicellular biological processes, providing valuable insights into mechanisms of disease. However large amounts of data generated in this process can be difficult to interpret, therefore I worked together with other members of the laboratory group who have an interest in bioinformatics (Carolin Haas, Gabriele Pollara and Lucy Bell), to develop transcriptional modules to identify different cell types and inducible TNF activity. Subsequently we have

validated this method of using transcriptional modules in tissue as well as blood [310].

Four individual modules of inducible TNF activity were developed, derived from our own experiments and published data [311, 312]. Two modules were derived from monocyte-derived macrophages (MDM) – both exogenous TNF stimulation and endogenous TNF production through lipopolysaccharide (LPS) stimulation, one derived from whole blood stimulated with TNF and lastly a module derived from published data from primary human keratinocytes [311, 312] (this was particularly important for the skin samples, as the majority of keratinocytes are found within the epidermis). I also compared a module derived from whole blood of RA patients, and compared this to the whole blood module from HV to show that similar patterns of TNF activity could be seen in the unstimulated and TNF-stimulated blood, suggesting that the disease from which the modules are derived from is unimportant in this context. Despite the different methods, cell types and modest overlap between gene lists of modules, a similar pattern of TNF activity was seen and cross-validation of each gene list highlighted the context specificity of the functional gene modules. I would have also liked to test these modules on endogenous production of TNF in whole blood, in the context of general inflammation following stimulation with LPS.

Gene expression profiling of monocytes [170], peripheral blood [171-174] or synovial tissue [175-178], has been used extensively in RA before and after anti-TNF treatment, in order identify biomarkers that may be used to stratify likelihood of beneficial responses to treatment. However in the context of anti-TNF therapy increasing the risk of active TB, it has not been shown that downregulation of TNF activity persists during an acute inflammatory event, in patients with otherwise stable RA disease. Therefore this project aimed to study TNF activity in vivo in RA patients treated with anti-TNF therapy at the site of a standardised experimental challenge (the TST). In order to fully understand what effect anti-TNF therapy has on the immune response to TB, it would have been even better if samples were taken from the pulmonary TB granuloma itself, reflecting the exact cells which are involved in the host response to TB. The TST is a good representation of all of the components of the integrated innate and

adaptive immune responses to TB, but the skin and pulmonary resident cells differ, thereby offering slightly different immune responses. Cases of active TB during anti-TNF therapy are now declining, as physicians are more thorough at screening for LTBI [191], and therefore these cases now occur infrequently. The TST is a simple, well-tolerated routine clinical procedure, which is easily amenable to biopsy and thus provided the most appropriate method to study TNF activity.

Peripheral blood samples from RA patients showed that TNF bioactivity represented by the expression of TNF modules was not significantly different between those treated with methotrexate alone, or anti-TNF therapy. Given that these patients all had quiescent RA disease, it is more likely that the reported diminution of pro-inflammatory mediators may simply represent control of active disease and not due to a targeted reduction in steady state expression of TNF-dependent gene expression. However following TNF stimulation of whole blood, significant attenuation of both TNF gene expression and TNF activity was seen across all four transcriptional modules, confirming that TNF activity is reduced in the context of an acute inflammatory challenge.

I would have liked to extend these observations further, by also stimulating whole blood with LPS, to see whether endogenous TNF responses are blocked by anti-TNF therapy, and whether TNF activity is attenuated by anti-TNF therapy, in response to whole blood stimulated with mycobacterial antigens.

10.3   THE EFFECT OF ANTI-TNF THERAPY ON IN VIVO PPD RESPONSES IN THE SKIN

Given that I have confirmed attenuation of TNF activity in peripheral blood of RA patients receiving anti-TNF therapy following TNF stimulation, I wanted to assess whether this altered response was also seen in vivo, at the site of a standardised immunological challenge.

Clinical inflammation in the TST has been widely used as a surrogate for T cell memory for mycobacterial antigens [340], but transcriptional profiling of biopsies from the injection site reflects all the components of integrated innate and adaptive immune responses, each of which can be quantified with independently derived transcriptional modules [309]. Importantly, this approach also revealed

immune responses in the absence of clinically evident inflammatory induration, allowing unprecedented sensitivity to measure immune responses that were previously described as anergic [309, 339].

I selected RA patients on anti-TNF therapies (adalimumab and etanercept), and compared these to patients receiving standard treatment (methotrexate), and to a group of healthy volunteers. Significant ethnic and age differences occurred between the HV and RA patients, which may have confounded the results. HV patients were younger and had a more multicultural demographic. Most RA patients were older and of a White British demographic. It has been previously described that increased age (more than 65 years) may attenuate clinical TST responses [378, 379], but given that our group has previously shown genome wide transcriptional profiling of the TST site is more sensitive for measuring immune responses that clinical responses [309, 339], this gave confidence the methodology.

TNF gene expression was significantly upregulated in the TST of healthy volunteers, which correlated well with modular expression of TNF activity. Matrix metalloproteinase (MMP)9 was chosen as an immunohistochemistry marker of TNF expression, as this is associated with TNF activity [346], and MMP9 featured in the transcriptional TNF module derived from keratinocytes. MMP9 was consistently increased in the TST and was strongly correlated with TNF gene expression, in 60% of healthy participants. These results validated that TNF bioactivity was detectable in the TST.

Interestingly in all RA patients there was upregulation of TNF gene expression, increased modular expression of TNF activity and increased MMP9 expression in the TST, despite treatment with anti-TNF therapy. Given that we have previously seen anti-TNF agents suppress TNF activity in peripheral blood, the most likely explanation is that anti-TNF therapy does not reach sufficient concentration within the tissue microenvironment to neutralise the level of TNF produced in an acute cell mediated immune response, modelled by the TST. It is likely that differential sensitivity of acute and chronic inflammation generates the therapeutic window in which anti-TNF agents have provided such effective treatments for chronic

inflammatory disease. In such a model, current anti-TNF therapies may be ineffective in acute inflammatory pathology, and strategies that effectively inhibit TNF activity in acute inflammation may incur much greater risk of acute infections.

Given that the saline control samples (albeit only three participants in each group), appeared to have a lower level of TNF activity in RA patients on anti-TNF therapy, compared to those treated with methotrexate, it is possible that the therapeutic effects of TNF inhibition may be achieved by neutralising lower levels of TNF in foci of chronic inflammation or by neutralising the endocrine functions of circulating TNF. However as this was not the expected result, only small numbers of control samples were collected per treatment group, and it would be useful to expand these numbers.

The most significant cell type to infiltrate the TST was T cells, which is in keeping with published literature [339]; this was confirmed by cell-specific transcriptional modules and validated by immunohistochemistry to quantify cellular infiltration, giving further confidence to our methods. Further work is also being carried out by other members of the group to look more specifically at CD4^{+} and CD8^{+} cell infiltration in the TST, both by transcriptional modular analysis and by histology.

10.4   THE EFFECT OF TNF BLOCKADE ON IN VITRO MYCOBACTERIAL GROWTH

TNF plays an important role in the host response to TB, as it stimulates the production of chemokines (CCL2, 3, 4, 5, and 8) in macrophages and T cells and induces vascular adhesion molecules, promoting a focused accumulation of immune cells at the site of infection [284, 380]. It is also able to increase the phagocytic capacity of macrophages and enhance intracellular killing via the generation of ROS and RNI in mice [381], and aiding mycobacterial control by inducing apoptosis [355-358]. However few studies have looked directly at the effect of TNF blockade on intracellular mycobacterial growth, therefore I used an in vitro model of Mtb-infected macrophages to answer this question.

By establishing a novel assay using flow cytometry and microscopy of fluorescent Mtb, I was able to determine intracellular and extracellular Mtb growth in monocyte-derived macrophages. Together with other members of our laboratory

group (Elspeth Potton, Gabriele Pollara, Jennifer Roe and Meera Mehta) this assay was refined to incorporate cell number quantified by microscopy, which was found to decrease over time and potentially confound rates of intracellular mycobacterial growth. As the virulent Mtb (H37Rv mCherry) used to infect macrophages was grown in liquid culture at log-phase, mycobacterial fluorescence was also normalised to the infecting inoculum, which was found to vary slightly between experiments. In this assay I was also able to quantify extra-cellular H37Rv mCherry that had been released by Mtb-infected cells, either as a mechanism of bacterial survival or following macrophage apoptosis. Previously this has not been extensively studied, but was important for quantifying total mycobacterial growth. Extracellular mycobacteria may subsequently infect other cells in the culture, and other members of the group are currently studying this.

It was not clear what effect anti-TNF therapy would have on numbers of Mtb-infected macrophages in culture, given that TNF is known to induce macrophage apoptosis in Mtb-infected cells [355-358] and anti-TNF therapy can stimulate apoptosis of key immune cells, including monocytes [142]. I found that in RA patients, there was significantly more loss after 120 hours which was compounded by Mtb infection and in vitro addition of anti-TNF therapy. I would have liked to further investigate whether this was indeed due to increased apoptosis or due to technical handling of the culture.

Although it appeared that in RA MDM, intracellular and extracellular H37Rv mCherry was greater in the context of TNF blockade, this was not significantly different from the IgG1 isotype control, or the control sample without anti-TNF therapy. This may have been explained by significant variation of Mtb growth and cell number between donors, although six participants were recruited per disease group and results were normalised to cell number and infecting inoculum. It would be useful to repeat these experiments again to see whether the results do reach significance. Interestingly in MDM from HV, anti-TNF therapy had no impact on mycobacterial growth. Although we have confidence that ETN blocks TNF activity in MDM from our transcriptional modules of TNF activity, it may have been useful to quantify serum TNF levels throughout the experiment, and further study Mtb-infected macrophages for TNF activity, after addition of anti-TNF therapy. Thus we

have not found a clear implication that anti-TNF therapy increases intracellular or extracellular mycobacterial growth in HV or RA MDM.

Further investigations of Mtb intracellular growth should also include the addition of TNF to Mtb-infected macrophages to see whether this reduces intracellular and total mycobacterial growth, and what impact this has on cell number.

11 CONCLUSIONS

By studying data from the British Society of Rheumatology Biologics Registry (BSRBR), I have been able to confirm that anti-TNF therapy increases the risk of reactivation of latent TB infection (LTBI) in patients with rheumatoid arthritis, but have not found evidence of increased risk of new active TB infection in these patients.

Anti-TNF therapy does not block TNF gene expression or TNF activity in whole blood of RA patients with stable inflammatory disease. However following about inflammatory response (or in this case, stimulation with TNF), TNF activity is reduced in those receiving TNF inhibitors. During a prototypic cell-mediated immune response at the site of immune challenge in vivo, anti-TNF therapy in RA patients does not inhibit inducible TNF function, suggesting that anti-TNF agents do not reach sufficient concentration within the tissue microenvironment to neutralise the level of TNF produced in an acute cell mediated immune response or their therapeutic effects are limited to regulating TNF activity in chronic inflammation or by alternative non-canonical pathways.

A virulent strain of Mtb constitutively expressing a fluorophore, H37Rv mCherry, can be accurately measured by flow cytometry to measure Mtb growth inside and outside of macrophages. Using this technique, I found that addition of anti-TNF therapy in vitro had no significant effect on mycobacterial growth, but may increase cell death in Mtb-infected cells.

12 FUTURE WORK

12.1 INVESTIGATING INDUCIBLE TNF ACTIVITY IN RHEUMATOID ARTHRITIS PATIENTS TREATED WITH ANTI-TNF THERAPY

Anti-TNF therapy reduced TNF activity in whole blood following TNF stimulation, compared to RA patients treated only with methotrexate. I would like to expand these experiments to incorporate a larger number of subjects, to see if there is a significant difference between ADA and ETN – as ADA did significantly reduce TNF activity whereas ETN did not. This may go some way to explain the increased risk of TB with anti-TNF antibodies. I would also like to induce an endogenous TNF response in the context of a broad inflammatory response, following whole blood stimulation with lipopolysaccharide (LPS), and also to mycobacterial antigens. We have already shown that ETN inhibits TNF activity in MDM, but would be important to demonstrate that endogenous TNF responses can also be blocked in whole blood, as this may have more direct clinical relevance to patients receiving anti-TNF therapy.

If it were possible to study lung biopsy specimens from RA patients receiving anti-TNF therapy with active TB, it would also be important to see whether anti-TNF therapies reach sufficient concentration in the lung to block TNF bioactivity. However, as the rates of active TB during anti-TNF therapy are declining, it may be difficult to access this tissue.

12.2 INVESTIGATING THE EFFECT OF ANTI-TNF THERAPY ON THE IMMUNE RESPONSE TO TB IN VITRO

The role of TNF in the host response to TB is thought to mainly be activation of macrophages, apoptosis of infected macrophages and granuloma formation with the aim of controlling Mtb growth and limiting spread of disease.

However TNF blockade in Mtb-infected macrophages in vitro did not result in any significant change to mycobacterial growth in HV or RA MDM. I would also use this assay to assess whether addition of in vitro TNF limits mycobacterial growth and also to further study whether macrophages are apoptosing once infected.

As our laboratory group has previously described differences in regulatory T cell (Treg) function in patients responding to anti-TNF antibody therapy [86, 94, 151], I would also like to extend experiments in order to co-culture Mtb-infected macrophages with autologous Treg, in the context of anti-TNF therapy to see whether this increases mycobacterial growth further.

Monocyte-derived macrophages which have been differentiated in macrophage colony stimulating factor (M-CSF) are thought to have a more anti-inflammatory phenotype, compared to this differentiated in granulocyte macrophage colony stimulating factor (GM-CSF), which are more likely to mimic alveolar macrophages [372-374]. Although macrophages are very plastic, particularly in response to stimulation, I would like to include further experiments using GM-CSF in differentiation media to see if they are able to control intra- and extracellular mycobacterial growth more tightly.

In addition, RA monocytes express increased levels of transmembrane TNF (tmTNF) [72], and our group have demonstrated that when anti-TNF antibody binds to tmTNF on monocytes, it leads to enhanced expression of tmTNF [152, 153], which subsequently drives expansion of regulatory T cells by binding to TNFR2 on their cell surface [153]. It is not clear whether this increase in tmTNF remains after differentiation from monocytes to macrophages and what impact this has on T cell binding and thus control of mycobacterial TB growth. In the first instance it would be interesting to compare whether expression of tmTNF on macrophages is comparable to that of monocytes in rheumatoid arthritis and healthy volunteers.

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